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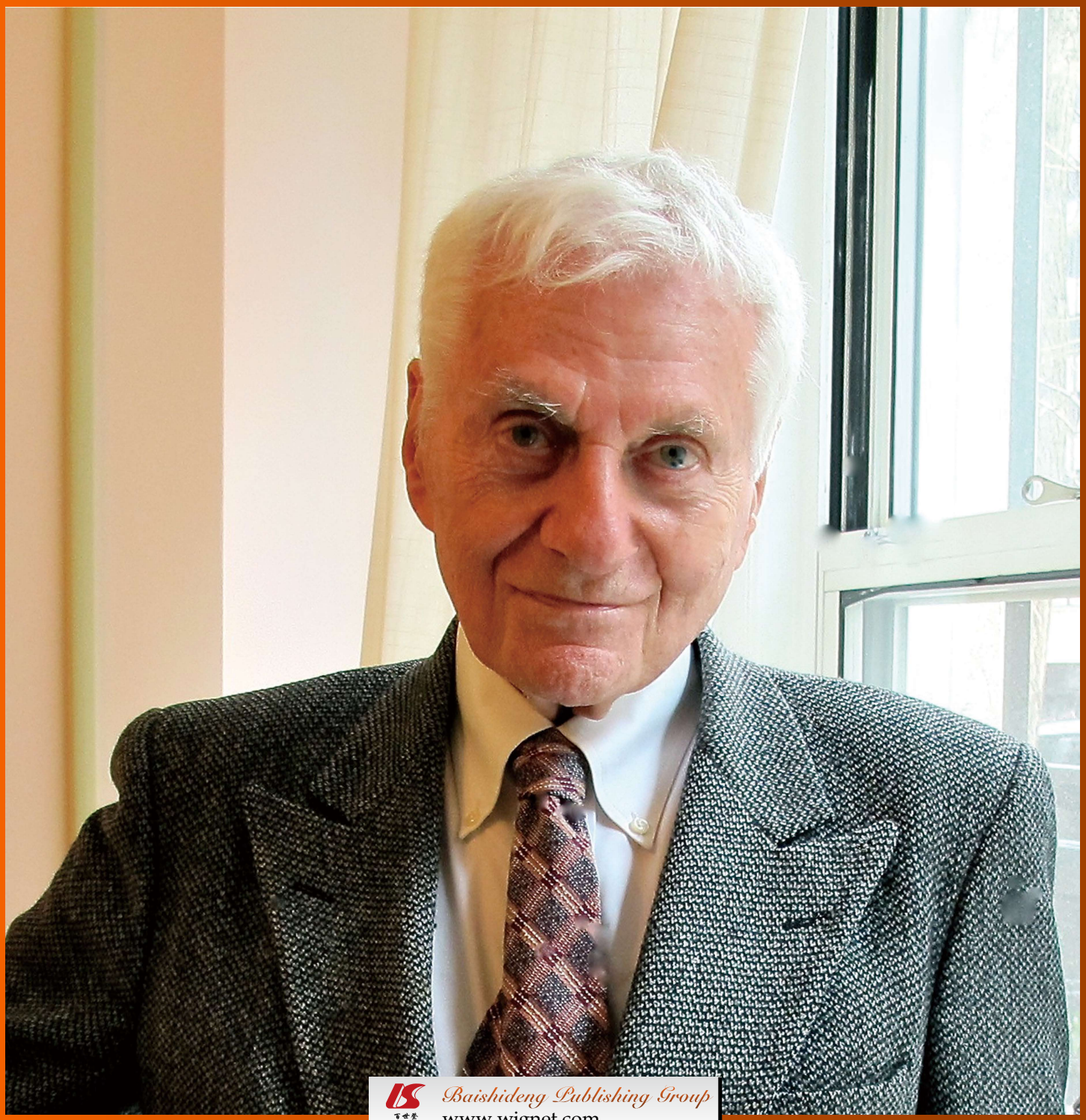

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FRONTIER

Expert opinion: Experience with 6-mercaptopurine in the treatment of inflammatory bowel disease

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Abstract

Arbitrarily, modern day treatment of inflammatory bowel disease begins with the introduction of immunosuppressives for ulcerative colitis. Clinical improvement with sulfasalazine had been meaningful but modest. Treatment with adrenocorticotropic hormone and corticosteroids led to clinical responses never before realized but it took much too long to recognize that they were not capable of maintaining remission, that adverse reactions were subtle but potentially devastating and that some other agent would be necessary to capitalize on their transient advantage. This of course was true in the treatment of Crohn's disease as well. Not much was ever made of the role of sulfasalazine for Crohn's disease, but with the severing of the diazobond and the elimination of the sulphur component, the 5-aminosalacylic acid (5-ASA) products clearly led to clinical improvement, especially in cases of Crohn's colitis and those with ileitis where the 5-ASA product was released in the terminal ileum and more proximal in the small bowel as well as in ulcerative colitis. The induction of remission was first demonstrated by 6-mercaptopurine (6-MP) with case reports and uncontrolled trials in patients with ulcerative colitis, but its placebo controlled trial for Crohn's disease firmly established its role in inducing remission. No subsequent trial has confirmed its similar role for ulcerative colitis, but nevertheless clinicians know well that 6-MP works at least as well and probably more effectively for ulcerative colitis than for Crohn's disease. What changes have taken place utilizing 6-MP in the management of inflammatory bowel disease since its introduction in the 1960's and 1970's and its trial for Crohn's disease published in the *New England Journal of Medicine* in 1980?

Key words: 6-Mercaptopurine; Crohn's disease; Ulcerative colitis

Core tip: Calculation of dose, utilization of serological tests, maintenance therapy, desensitization to 6-mercaptopurine (6-MP), toxicity to 6-MP, post-operative prevention, extraintestinal manifestations, perirectal fistulas and other fistulas, pregnancy, role of biologicals in management, brand name *vs* generic 6-MP and azathioprine.

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CALCULATION OF DOSE

In the original controlled study, the dose of 6-mercaptopurine (6-MP) chosen by Present and myself was based on weight at 1 ½ mg per kilogram. This precedent has subsequently been used in almost all other studies around the world when the immunosuppressive of choice was 6-MP rather than Azathioprine. Despite this and due to my own experience based on the wide range of leukopenia as influenced by dose, I changed my preference from dose by weight and started all patients at 50 mg/d. Since

in both cases my policy was to have blood drawn for a complete blood count (CBC), at one, two and three weeks, a rapid fall in the white blood cell count (WBC) would be recognized early and the drug could be reduced or stopped accordingly. On the contrary should there be no fall in the WBC and there also be no clinical improvement in the inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), I would have the opportunity to increase the dose within the 3 wk period[1-5].

If the patient were still on steroids when the 6-MP was started and there was clinical improvement, I have reduced the steroid dose early to minimize its duration, also keeping in mind that the WBC was elevated because of the steroids and their reduction might accelerate the leukopenia. Reintroduction of 6-MP after stabilization on recovery from the leukopenia would be by 25-50 mg less than before. Some patients tolerate only as little as ½ tablet or 25 mg/wk while others require an increase up to 300 mg/d, the low and the high doses being equally effective for the individual case.

This exercise has been modified by the availability of biologicals since the severity of disease and lack of response to early doses of 6-MP will serve to accelerate the decision to add the biological to the therapy[6-8].

UTILIZATION OF SEROLOGICAL TESTS TO INFLUENCE THE DOSE OF 6-MP

Since these tests were not available in the 1960's and 1970's, we were permitted a prolonged period of experience without them and then later learned that I never had to utilize them. By the time I would decide to start 6-MP the first CBC was drawn and interpreted before there was even time to receive a report on thiopurine methyltransferase, TGN or TPPT. Furthermore, a favorable clinical response was documented as early as possible since the blood counts were entered on a monitor sheet followed by directions by me when the patient called as told to do so. If the patient did not call on time, it was the practice of my office to call them should a change in dose be warranted. With increased experience, I would also get liver function tests including a GGTP early on and then periodically to recognize mild abnormalities consistent with an acceptable "transaminitis" versus a progressive abnormality of true liver damage. One monitor sheet included a column for WBC, Hb, Hct, and platelets and another for bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvate transaminase, alkaline phosphatase, GGTP and Amylase[9-12].

MAINTENANCE THERAPY WITH 6-MP FOLLOWING SUCCESSFUL INDUCTION

As an outcome of the original controlled trial, we learned that the mean time to induction of remission was three months. Even then, however, it was clear that some patients responded faster, even as little as three weeks, and others might take up to a year and still be successful. The differences were modified by recognizing the success of a small dose of 6-MP leading to that dose becoming the maintenance dose, and the failure of the initial dose leading to a relatively rapid increase until the maintenance dose was found without requiring a whole year or even six months. Again, should the patient be on steroids when the 6-MP was started, tapering and elimination of the steroids was part of the goal, always mindful that any reduction of steroids increased the risk for leukopenia.

The role of immunosuppressives in top down *vs* bottom up straddles these two approaches to therapy. This is because 6-MP has maintenance as well as induction value as opposed to steroids which work quickly but have no maintenance value and infliximab which also works quickly and does have maintenance value. The 6-MP, however, is slower in its action than both of the above in most cases. Therefore 6-MP will be more appropriate as a Step Up drug unless used from the outset as a Top Down in conjunction with infliximab as done in the Sonic Trial.

At the other end of the spectrum, after prolonged remission on 6-MP [± a 5-aminosalacylic acid (5-ASA) product but no biological as yet], some patients wanted to stop the drug, others refused to stop it remembering the severity of their disease before its introduction, and others accepted my recommendation based on clinical judgment. The time between CBC's and LFT's was already extended due to persistence of normality, so that some patients reached an interval of three months. I have chosen not to extend the interval beyond this period. If I then chose to reduce the dose on clinical grounds (usually because of patients' persistent fear of toxicity), I would deduct 25-50 mg. from the daily dose and keep that level for a trial of one year before considering a further reduction. This means that some patients would be maintained on one tablet/day (reduced from 2/d or 1 ½/d) and others on ½ every other day reduced from ½/d or ½ on two out of three days.

At this time I will add that in a few cases I eventually stopped the 6-MP entirely. I have not yet analyzed the results but have witnessed many examples of recurrence on both stopping and reducing the dose of the 6-MP[13-16].

DESENSITIZATION TO 6-MP IN THE COURSE OF TREATMENT OF IBD

Next to leukopenia, allergic reactions to 6-MP are the next most common adverse effects. Whether nausea and malaise are also on an allergic basis has not been clear. In my own experience, the most common allergic reactions are fever, skin rash, joint pains and back pain. Unfortunately, the 6-MP has often been terminated on this basis and both CD and UC have frequently progressed in severity thereafter, leading to surgical resections which might not have been necessary. Just as we have found earlier that desensitization to sulfasalazine not only is frequently successful, and treatment with that drug resulted in remissions of IBD, particularly UC, desensitization has been fruitful for 6-MP. This has been done by starting

with as little as 1/8 tablet and increasing the dose every few days or switching to Azathioprine at either full dose or the same fractions. In my experience the only allergic reaction to which desensitization has rarely been successful has been pancreatitis. Once desensitization to 6-MP has been accomplished, remission has been maintained on this drug for many years[17,18].

TOXICITY TO 6-MP IN THE COURSE OF TREATMENT OF IBD

Other than leukopenia and allergic reactions to 6-MP, concern about neoplasm has prevailed. After many years of experience, I am convinced that malignant tumors such as breast, lung, liver, pancreas, kidney, prostate and brain are not more common in those treated with 6-MP than in the entire IBD population or the general population. The problem of lymphoma has warranted intense observation and it is statistically significantly increased but still rare. I accept the conclusion that a lymphoma occurs in somewhere between 1:2000 and 1:4000 cases. If it occurs its prognosis is no different than lymphomas in IBD patients not treated with 6-MP or Azathioprine or no IBD. The exception to this rule is the hepato-splenic lymphoma which carries the worst prognosis of the lymphomas and it occurs in children, particularly male children who have the most virulent IBD, so that even in this group of patients it is challenging not to use 6-MP as well as to using it. Most patients with this type of lymphoma have been on combination therapy (6-MP or Azathioprine plus IV infliximab) but the onus is on the immunosuppressive since the lymphoma rarely occurs with infliximab alone. The one neoplasm which theoretically might be increased with immunosuppressive therapy is colon cancer superimposed on ulcerative or Crohn's colitis. My own studies and those of others have shown that this is not the case. If anything, treatment with immunosuppressives has reduced the risk of colon cancer, probably as a result of eliminating inflammation due to successful therapy.

Unfortunately, skin cancers are common in IBD patients treated with immunosuppressives. While basal cells can be successfully resected and don't often lead to terminating 6-MP, this is not so true of squamous cell carcinomas which I see fairly commonly in patients who have received the drug for many years. I have rarely seen a melanoma in patients on 6-MP.

Opportunistic infections are rare. When they occur they correlate best with situations when the disease is not controlled by the immunosuppressive drug and usually when the patient is still being treated with steroids.

Pancreatitis usually occurs within three weeks of onset of treatment with 6-MP. In our original studies, we encountered pancreatitis in 3% of patients. I rarely see it anymore.

Thrombocytopenia is fairly common in patients on 6-MP and if it occurs it is usually in conjunction with leukopenia. Anemia due to 6-MP is rare and if it occurs is most likely accompanied by a pancytopenia and requires stopping the immunosuppressive therapy[19-26].

POST OPERATIVE PREVENTION WITH 6-MP

Trials of available drugs for prevention of recurrent ileitis after ileo-colic resection have been disappointing. My own study of 6-MP for this indication showed statistically significant but not impressive results. Nevertheless, I have had many patients who started 6-MP following surgery who have remained without clinical recurrence for many years. It is my impression that endoscopic recurrence may occur despite taking the 6-MP, but its progress to a point of clinical recurrence is extremely retarded. I have also reassessed the results of my own study and learned that when the 6-MP is started in the immediate peri-operative period, protection against the recurrence is far more effective. This is an area where infliximab is proving to be more effective than immunosuppressives, again best when started immediately postoperatively. More studies in the area of which drug, both, and when are needed to resolve this question[27-29].

THE ROLE OF IMMUNOSUPPRESSIVES FOR EXTRAINTESTINAL MANIFESTATIONS

Before the advent of biologicals, treatment of pyoderma gangrenosum, erythema nodosum, arthritic manifestations and uveitis were often successful with the introduction of 6-MP. If however, the extraintestinal manifestation occurs while the patient is already taking an immunosuppressive, the need to introduce a biological is clear.

PERIRECTAL FISTULAS AND OTHER FISTULAS

Many of my own studies have demonstrated the favorable effect on all fistulas with 6-MP treatment. Perirectal fistulas and abscesses often require incision and drainage in conjunction with 6-MP but are more likely to be recurrent without immunosuppressive therapy at the same time. Infliximab also has been very effective in closing fistulas. Nevertheless, it is fairly common to see persistent drainage from fistulas despite treatment with either drug alone and even with the combination. Fortunately, the severity of the residual fistula is not great and if the primary CD has been brought into remission, the patient tolerates the drainage well[30,31].

PREGNANCY AND IMMUNOSUPPRESSIVES

The issue of 6-MP and AZA before and during pregnancy prevails since the most common years of onset

of Crohn's disease and ulcerative colitis are during the ages of greatest fertility and Crohn's disease occurs more often in females than males. Furthermore, consideration of continuing immunosuppressives during pregnancy is markedly diluted as an issue since pregnancy usually takes emotional priority over treatment of the disease in female IBD patients who want to stop all medications and so often the obstetrician is encouraging them to do so.

The evidence favoring continuing 6-MP/AZA during the pregnancy is based on the following: (1) The largest reported study on pregnancy and adverse outcomes possibly attributed to 6-MP from Mount Sinai has concluded that these drugs are safe; (2) Most adverse reactions to 6-MP/AZA occur early, soon after the drug is started. Therefore, the coincidence of any other toxicity to 6-MP in pregnancy most likely must be attributed to active disease; and (3) If the most virulent factor with toxic complications during pregnancy is active Crohn's disease and if the patient is in a remission just achieved by the drug, it should not be stopped. On the other hand, in a study from Lenox Hill Hospital there was a 23% incidence of spontaneous abortions (*vs* 13% in IBD controls), a 3% incidence of ectopic pregnancies (compared with none in IBD controls) and finally an abnormal amniocentesis in 2 patients (and none in the IBD controls).

Statistically speaking, no one is yet certain of the risk or the safety of immunosuppressives taken before or during pregnancy and therefore no conclusion should yet be drawn. Logically there must be a compromise solution: (1) Given that the most important issue is active Crohn's disease at conception, if the patient has already been started on the immunosuppressive drug it should be continued and the dose even increased if the clinical severity of the disease warrants it; (2) If the IBD is in remission and has been for months or for years, I find no contraindication to stopping the drug at or before the diagnosis of pregnancy since our experience has shown that any exacerbation is not likely to occur immediately or for that matter even for months, by which time the pregnancy may be ended or at least the fetus is protected through the first trimester when theoretically it would be most susceptible to any danger. Should an exacerbation occur earlier in the pregnancy, the choice may be made to reintroduce the drug; (3) The risk of toxicity to the pregnancy when the father is the one who has the IBD and is taking 6-MP/AZA raises a special consideration. If the male has been in remission, it might be prudent to stop the drug for 1 to 3 mo before conception. Since the timing of the pregnancy is so infrequently controlled, this opportunity does not occur often; and (4) Decisions whether to continue 6-MP/AZA in pregnant women and their husbands who are taking the drug for IBD require rigorous clinical judgement. For example, if the woman has been in remission for a long time, it seems reasonable to stop the drug until delivery since recurrence is very unlikely. If recurrence does develop, then the drug can be restarted at that time. If either the pregnant female patient or the husband with IBD have active Crohn's disease or have been in remission only briefly following a severe attack, I recommend continuing the drug. This is an area where rules should not be rigid[32-36].

THE ROLE OF 6-MP SINCE THE AVAILABILITY OF BIOLOGICALS; WHEN TO ADD ONE TO THE OTHER, WHEN TO TERMINATE ONE OR THE OTHER AND WHICH ONE

Some of the most challenging therapeutic decisions have been raised since the publication of articles suggesting that once a patient with Crohn's disease is in clinical remission while being treated with both infliximab and 6-MP/AZA, there is no advantage to continuing the immunosuppressive drug. These studies do not adequately allow for the duration of treatment with the 6-MP, when it was started in reference to infliximab, or the duration and dose of infliximab required to bring the patient into remission. Furthermore, it does not allow for the conclusions of the Study of Immunomodulator Naïve Patients in Crohn's Disease which demonstrated that the therapeutic efficacy of the combination of infliximab and 6-MP/AZA is greater than either drug alone.

The following are the my suggested options for changing therapy for Crohn's disease and ulcerative colitis in regard to either 6-MP or AZA alone, infliximab or other biological alone, and 6-MP/AZA and a biological together.

No response or beginning failure with 6-MP/AZA alone

Increase the dose if WBC or platelets permit; add a biological; add a 5-ASA product (this is a particularly good opportunity to add a once daily dose product for compliance reasons.); surgery, usually the last resort, but influenced by location and specific complication of Crohn's disease.

No response or failure with a biological

Increase the dose, decrease the interval between infusions or injections; add 6-MP or AZA; add a 5-ASA product; change the biological; Measure serum infliximab and antibody levels for guidance; brief rescue therapy with intravenous corticosteroids; Surgery, usually the last resort, but influenced too by location and specific complication of the Crohn's disease.

Failure with combined therapy of immunosuppressive and biological

Increase the dose of the immunosuppressive if WBC or platelet counts permit; decrease the interval between infusions or injections; add a 5-ASA product; brief rescue therapy with intravenous corticosteroids; measure serum infliximab and antibody levels for guidance; stop biological if degree of immunogenicity is high and accompanied by allergic symptoms such as joint pains; stop 6-MP or

AZA if complications suspected of being attributed to these drugs are evident, such as nausea, malaise, fever, and worsening liver or pancreatic function tests; surgery.

Eliminating 6-MP/AZA after remission with combined therapy of immunosuppressives and biologicals

Complications of drug or disease; reduce the dose - especially for persistent leukopenia; patients' fear of late complications; in some cases of pregnancy or anticipated pregnancy; continuation influenced by earlier severity of the disease.

Eliminating biologicals (when used alone) after remission

Fear of complications of the drug; lack of compliance; now substitute 6-MP/AZA; first extend interval for infusion or injection; first reduce the dose; add a 5-ASA product if not already done.

Eliminating the biological or immunosuppressive after remission with both

To be considered preferably only after 1 full year of maintenance therapy and full dose of both after remission achieved; first reduce dose of the immunosuppressives; eventually eliminate the 6-MP/AZA or the biological; the author's preference is to eliminate the 6-MP/AZA and continue the biological; later reduce the dose of the biological as well.

Once remission of the IBD has been maintained for at least a year, there are many considerations. While some patients do not wish to change the therapeutic program because of its success, others are fearful of complications of either or both drugs and are anxious to eliminate or reduce. In some cases the specific indication for starting the program remains tolerable but not eliminated, in which case I encourage the patient to persist. In other cases where the indication for starting one or both drugs is gone and indeed mucosal healing has been accomplished as well (mucosal healing to me requires histological healing), I undertake a dose reduction.

Despite the efforts made in rationalizing the reduction of 6-MP *vs* infliximab, the subsequent management is currently influenced by the subsequent course of the primary disease rather than patient hardships or drug complications. I have witnessed exacerbations of Crohn's disease and ulcerative colitis following elimination or reduction of both drugs. The course of management is then clear by reinstituting the appropriate drug, preferably to the dose of 6-MP at which the longest remission was maintained or the infliximab at the dose and frequency of infusions in which remission was achieved.

One other option for failure or intolerance to 6-MP or lack of response to Remicade is the substitution of a different biological. My experience is biased by the long period of time with the availability of only the Remicade. With the subsequent introduction of Adalimumab and Certolizumab pegol, I had already learned how to use the inflximab well and had no need to change, or if the infliximab had failed so then did the other biological that I then tried. Furthermore, I don't consider self administration of the biological an advantage since the patient has been known to alter the dose or the frequency for whatever the rationale whenever the guidance of the managing gastroenterologist is reduced or otherwise modified. Rapport between patient and doctor remains an influential factor in successful therapy even though the scientific evidence for this might be lacking[37-39].

BRAND NAME *VS* GENERIC 6-MP AND AZATHIOPRINE

While there are no controlled trials to resolve this issue, I have seen more recurrences of IBD after switching to the generic than when continuing with the brand (Purinethol or Imuran). Therefore, I continue using the brand name when feasible.

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REVIEW

Role of microRNAs in the immune system, inflammation and cancer

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Abstract

MicroRNAs, a key class of gene expression regulators, have emerged as crucial players in various biological processes such as cellular proliferation and differentiation, development and apoptosis. In addition, microRNAs are coming to light as crucial regulators of innate and adaptive immune responses, and their abnormal expression and/or function in the immune system have been linked to multiple human diseases including inflammatory disorders, such as inflammatory bowel disease, and cancers. In this review, we discuss our current understanding of microRNAs with a focus on their role and mode of action in regulating the immune system during inflammation and carcinogenesis.

Key words: MicroRNAs; Immune response; Inflammation; Inflammatory bowel disease; Colorectal cancer

Core tip: MicroRNAs (miRNAs), a key class of gene expression regulators, have emerged as crucial players in various biological processes such as cellular proliferation and differentiation, development and apoptosis. A better understanding of the function of miRNAs is providing new insights into the molecular basis of human pathologies, and new biomarkers for disease diagnosis and therapy.

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INTRODUCTION

MicroRNAs (miRNAs, miR) are small (approximately 20-22 nucleotides), non-coding RNAs that post-transcriptionally regulate gene expression by binding to the 3'-untranslated region of target mRNAs, leading to mRNA degradation or translational inhibition[1].

Since the identification of the first miRNA, *lin-4*, in *Caenorabditis elegans* in 1993[2,3], thousands of miRNA genes have been identified in animal and plant genomes[4]. As a class, miRNAs account for about 1%-2% of genes in worms, flies, and mammals[5]. Each miRNA can target hundreds of mRNAs within a given cell type, and a single mRNA is often the target of multiple miRNAs, and thus over half of the human transcriptome is predicted to be under miRNA regulation, embedding this post-transcriptional control pathway within nearly every biological process[5].

Given its fundamental biological roles, it is not sur-

prising that miRNA expression is tightly controlled and that its deregulation can lead to various diseases. In this review, we summarize our current knowledge about the physiological role of miRNAs in mammalian biology and the manner in which miRNA activities contribute to diseases including inflammatory disorders and cancer.

MIRNA BIOSYNTHESIS AND REGULATION

Biosynthesis

Our knowledge of miRNA biogenesis and regulation has been greatly expanded in recent years[1]. The canonical miRNA biogenesis takes place in a multi-step process and involves two RNAse Ⅲ endonucleases, Dicer and Drosha. MiRNAs are encoded by genomic DNA and are most commonly transcribed by RNA polymerase Ⅱ, which generates a primary miRNA (pri-miRNA) transcript. Within the primary transcripts, miRNAs form stem-loop structures, which contain the mature miRNA as part of an imperfectly paired double-stranded stem connected by a short terminal loop. Pri-miRNAs are then processed by a microprocessor complex, a multiprotein complex with the two core components, Drosha and Di George Syndrome critical region 8 (DGCR8)[6-8]. This results in the formation of a hairpin-shaped RNA molecule of 70-100 bp called miRNA precursor or pre-miRNA, which is then exported into the cytoplasm in a process involving the nucleocytoplasmic shuttle Exportin-5 and in a Ran-GTP-dependent manner[9-11]. In cytoplasm, the pre-miRNA hairpin is cleaved by the endonuclease DICER into an imperfect miRNA:miRNA* duplex of 21-23 nucleotides in length[12]. After separation of the two strands of the duplex, one of the strands (the mature miRNA) is transferred into an Argonote (Ago) protein located in the RNA-induced silencing complex (RISC or miRISC), which is involved in the repression of gene expression by leading miRNAs to specific target mRNAs, whereas the other strand (the star-strand) is degraded. It has been shown that strand selection and RISC assembly in mammals are accomplished by a complex that contains Dicer, Ago and the double-stranded RNA binding protein TRBP[13-15]. MiRNAs target mRNAs by interacting with sites of imperfect complementarity. Short "seed" sequences at the 5'-ends of miRNAs (nucleotides 2-8) are critical, and in some cases fully sufficient, for target selection[16,17].

Regulation

Although there have been recent advances in our knowledge of the biogenesis of the miRNA pathway, relatively little is known about the mechanisms regulating the activity of the pathway's components. Several recent studies indicate that the regulation of miRNA expression and function occurs at three levels: transcription, processing and subcellular localization[17,18].

The first, and one of the most important, mechanisms controlling miRNA abundance is the regulation of pri-miRNA transcription, which could be positively or negatively regulated by different factors such as transcription factors, enhancers, silencers and epigenetic modification in miRNA promoters[16]. For example, the oncogene c-myc can bind to the promoter of the miR-17-5p cluster, thereby up-regulating expression of the miRNAs encoded by the cluster[19,20]. Similarly, the tumour suppressor p53 has been shown to upregulate the transcription of miR-34 family members, inhibiting important factors of cell proliferation and survival, such as Bcl2 and Cdk4 and 6[21-24]. A region of miRNA genes is located within CpG islands involving the epigenetic control of miRNA transcription. It is estimated from recent works that 5%-10% of mammalian miRNAs are epigenetically regulated[19,25-27].

Several post-transcriptional regulatory mechanisms that affect miRNA processing at different stages, from the pri-miRNA transcripts to the delivery of mature miRNAs to their target mRNAs, have recently been investigated[18]. For example, p53 can form a complex with Drosha, which increases the processing of pri-miRNAs to pre-miRNAs[28]. Histone deacetylase I can enhance pri-miRNA processing by deacetylating the protein DGCR8 of the microprocessor complex[29]. Cytokines such as interferons have been shown to inhibit Dicer expression, decreasing the processing of pre-miRNAs[30].

MICRORNAS AND IMMUNE SYSTEM

The immune system has evolved to maintain self-tolerance and to recognize efficiently specific pathogens. The innate immune system acts as a first protector providing an immediate response to pathogens, and propagation of the innate response activates the adaptive immune system. Both innate and adaptive immune responses are highly regulated, and recent studies have shed light on the role of miRNAs in this intricate system[31,32]. The role of miRNAs in immune responses will be discussed in this section.

MicroRNAs and innate immune response

The innate immune system is activated *via* recognition of pathogen-associated molecular patterns by toll-like receptors (TLRs)[33], which will recruit adaptor proteins to the receptor, followed by activation of downstream signalling pathways such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway[34]. This signal transduction ultimately leads to induction of immune gene expression.

The first study examining the effect of lipopolysaccharide (LPS)-mediated activation of TLR signalling on miRNA production identified miR-155, miR-146a and miR-132, which are induced in human macrophages by LPS[35]. Further analysis showed that miR-155 is induced by LPS, cytokine IFN-β and various TLR ligands in murine macrophages[36,37]. MiR-155, once induced, is involved in the activation of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), enzyme linked immunosorbent assay pathway *via* targeting the Fas-associated

death domain protein, I B kinase ε, and receptor (TNF receptor superfamily)-interacting serine-threonine kinase 1[37]. MiR-155 plays a role in the innate immune response by regulating suppressor of cytokine signalling (SOCS)-1, a negative regulator of dendritic cell antigen-presenting capacity[37-39]. Likewise, miR-155-deficient dendritic cells exhibit impaired antigen presentation and therefore are unable to activate T cells to promote inflammation[39]. One study demonstrated that in human myeloid-derived DCs, knockdown of miR-155 expression significantly increased protein expression of the pro-inflammatory cytokine IL-1[40]. The same study also showed that miR-155 directly inhibited expression of the pro-inflammatory signalling protein TAK1-binding protein 2 (TAB2, also known as MAP3K7IP2), which could be a mechanism underlying its anti-inflammatory property[40]. In contrast, other studies have shown that miR-155 can enhance inflammatory responses. Overexpression of miR-155 in mouse bone marrow leads to a myeloproliferative phenotype that is similar to that observed transiently after LPS stimulation[41]. MiR-155 can negatively regulate SHIP1, an important negative regulator of phosphoinositide 3-kinase (PI3K) and the downstream AKT pathway[42,43]. SHIP1, which is similar to SOCS1, is a negative regulator of TLR4 signaling[44], and hence repression of SHIP1 by miR-155 may counter this negative regulation and increase downstream AKT signalling.

Like miR-155, miR-146a is induced by LPS, TNF-α and IL-1β in a NF-κB-dependent manner. MiR-146a in turn inhibits expression of two components of the TLR4 signaling pathway, IL-1 receptor associated kinase and TNF receptor-associated factor-6[35]. Thus, miR-146a functions as a negative feedback regulator of the TLR/NF-κB pathway. MiR-155 and miR-146 expression is increased in macrophages in response to LPS stimulation, while miR-125b expression is decreased. MiR-125b can target TNF-α mRNA, and a decrease in its expression leads to elevated TNF-α production and consequently increased inflammatory response[37].

Macrophage inflammatory response to infection involves the upregulation of several miRNAs, such as miR-21, miR-9 and miR-147[45-47]. These miRNAs can also be induced by TLR signaling, and can negatively regulate activation of inflammatory pathways in myeloid cells. MiR-9 represses NF-κB subunit 1 (NFKB1/p50 unit) and helps to maintain a constant level of NF-κB1 protein expression during TLR4-mediated activation of monocytes and neutrophils[46]. MiR-147 has been shown to attenuate TLR2, TLR3 and TLR4-mediated production of inflammatory proteins such as TNF-α and IL-6[47]. Induction of miR-21 inhibits PDCD4, an IL-10 inhibitor, thereby derepressing IL-10. IL-10 in turn inhibits miR-155, allowing SHIP1 to be derepressed and inhibit TLR signaling[45,48]. Hence, immune responses are highly regulated by TLRs-mediated upregulation of different miRNAs.

In addition to miRNA induction by TLR signaling, recent studies have also reported inflammatory repression, such as miR-155 repression, in response to anti-inflammatory cytokine IL-10[49].

MicroRNAs and adaptive immune response

In addition to their role in regulating the innate immune system, miRNAs have been implicated in adaptive immunity by controlling the development and activation of T and B cells.

T cells

Specific miRNA expression profiles have been reported in different T cell subsets and stages of development[50-52], suggesting that miRNA-mediated regulation of signaling networks in T cells, and probably other immune cells, is dynamic and highly regulated. Interestingly, miRNA profiling in naive, effector and memory $CD8^+$ T cells has revealed that a few highly expressed miRNAs are dynamically regulated during antigen-specific T-cell differentiation[52]. Mice exhibiting T-cell specific deletion of Dicer had lower numbers of mature T cells with abnormally developed T-cell subsets than wild-type mice, indicating that miRNAs are required for T cell development[53,54]. Two specific miRNAs have been implicated in T cell development, and probably account for some of the phenotype of *Dicer*-deficient T cells. The miR-17-92 cluster suppresses expression of pro-apoptotic proteins, including BCL-2-interacting mediators of cell death (BIM or BCL2L11) and phosphatase and tensin homologue. This miRNA cluster is thought to increase T cell survival during development and is expressed during the double negative 2 stage of thymopoiesis[55].

The role of miRNAs in the differentiation of T cells into distinct effector T helper cell subsets has been recently reported. It was demonstrated that miR-326 regulates differentiation of T_H17 cells both *in vitro* and *in vivo*[56]. MiR-155 is implicated in regulatory T (T_{reg}) cell formation and function, since forkhead box P3 (FOXP3), a transcription factor that is required for the development and function of T_{reg} cells, may directly regulate the expression of this miRNA[57]. Furthermore, miRNA-155-deficient mice are immunodeficient, indicating the implication of miR-155 in homeostasis and the immune system[39]. Similarly, using genetic deletion and transgenic approaches, Thai and colleagues showed the important role of miR-155 in the mammalian immune system, specifically in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell-dependent antibody response[58]. Certain miRNAs, such as the miR-17-92 cluster, might be involved in the development and function of T follicular helper cells (specialized T cells that provide selective signals to supporting geminal center B cells), which are essential for long-lived antibody responses[59,60]. In addition, miR-181a, which is increased during early T cell development and down-regulated in mature CD4 T cells such as Th1 and Th2 effector cells, can enhance TCR signaling strength by inhibiting multiple phosphatases that negatively regulate the TCR signaling cascade[61]. Finally, conditional deletion

of *Dicer* or *Drosha* in T_{reg} cells led to lethal autoimmune inflammatory disease, accompanied by impaired development or function of T_{reg} cells, indicating the role of miRNAs in T_{reg} cell biology[62-64].

B cells

Distinct miRNA profiles in naive, germinal central and post-germinal central B cells have been reported[65-67], suggesting the implication of miRNAs in B cell development and maturation. A pioneer study showed that miR-181 is highly expressed in B cells of mouse bone marrow, and its ectopic expression in hematopoietic stem and progenitor cells resulted in an increase in the percentage of B-lineage cells but not in T cells or myeloid cells[68], indicating the role of lineage-specific miRNAs in regulating lymphocyte development. Conditional deletion of *Dicer* in B cells completely arrested B cell development in mice, which is thought to be due to dysregulated expression of the pro-apoptotic protein BIM, probably during the selection of effective antigen receptors[69]. Notably, B cells lacking miR-17-92 family and *Dicer*-deficient B cells exhibited similar gene expression profiles[70], suggesting that this miRNA cluster could play a determining role in the regulation of B cell development.

Recent studies have explored the role of miR-150, a miRNA specifically expressed by mature lymphocytes, in B cell differentiation[51,71,72]. MiR-150 expression increases during B-cell maturation in bone marrow, and its constitutive expression blocked B cell development at the transition from the pro-B-cell to pre-B-cell developmental stage, leading to severe defects in the production of mature B cells[71]. MiR-150-deficient mice exhibited a 2-fold increase in splenic B-1 cell numbers, with a relative decrease in those of B-2 cells, but had no apparent defect in the development of other lymphoid-derived T- and B-cell types[72]. Mice expressing a miR-150 transgene early in life also had dramatically impaired B cell development with normal T cell levels. These defects in miR-150 gain- and loss-of-function were further shown to be due to dysregulation of c-Myb, a target of miR-150 and a transcription factor that controls multiple steps of lymphocyte development[72]. MiR-155-deficient B cells showed defects in antibody class switching and differentiation into plasma cells, resulting in an impaired humoral response to T cell-dependent antigenic stimulation[39,58,73]. The constitutive expression of miR-34a blocked B cell development at the pro-B to pre-B cell transition, leading to a reduction in mature B cells[74]. This block appeared to be mediated by miR-34a-inhibited expression of the transcription factor Foxp1[74], which is an essential regulator of B cell development[75]. Together, these studies show the important role of miRNAs in normal B cell development.

MICRORNAS AND INFLAMMATORY BOWEL DISEASE

As miRNAs play a critical role in the regulation of the immune system, failure of miRNA regulation is associated with several human disorders such as inflammatory bowel disease (IBD) (Table 1), which is a chronic inflammatory gastrointestinal disorder. Although the etiology of IBD remains largely unknown, extensive studies in the last decades have suggested that it involves environmental and genetic factors that lead to dysfunction of the epithelial barrier with consequent deregulation of the mucosal immune system and responses to gut microbiota[76].

Distinguished miRNA expression profiles have been recently described in tissues of patients with active and inactive UC, CD, irritable bowel syndrome (IBS), infectious colitis (IC), and microscopic colitis (MC)[77]. Wu and colleagues demonstrated that active UC was associated

Table 1 MicroRNAs dysregulated in ulcerative colitis and/or Crohn's disease

	Up-regulated	Down-regulated	Source, reference
UC *vs* healthy	miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, let-7f, miR-21, miR-155	miR-192, miR-375, miR-422b	Sigmoid colon[77]
	miR-21, miR-155		Colon[78]
	miR-7, miR-31, miR-135b, miR-223, miR-29a, miR-29b, miR-126*, miR-127-3p, miR324-3p	miR-188-5p, miR-25, miR-320a, miR-346	Colonic mucosa[79]
	(*miR-196a, miR-29a, miR-29b, miR-126*, miR-127-3p, miR324-3p*)[1]	(*miR-188-5p, miR-25, miR-320a, miR-346*)[2]	
	miR-28-5p, miR-151-5p, miR-199a-5p, miR-340*, miRplus-E1271, miR-103-2*, miR-362-3p, miR-532-3p	miR-505*	Peripheral blood[80]
CD *vs* healthy	miR-9, miR-126, miR-130a, miR-181c, miR-375, miR-26a, miR-29b, miR-30b, miR-34c-5p, miR-126*, miR127-3p, miR-133b, miR-155, miR-196a, miR324-3p, miR-21, miR-22, miR-29c, miR-31, miR-106a, miR-146a, miR146b-3p, miR-150 (*miR-9*, miR-30a*, miR-30c, miR-223 miR-25a, miR-29b, miR-30b, miR-34c-5p, miR-126*, miR127-3p, miR-133b, miR-155, miR-196a, miR324-3p, miR-21, miR-22, miR-29c, miR-31, miR-106a, miR-146a, miR146b-3p, miR-150*)[1]		Colonic mucosa[79]
	miR-199p-5a, miR-362-3p, miR-340*, miR-532-3p, miRplus-E1271	miR-149*, miRplus-F1065	Peripheral blood[80]
UC *vs* CD		(*miR-150, miR-196b, miR-199a-3p, miR-199b-5p, miR-223, miR-320a*)[2]	Colonic mucosa[79]
	miR-28-5p, miR-103-2*, miR-149*, miR-151-5p, miR-340*, miR-532-3p, miRplus-E1153	*miR-505**	Peripheral blood[80]

[1]miRNAs upregulated specifically in non-inflamed colonic mucosa; [2]miRNAs downregulated specifically in non-inflamed tissue colonic mucosa. UC: Ulcerative colitis; CD: Crohn's disease.

with the differential expression of 11 miRNAs (3 significantly decreased and 8 significantly increased in UC tissues). MiR-192, the expression of which is decreased in active UC, was predominantly localized to colonic epithelial cells, and targeted macrophage inflammatory peptide (MIP)-2α, a chemokine expressed by epithelial cells[77]. In colonic epithelial cells, TNF-α-induced MIP-2α expression was inhibited by a miR-192 mimic. In contrast, miR-21 is significantly increased in patients with active UC compared to healthy subjects. In inactive UC patients, miR-375 and miR-422 expression was increased, while that of miR-192 was unaltered compared to healthy subjects[77]. Inactive UC showed similar expression levels of miR-375, miR-422b, and miR-23a to IBS and IC tissues. The miRNAs differently expressed in active UC were not dysregulated in MC and CD. This study highlights the specific miRNA expression patterns in active and inactive IBD tissues, and suggests that miRNAs could regulate expression of proteins implicated in the pathogenesis.

Another study showed the upregulated expression of several miRNAs in active UC compared to healthy colonic biopsies, suggesting that upregulation of miRNAs may be responsible for the development of intestinal inflammation in UC[78]. MiR-21 was found among the upregulated miRNAs, which is consistent with the findings of Takagi *et al*[78].

Of interest, Fasseu and colleagues identified restricted subsets of miRNAs abnormally expressed in inactive colonic mucosa of IBD patients[79]. This elegant study identified 14 (in UC) and 23 (in CD) miRNAs with significantly altered expression (> 5-fold increase or < 0.05-fold decrease) in quiescent colonic mucosa compared to healthy control tissues. Eight of the miRNAs (miR-26a, -29a, -29b, -30c, -126*, -127-3p, -196a, -324-3p) were commonly dysregulated in non-inflamed UC and CD. Six miRNAs (miR-196b, -199a-3p, -199b-5p, -320a, -150, -223) displayed significantly distinct dysregulation of expression between non-inflamed UC and CD colonic biopsies. Interestingly, several miRNA genes with dysregulated expression mapped within acknowledged IBD-susceptibility loci. In addition, significant dysregulated expression of four and five miRNAs specific to inflamed UC or CD tissues, respectively, compared to healthy controls was observed[79]. This study sheds light on the role of miRNAs as contributors to IBD susceptibility, in particular their implication in the onset and/or relapse of inflammation from quiescent mucosa of IBD patients.

There have been recent reports of differential miRNA expression profiles in the peripheral blood of IBD patients[80]. Four miRNAs (miR-199a5p, -362-3p, -532-3p and miRplus-E1271) were upregulated and one miRNA (miRplus-F1065) was downregulated in the peripheral blood of patients with active CD, but not inactive CD, compared to healthy controls[80]. Both active and inactive CD patients had increased expression of miR-340 and decreased expression of miR-149 in the blood. Expression of three miRNAs (miR-103-2, 262-3p, 532-3p) was increased in the blood of both active and inactive UC patients. In addition, a subset of 11 miRNAs can distinguish active CD from active UC[80]. This study importantly supports the evidence that distinct peripheral blood miRNA profiles in different circulating immune cell types are associated with IBD.

Efforts have been made to understand the mechanisms underlying the implication of miRNAs in the pathogenesis of IBD. The potential association between single nucleotide polymorphisms (SNPs) in pre-miRNA coding regions and IBD susceptibility has been analyzed. A study in a Japanese cohort of 170 UC patients and 403 healthy controls revealed the association of three SNPs (rs11614913, rs2910164, and rs3746444) in coding regions of pre-miR-196a2, pre-miR-146a and pre-miR-499[81]. Of particular interest, the CD-associated SNP C313T in immunity-related GTPase family, M (*IRGM*) gene caused a loss in binding of miR-196[82] (Figure 1). IRGM plays an important role in the immune system by its involvement in the autophagy process. In addition, miR-196 is overexpressed in the inflamed epithelium of CD patients and downregulates the IRGM protective variant (c.313C) but not the risk-associated allele (c.313T)[82]. Loss of tight regulation of IRGM expression by miR-196 resulted in defects in autophagy-mediated control of intracellular replication of adherent-invasive *Escherichia coli* (AIEC), leading to abnormal persistence of AIEC in host cells (Figure 1). This suggests that the association of IRGM with CD could arise from abnormal miRNA-mediated IRGM regulation, which affects the efficacy of autophagy, thereby contributing a synonymous polymorphism as a likely causal variant.

Intestinal microbiota is increasingly recognized as a risk for, and a causal factor of, IBD. Our recent studies showed that miRNAs are involved in the regulation of host gene expression by gut microbiota[83]. In another study, we showed that miRNAs play a role in determining the unique physiological characteristics of intestinal epithelial cells, such as their differentiation during migration along the crypt/villus axis[84]. In particular, expression of CD98, a transmembrane glycoprotein that regulates integrin signalling, cellular homeostasis and innate immune response in the gut[85], and its function are directly under the control of miRNAs during the differentiation of intestinal epithelial cells[86]. MiRNAs could also be involved in the upregulation of CD98 during intestinal inflammation and IBD[86]. The biological importance of miRNAs in the pathogenesis of IBD is becoming clearer, and targeting miRNAs in the gastrointestinal tract may be a promising approach for future therapeutic opportunities.

MICRORNAS AND COLORECTAL CANCERS

The transformation of a normal epithelium into a cancerous state involves modifications in several genes that are involved in different stages of carcinogenesis such as apoptosis, proliferation, limitless replicative potential of tumor cells, angiogenesis, migration and invasion[87].

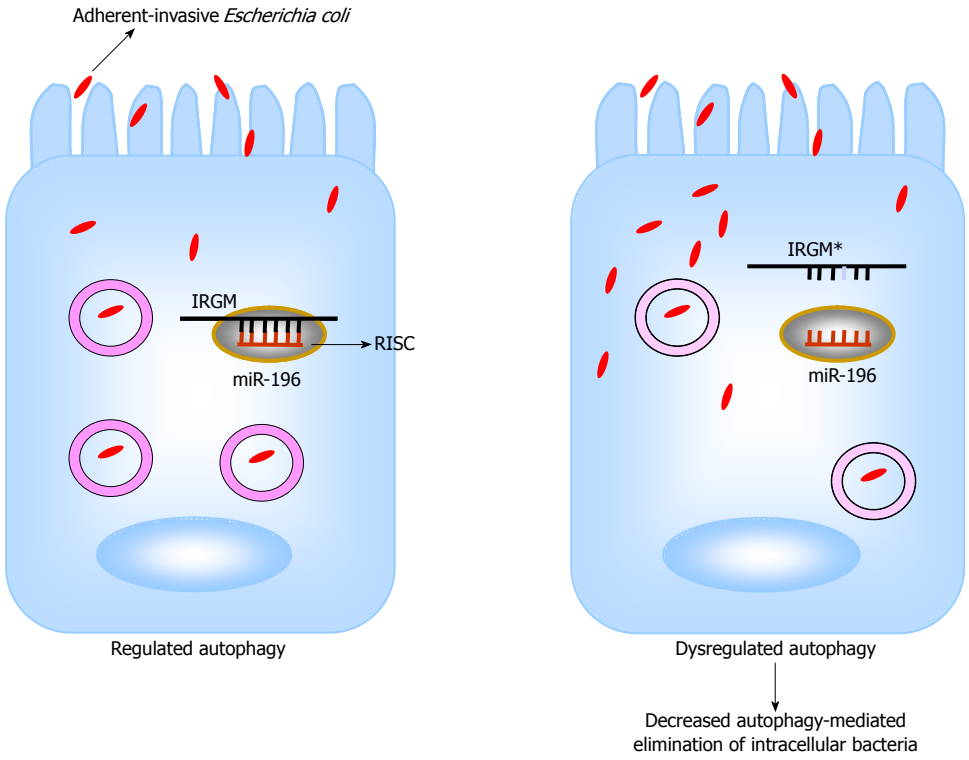

Figure 1 Hypothetical model for the involvement of miR-196 in the pathogenesis of Crohn's disease. MicroRNAs (miRNAs, miR) 196 normally targets immunity-related GTPase family, M (IRGM) mRNA within RNA-induced silencing complex (RISC) for a negative regulation (left panel). The IRGM risk allele (IRGM*) mRNA lacks the binding site for miR-196 and therefore is not regulated by this miRNA (right panel). During Crohn's disease, loss of tight regulation of IRGM* expression by miR-196 may lead to defects in autophagy with most intracellular bacteria replication occurring in dysfunctional vacuoles[82] (dotted cycle). This consequently results in abnormal persistence of pathogens in host cells, which could further worsen disease status[82].

Colorectal cancer (CRC) is one of the most common cancers worldwide. Its incidence is greater in industrial countries than in developing countries[88]. MiRNAs have been shown to play an important role in oncogenesis by regulating the expression of genes involved in cancer initiation, promotion and development[89]. Hundreds of miRNAs mapped to the human genome regions that are known to be altered in cancer, and a similar number of miRNAs are aberrantly expressed in cancerous tissues[90,91]. By analyzing miRNA expression profile (miRNome) of prostate, stomach, pancreas, lung, breast and colon tumors, Volinia and colleagues identified a solid cancer miRNA signature including those with well-characterized cancer association, such as miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155[92]. In particular, 21 miRNAs are up-regulated and 1 is down-regulated in colon tumors compared to normal tissue[92]. MiRNA profiles can identify different tissue and tumor types better than mRNA expression patterns, making them attractive targets for development as cancer biomarkers[93]. Distinguished miRNA profiles can even be found in the serum of patients with cancers. The functions of such circulating miRNAs have not been identified, but profiling of serum miRNAs might be a powerful approach for early cancer diagnosis. The cancer-associated miRNAs may function as oncogenes or tumor suppressors depending on their role in carcinogenesis. Some of the best examples of such miRNAs will be discussed in this section (Figure 2).

Oncogenic miRNAs

MiR-21 is one of the most up-regulated miRNAs in various cancers, including CRC[92,94], and was identified as an independent predictor of overall survival in the validation set containing tumor samples from 113 patients with CRC[95]. It has been shown that miR-21 is involved in invasion, intravasation and metastasis processes by targeting the tumor suppressor PDCD4[96], and in CRC tissues expression of miR-21 is inversely correlated with that of PDCD4 compared to normal tissue[97]. Shibuya and colleagues suggested that miR-21 expression may predict poor prognosis in CRC[98]. Likewise, these authors also examined the prognostic value of miR-155 in CRC since its expression is up-regulated in tumor tissues compared to normal adjacent tissues from CRC patients[98]. MiR-155 was previously shown to target the tumor protein 53-induced nuclear protein 1 (TP53INP1), a pro-apoptotic stress-induced p53 target, and significant reduction or loss of TP53INP1 expression was detected during adenocarcinoma progression[99].

MiR-17-92 and miR-106b-25 clusters are known to be up-regulated in CRC stromal tissues compared with normal stroma[100]. They include, respectively, multiple mature miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b1, miR-92-1[101], and miR-106b, miR-93 and miR-25[102]. These miRNA clusters play an important role

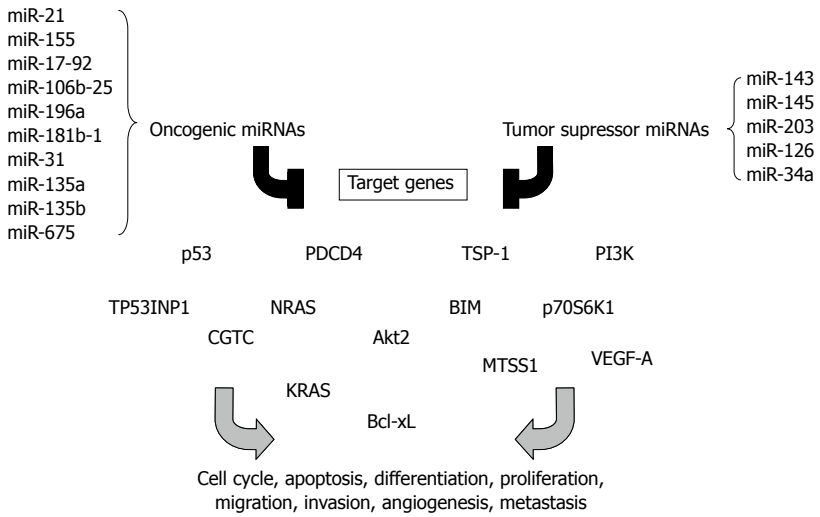

Figure 2 Overview of "oncogenic" and "tumor suppressor" microRNAs related to colorectal cancer described in this review, their targets and different carcinogenesis pathways in which they have been implicated.

during carcinogenesis[92,100,103,104]. An anti-apoptotic effect of miR-17-92 appears to be one of the mechanisms underlying its procarcinogenic role in CRC development and progression[105]. Abrogation of miR-92a leads to cell apoptosis, and there is a correlation between the miR-17-92 overexpression in tumors of CRC patients and the downregulated expression of BIM, a member of the Bcl-2 family that promotes apoptosis[105]. Some works have reported that there is an interconnection between the expression of miR-17-92 cluster and angiogenesis, which occurs later in tumor development and is one of the most important stages in carcinogenesis. Dews and colleagues demonstrated that the anti-angiogenic factors thrombospondin-1 (tsp-1) and connective tissue growth factor (CTGC) are down-regulated by this cluster in intestinal epithelial cells expressing constitutively the oncogene c-myc[106], which was shown to be involved in regulation of miR-17-92 expression[20]. MiR-18 targets tsp-1 and miR-19 modulates the expression of CTGF[107].

Other miRNAs have also been identified as causal factors in colon carcinogenesis. For example, miR-196a had higher expression level in CRC tissues than in normal epithelial tissues[108]. MiR-196a exerts a pro-oncogenic influence in CRC as a high level of its expression promotes the oncogenic phenotype of colorectal cancer cells such as increased cell detachment, migration and invasion[109]. MiR-31 is often up-regulated in CRC and its high expression associated with advanced tumor stage but the clinical significance is unclear[110]. MiR-181b-1, miR-135a, miR-135b, miR-675 are also known to be up-regulated in CRC tumors[111]. MiR-135a is able to promote the growth and invasion of CRC cells by targeting the metastasis suppressor 1[112].

Tumor suppressor miRNAs

Mir-143 and miR-145 are among the best examples of tumor suppressor miRNAs. The expression of these miRNAs is down-regulated in CRC tumors, and in other cancers such as breast, prostate, cervical and lymphoid cancer[113-115]. Many studies have reported that down-regulation of miR-143 and miR-145 correlates with poor prognosis[110,115,116]. The expression and post-transcriptional maturation of these miRNAs were recently shown to be enhanced by the tumor suppressor p53 in response to DNA damages in CRC cell lines[28,117]. In particular, miR-143 is involved in inhibition of oncogene KRAS expression[118]. MiR-145 is reported to inhibit tumor growth and angiogenesis by directly targeting p70S6K1[117], which is activated by mTOR, and its overexpression in cancer cells induces tumor angiogenesis[119-121]. Another study reported that this miRNA is able to inhibit tumor growth and angiogenesis in breast cancer by targeting N-RAS and VEGF-A, which are key players in carcinogenesis[122].

It was recently demonstrated that miR-34a is down-regulated in colon tumors and also in circulating blood[123]. Furthermore, ectopic expression of miR-34a in CRC cell line reduces cell proliferation, demonstrating that this miRNA has a tumor suppressive function in colon carcinogenesis[124]. Several studies conducted in 2007 revealed that miR-34a can target p53, leading to apoptosis and cell cycle arrest[21-24,125]. MiR-203 is identified as another tumor suppressor miRNA. Its low expression was found *in vitro* in CRC cell lines and was correlated with tumor size in CRC. MiR-203 can inhibit proliferation of cancer cell lines[126]. Li *et al*[127] showed that miR-203 overexpression significantly decreased cell proliferation and survival and induced cell apoptosis in the p53-mutated CRC cells. The tumor suppressive role of miR-203 was mediated by negatively regulating Akt2 expression *via* mRNA degradation. In addition, overexpression of miR-203 decreased expression of the anti-apoptotic gene Bcl-xL, leading to a resistance to apoptosis[127]. MiR-126 is specifically expressed in endothelial cells and is known to be down-regulated in CRC compared to normal tissue *via* an unknown mechanism[128]. *In vitro* studies suggested that a loss in negative regulation of p85 subunit of PI3K

by miR-126 could lead to a selective growth advantage during colon carcinogenesis[129].

CONCLUSION

MiRNAs are a class of gene regulators that have recently emerged as key players in the innate and adaptive immune system. Changes in miRNA expression are observed in many human diseases such as inflammatory bowel disease and cancers. Dysregulated miRNA expression profiles in IBD have been reported and could be used as diagnostic biomarkers but further studies are needed to examine the mechanism of their action in the etiopathogenesis of this disease and their clinical utility. Emerging evidence suggests that miRNAs play important roles in the pathogenesis of a limited range of human cancers. Some miRNAs may be directly involved in cancer development by controlling cell differentiation and apoptosis, while others may be involved in cancers by targeting cancer oncogenes and/or tumor suppressors. Given the critical role of miRNAs, current studies are focusing on their association with CRC incidence and prognosis and on the possibility of using circulating miRNAs or fecal miRNA expression as noninvasive early detection biomarkers. These data suggest that miRNAs may be potential molecular classifiers, early detection biomarkers, and therapeutic targets for CRC. Finally, miRNA-based cancer therapy has been limited to targeting a single miRNA[130,131]. However, it has been recently shown that the small molecule enoxacin, a fluoroquinolone used as an antibacterial compound, enhances the miRNA-processing machinery by binding to TRBP[132]. Thus, if most cancers are characterized by a dysregulation of global mature miRNA expression, restoration of the global miRNome may be an attractive approach in cancer therapy. In conclusion, a better understanding of the function of miRNAs is providing new insights into the molecular basis of human pathologies, and new biomarkers for disease diagnosis and therapy.

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REVIEW

Colon cancer stem cells: Controversies and perspectives

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Abstract

Tumors have long been viewed as a population in which all cells have the equal propensity to form new tumors, the so called conventional stochastic model. The cutting-edge theory on tumor origin and progression, tends to consider cancer as a stem cell disease. Stem cells are actively involved in the onset and maintenance of colon cancer. This review is intended to examine the state of the art on colon cancer stem cells (CSCs), with regard to the recent achievements of basic research and to the corresponding translational consequences. Specific prominence is given to the hypothesized origin of CSCs and to the methods for their identification. The growing understanding of CSC biology is driving the optimization of novel anti-cancer targeted drugs.

Key words: Colon cancer stem cells; Colorectal cancer; CD133; Therapy; Chemoresistance

Core tip: According to the "cancer stem cell" (CSC) theory, tumor growth and spread are driven by a minority of cancer cells which exhibit characteristics similar to normal stem cells. Although CSCs have been implicated in colon carcinogenesis, due to the complexity of their biology and unsolved technical issues, an unequivocally approved identification and isolation strategy is still a matter of debate. Several markers have been used to identify colon CSCs but the function of these proteins in CSC biology has not yet been clarified. Moreover, the possibility that CSCs might contribute to the failure of existing chemotherapies to eradicate malignant tumors, indicate that targeting of CSCs may represent a promising strategy to eradicate chemoresistant cancers. Aim of this review was to acquire more information on the biology of human colon CSCs and shed light on the role of this cells in the onset and the maintenance of colon cancer.

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INTRODUCTION

The intestine epithelium is subjected to a rapid and continuous regeneration, supported by crypt intestinal stem cells (ISCs). This feature severely increases the risk for malignant conversion[1]. Indeed, colorectal cancer (CRC) is one of the most common type of neoplasm worldwide, representing the second leading cause of morbidity and mortality from cancer in both Europe and the United States. This means that CRC can also be considered one of the main national emergencies, both in terms of morbidity and in terms of social and economic costs[2].

Studies on CRC pathogenesis have been originally focusing on the clonal selection process, a model of carcinogenesis postulated in 1975[3]. The characterization

of the genetic mechanisms underlying this process has been performed, for the first time, in the early 90s by Bert Vogelstein, who developed the molecular model of CRC progression known as "Vogelstein model". According to it, the CRC develops from epithelial cells lining the gastrointestinal tract, which undergo sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal[3,4]. This pathogeneic model still represents a paradigm of tumor growth and provides a standard rationale to dissect the molecular mechanisms responsible for CRC. Though, current anticancer treatments are often unable, even those based on molecular targeted strategies, to eradicate the disease. Otherwise, the cellular origin of human cancers is still controversial and the mechanisms responsible for the complexity and heterogeneity of tumors remain to be defined[3,4]. In recent years, converging evidence has suggested that human cancer can be considered as a stem cell disease. Therefore, the "stochastic" theory for the cellular origin of cancer, based upon the assumption that all cancer cells are equally malignant and able to give rise to tumors, has been abandoned in favor of the "hierarchical" theory. The latter assumes that: (1) tumors are hierarchically organized; and (2) only a rare subpopulation of undifferentiated cells at the apex of this hierarchy have the unique biological properties necessary for tumor initiation, maintenance and spreading. Given the similarities between tumor-initiating cells and normal stem cells (SC), the tumor-initiating cells have been termed "cancer stem cells" (CSCs)[5]. In its simplicity, this hypothesis suggests that tumorigenesis is an "aberrant organogenesis", supported by a minority of cancer cells, that by consecutive genetic changes, can differentiate to give rise to different phenotypes in the neoplastic population[6].

The hierarchical model implies that within tumors there are cells with different tumorigenic potential: cells that have lost the ability to propagate the tumor and cells that retain their clonogenic ability. Indeed, biologically distinct populations of CSCs have been identified within hematological malignancies[7] and in most solid tumors, including colon cancer[8,9]. The origin of CSCs is still unclear but the discovery of stem cells in the majority of normal tissues, including colon crypts, support the hypothesis that normal SC might represent a possible target for tumorigenic mutations, due to both their long life and their capacity of self-renewal[10].

Cancer stem cells theory has profound translational implications. Current treatments are hardly able to completely eradicate cancer cells, being often complicated by the occurrence of tumor recurrence and/or metastasis and are burdened by toxicity issues. The failure of chemotherapy may at least in part lie in its capacity of targeting the bulk of cancer without affecting stem cells. These can on turn replicate after treatment and, eventually, develop selective features responsible for the occurrence of drug-resistance, which usually characterize and complicate the course of the disease[11-13].

Several studies have suggested that the CSC fraction may be identified within a variety of human cancers, including CRC, by the expression of the CD133 surface marker[8,9,14,15]. CD133 (also known as prominin-1 in rodents or AC133 in humans), a 120 kDa transmembrane and cell surface protein, has been shown to characterize normal and cancer stem cells in several human tissues, including the colonic mucosa. Hence, CD133 has been used to identify and isolate tumor initiating cells from human colon cancers. CD133+ cells are able to maintain themselves as well as to differentiate and re-establish tumor heterogeneity upon serial transplantation *in vivo*[8,9]. However, despite constant research efforts, the molecular mechanisms and signaling pathways that regulate the behavior of CD133-expressing cells remain unknown. Aim of this review was to acquire more information on the biology of human colon CSCs and shed light on the role of CD133+ tumor-initiating cells in the onset and the maintenance of colon cancer.

CANCER STEM CELLS: PROPERTIES

It is widely accepted the concept that tumor is an heterogeneous entity derived from a small subpopulation of undifferentiated cancer cells, the CSCs. CSCs are defined as cells having three unique properties: the capacity of self-renew indefinitely, the ability to recreate the full repertoire of cancer cells of the parent tumor and the expression of a distinctive set of surface biomarkers[16]. It must be emphasized that self-renewal is not synonymous with proliferation. Self-renewal involves the ability of SC populations to precisely maintain their numbers through a combination of symmetric and asymmetric SC division, giving rise to one or both daughter cells identical to the mother and retaining SC properties[17]. In the case of CSCs, mechanisms involved in self-renewal are deregulated and seem to lead to CSC overpopulation. The underlying mechanisms for generating excess of CSC numbers are believed to relate to increases in symmetric CSC division (which produces two CSC daughters) relative to asymmetric CSC division (which produces one CSC daughter and one non-CSC daughter). Many authors have documented this concept quantitatively, using mathematical modeling[17] or fluorescent dye assay[18]. These authors have showed that only increased symmetric division of CSCs could account both for the biologic observation that there is an exponential increase in CSC populations in tumoral tissues and for the known long lag phase, which is typical in the development of many cancers, including colorectal cancer[17]. Although CSCs have the capacity for self-renewal, they are relatively quiescent; that is, they have proliferative capacity but are often not cycling. Indeed, they have been shown to have significantly longer cell cycle times than proliferating non-SCs. This is presumably due to the arrest of SCs at a G0-like cell cycle phase or checkpoint[19].

Another property of CSCs is their potential for multilineage differentiation. This is consistent with the concept that CSCs, like normal SCs, give rise to a hierarchical

organization of cell populations that underlie organogenesis[20].

CSCs also display alterations of DNA repair, due to the presence of cytoprotective properties (including telomerase activation and high expression of anti-apoptotic factors), and a relatively low proliferative potential. In addition, they express high levels of proteins belonging to the ABC membrane transporters family, involved in chemotherapeutic resistance (*i.e.*, to paclitaxel, cisplatin, 5-fluoruracilee, mitoxantrone, methotrexate, anthracyclines, etoposide, vinca alkaloids, camptothecins, topotecan, imatinib)[20]. These unique properties of CSCs would explain the failure of many antitumor therapies, which affect rapidly dividing cells, determining only a reduction of tumor cells number, while CSCs divide slowly and are not sensitive to the cytotoxic drugs. New insight in the molecular mechanisms that underlie these processes were obtained by studying the intracellular regulator of gene expression[21].

On this regard, Bitarte and colleagues showed that the micro RNA miR-451 was downregulated in colonspheres, obtained from different colon carcinoma cells, versus parental cells. The expression of miR-451 caused a decrease in self-renewal, tumorigenicity, and chemoresistance to irinotecan of colonspheres. Authors demonstrated that miR-451 downregulation allows the expression of the target gene macrophage migration inhibitory factor, which induces cyclooxygenase-2 (COX-2) expression. In turn, COX-2 allows Wnt activation, which is essential for CSC growth. Furthermore, miR-451 restoration decreased expression of the ATP-binding cassette drug transporter ABCB1 and resulted in irinotecan sensitization. These findings correlated well with the lower expression of miR-451 observed in patients who did not respond to irinotecan-based first-line therapy compared with patients who did[22]. Moreover, various signaling pathways have already been identified and described in CSCs. It is known that standard pathways for self-renewal of normal stem cells, such as Wnt, Notch and Hedgehog signaling, are also present in CSCs and have an important role in their function. Targeting critical steps in those pathways, however, will be complicated by intense cross-talks as well as main safety issues related to the pleiotropic effects of these signaling molecules[23]. Nevertheless, there are already several reports indicating that CSCs can be selectively targeted without damaging normal stem cells[24]. These and other findings could reasonably pave the way to the development of novel, more efficient and less toxic anti-cancer medications.

CANCER STEM CELLS: DEFINITION

In operational terms, CSCs can only be defined experimentally on the basis of their ability to regenerate continuously the tumor. The implementation of this approach, explains the use of alternative terms in the literature. For example, the term "tumor-initiating cells" is frequently used to describe putative CSCs. However, both these terms (cancer stem cell and tumor-initiating cells) can cause confusion about the cell type to which they relate[16]. In fact, the term CSCs might suggest that these cells arise from normal stem cells, which have acquired a number of genetic mutations sufficient to induce malignant transformation. This assumption is probably true in many cancers, but may not be the case of all tumors. It is plausible, indeed, that in some tumors, a number of differentiated cells can acquire the capacity for self-renewal, through multiple mutagenic events, and thus "reacquire" stem-like properties[25].

On the other hand, the term "tumor-initiating cells", according to experimental evidence, refers to the ability of these cells to initiate tumors when transplanted in a xenograft model. In this case, it could be incorrectly inferred that the cell that gives rise to the xenograft tumor is the same cell in which the first oncogenic mutation occurred. This is unlikely, since it is clear that the cells that drive aberrant growth at one precise moment may differ from those acting during different stages in tumor evolution or during metastasis. Furthermore, both genetic and epigenetic instability can induce cellular heterogeneity within the stem and non-stem cell populations of the tumor[26]. It has been argued that species differences alone might account for the selective growth of subpopulations of cells in xenotransplantations. Indeed, the great majority of cells in a mouse lymphoma were shown recently to possess tumor initiating capacity when allografted into syngenic mice[27].

WHICH IS THE ORIGIN OF COLON CANCER STEM CELLS?

Although, as mentioned earlier, the sequence of events in CRC has been intensively studied, the cell of origin for cancer formation is still poorly known. Two possible hypotheses have been suggested: the so called "bottom-up" and the "top-down" theories. The first proposes that an ISC, either a progenitor or a differentiated cell, is the first transformed cell, as a consequence of anomalous differentiation, giving rise directly to cancer cells or reprogramming itself, acquiring SC behavior before inducing cancer[28-30] (Figure 1).

ISCs represents the ideal target for neoplastic transformation, due to their extended life span that alter their behavior as a result of genetic and epigenetic changes. Also, similar signalling pathways may regulate self-renewal in stem cells and cancer cell, so that the transformation of an SC would require fewer mutations compared to a progenitor cell[31,32]. Conversely, histological evidence suggested that colon cancer might arise from late progenitors or even an early differentiated cell[33], sustaining the "top-down model" of CRC development. By contrast, genetic observation recently support strongly the bottom-up theory. Indeed, the identification of specific genes expressed in the stem cells of the intestine has recently allowed to show that the cell of origin of adenomas, induced by a constitutively active Wnt signaling, is the stem

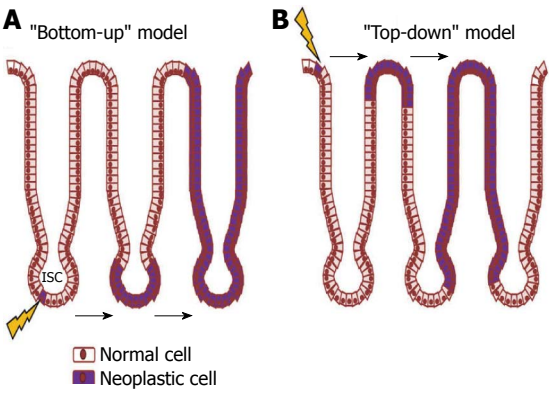

Figure 1 Schematic presentation of two possible ways of colon adeocarcinoma formation. A: "Bottom-up" theory: intestinal stem cells (ISCs, arrow) at the base of the crypt, within the stem cell zone, are the cell of origin of neoplasia as a consequence of anomalous differentiation; B: "Top-down" theory: a progenitor or differentiated cell is the first transformed cell that can acquire stem cells (arrow) behavior before inducing cancer.

Table 1 Summary of characteristics and controversies about colon cancer stem cells

Origin	"Bottom-up" theory[30-32,34,35]
	"Top-down" theory[33]
	CSCs can derive from epithelial non-stem cells that re-express stem cell markers[36]
	CSCs can derive from bone marrow cells[37-43]
Identification assays	Serial transplantation in immune-compromised mice[8,9,27]
	Expression of cell surface marker CD133[5,8,9,14,31,44-46,48,49]
	Side Population[52-61]
Therapeutic strategies	Induction of CSC differentiation by salinomycin or BMP4[72,73]
	Monoclonal antibodies directed against cell surface molecules, such as CD133, CD44, EGFR (cetuximab) and VEGF (bevacizumab)[74,76-79]
	Blockage of self-renewal pathways, including Wnt, Notch, PTEN, Hedgehog , EMT and IL-4 pathway by microRNA or selected small-molecule antagonists[65-70,80-86]
	Target the Warburg effect[87,88]

CSC: Colon cancer stem cell; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; EMT: Epithelial-to-mesenchymal transition.

cell of the small intestine[30,34,35]. This studies have demonstrated that ISC-specific deletion of both functional adenomatous polyposis coli (APC) alleles using Bmi1-, CD133- and (leucine-rich-repeat containing G-protein-coupled receptor 5) Lgr5- CRE recombinase mice leads to efficient tumor formation. Interestingly, Barker and colleagues also showed that tumor formation does not occur when APC is deleted in progenitors or differentiated cells[30]. These results show that the cell giving rise to adenomas in the small intestine is the stem cell. It is still pending, though, whether BMI-, LGR5-expressing cells or CD133-expressing cells of the tumor, are able alone to induce tumor progression and therefore are markers of the intestinal CSC.

Recently, Schwitalla *et al*[36] have suggested that these models do not exclude each other and that tumor-initiating mutations can occur in both Lgr5+ crypt stem cells or in more differentiated Lgr5-cells, as long as these initially negative cells dedifferentiate and re-express Lgr5. Indeed, the authors have demonstrated, in a genetic model of intestinal tumor initiation, that epithelial non-stem cells can re-express stem cell markers and be converted into tumor-initiating cells. This phenomenon is strictly dependent on the degree of Wnt activation and can only be observed when Wnt signaling is markedly elevated[37].

It has also been discussed that even cells from outside the tumor, for example, bone marrow-derived cells, might also serve as CSC's ancestors. This phenomenon has been firstly demonstrated in a murine model of gastric cancer induced by Helicobacter pylori, in which SC derived from bone marrow were able to generate the tumor[37,38].

Emerging evidence suggests that bone-marrow-derived mesenchymal stem cells (BM-MSCs) contribute to tissue regeneration in the colon partly by promoting neovascularization or arteriogenesis[39,40]. Although tumor stromal fibroblasts are mainly recruited from local tissue fibroblasts, it has been proposed that BM-MSCs are recruited into the stroma of developing tumors[41-43]. Several studies have demonstrated that BM-MSCs can selectively migrate to sites of mucosal damage and wound healing including colorectal cancers, where a number of tumor-related inflammatory reactions and abnormal tissue regeneration phenomena take place actively. It also has been shown that cancer cells release specific factors that induce BM-MSC mobilization and recruitment to the tumor stroma where they eventually contribute to the formation of a tumor-supportive microenvironment[44] (Table 1).

IDENTIFICATION OF COLON CANCER STEM CELLS: LIMITS AND CONTROVERSIES

The correct identification and isolation of the cells responsible for tumor formation is always challenging in cancer research. Although CSCs have been implicated in colon carcinogenesis, due to the complexity of their biology and unsolved technical issues, an unequivocally approved identification and isolation strategy is still a matter of debate[32]. The gold standard for identifying a CSC is the capacity to propagate tumors as xenografts in immuno-compromised mice. However, it has been argued that species differences alone might account for the selective growth of subpopulations of cells in these assays. Indeed, the great majority of cells in a mouse lymphoma were shown recently to possess tumor initiating capacity when allografted into syngenic mice[27]. Moreover, serial transplantation experiments with animal models are laborious and time-consuming, hence the need to develop reliable surrogate assays.

Several *in vitro* assays have been used to identify CSCs can derive, including sphere assays, surface cell markers and the Hoechst dye efflux properties, which identify the

so-called Side-Population (SP). Studies have also been performed to define putative CSC genetic signatures. However, each of these methods has potential pitfalls that complicate the interpretation of results[25].

It is clearly not sufficient to define a stem cell based only on surface markers. Moreover, none of the markers used to isolate stem cells in various normal and cancerous tissues is expressed exclusively by the stem cell fraction. Indeed most markers used for colon CSC isolation are chosen either because they are expressed in normal stem cells or as they were found to identify CSCs in other malignancies, either hematological or solid. The disadvantage of choosing markers in this fashion is that the functional effect of expression of the marker in CSCs is usually unknown.

For instance, focusing on CRC, several studies have suggested that the CSC fraction within colon cancer might be identified by the expression of the cell surface marker CD133[8,9]. CD133 is a trans-membrane glycoprotein, expressed by normal progenitors belonging to neuronal, hematopoietic, epithelial and endothelial lineages. In the last years, CD133 has become the "molecule of the moment", being recognized as a putative CSC marker for many human solid tumors, including liver, pancreas and colon neoplasms[14,45]. However, despite constant research efforts, the molecular mechanisms and signaling pathways that regulate the behavior of CD133-expressing CSC, remain unknown.

In particular, we demonstrated the existence of a population of self renewing cells expressing CD133 within primary and metastatic human CRC[5]. This antigen was expressed in significantly higher percentage in CRC samples, compared to the respective normal tissues. CD133-positive cells were also found in liver metastases (up to 10%), while they were hardly detectable in the healthy liver tissue[5]. In addition, CD133+ cells, isolated from different human colonic adenocarcinoma lines (CaCo-2, HT29, LoVo), were highly clonogenic *in vitro* and gave rise to tumors following transplantation in mice. Conversely, the CD133-negative fraction of all cell lines had a lower clonogenic potential in soft agar assays and did not generate tumors in secondary recipients[45], confirming the tumor initiating properties of CD133+ CSC. Interestingly, we also provided the original demonstration that modulation of CD133 expression in the CaCo-2 colon cancer cell line was associated with corresponding variations in the expression levels of both Endothelin-1 and nuclear receptor subfamily 4, group A, member 2[46], both known to play an important role in the proliferation and metastasis processes. This modulation was associated with a significant inhibition of the cells' clonogenic and migration ability, thus further confirming a role of the CD133 molecule in the definition of the CSC phenotype[46].

There are though still some controversies on the role of CD133 as a CSC marker in CRC; the opposing theories emerge from the evidence that most CD133 antibodies target glycosylation-dependent epitopes[35], whose presence is related to the differentiation stage of the cell. Experimental data from colon and glioblastoma cells suggested that the differential glycosylation of specific epitopes may mask the presence of CD133 on cells previously characterized as negative[47,48]. Moreover, CD133 has been found to be expressed by the full spectrum of undifferentiated and differentiated colonic epithelial cells, both in humans and in mice[49]. Shmelkov *et al*[49] have demonstrated that primary and metastatic colon cancers contain CD133+ and CD133- parenchymal tumor cells, and both types of cells are capable of tumor initiation, as observed in a xenotransplantation model. A similar lack of specificity has been also observed for other potential CSC markers of CRC, such as CD44, CD166, CD29, CD24, Lgr5, and nuclear beta-catenin[50]. In fact, the vast majority of cells that express these markers are not stem cells[51].

Another approach to identify CSCs is their presence within the so-called "Side Population". SP cells have been first described within the hematopoietic system. In particular, bone marrow stem cells contain a subpopulation that extrude the DNA-binding dye, Hoechst 33342, out of the cell membrane. Comparing the fluorescence intensity on the wavelength of blue against the red, the SP appears as a tail of cells with low fluorescence. This phenotype is attributed to the activity of the ABC membrane transporter proteins that can confer drug resistance to stem cells. These proteins can be blocked by inhibitors of efflux pumps, such as verapamil[52-54]. The SP fractions have been identified in various human tissues[55,56], cancer specimens[57,58] and tumor cell lines[59,60] and it has been suggested that they may represent the true stem cell population. However, as with cell surface markers, the SP phenotype is not synonymous with stemness. Indeed, a recent article claimed that both SP and non-SP cells, isolated from gastrointestinal cancer cell lines, displayed similar clonogenic and tumourigenic potential *in vitro* and *in vivo*, and showed identical expression of putative stem cell markers. Therefore, the Authors concluded that the SP does not enrich for stem cells in gastrointestinal cell lines[61]. Also, possible toxicity of the dye, in cells not capable of extrusion, should also be considered as a caveat to interpreting functional assays of SP cells.

Without a better understanding of normal tissue stem cells and their susceptibility to neoplastic transformation, it will be difficult to conduct conclusive studies of the existence and origins of CSCs (Table 1).

WHICH STRATEGY TO TARGET COLON CSCS?

According to the CSC model, the few self-renewing CSCs that mediate tumor growth are difficult to kill and their persistence might explain tumor recurrence after therapy[62]. Indeed, chemotherapeutics interfere with the ability of rapidly growing cells to divide, so CSCs may be spared, leading to tumor recurrence and metastasis[11-13]. Therefore, to assess the efficacy of therapeutics, it is

necessary to accurately distinguish tumorigenic from non-tumorigenic cancer cells and to understand which progression model occurs in the tumor. Unfortunately, the complex network of mechanisms that regulate SC renewal and carcinogenesis is not clear.

Chemotherapeutic resistance is exerted either through a shift from active state to quiescence or through a wide spectrum of protective mechanisms that characterize CSC biology; these include altered DNA damage repair, altered cell-cycle checkpoint control, malfunction of apoptosis, expression of drug transporters and detoxifying enzymes, and a high expression of proteins belonging to the ABC membrane transporters famil[63,64].

Moreover, the plasticity of CSCs and the epithelial-to-mesenchymal transition (EMT) complicate therapeutic approaches because participate in the acquisition of both *de novo* and acquired drug resistance[65]. Indeed, EMT can trigger reversion to a CSC-like phenotype, providing an association between EMT, CSCs and drug resistance[66]. Several key signaling pathways contribute to this process, such as transforming growth factor β and Wnt, that are well known to induce EMT and promote stem cell maintenance[67,68]. Recent studies have implicated microRNA functionality in these processes; indeed the dysregulation of microRNA expression is likely to be a major contributing factor in the etiology of some cancers[69,70].

It is therefore essential to renovate the therapeutic repertoire by designing new treatments that specifically target CSCs and, at the same time, also eliminate the non-CSC population, intervening in the process of EMT[65,71]. Novel therapeutic strategy based on targeting EMT pathways and CSC maintenance might be a promising tool for CRC defeat.

Moreover, CSCs can be functionally antagonized by inducing their differentiation. Differentiation therapy forces cells to shift into a mature phenotype, lose their self-renewal abilities, and therefore become vulnerable to conventional treatment. For instance, salinomycin, a highly selective potassium ionophore, was recently described as the first compound that can selectively eradicate the tumor through induction of terminal epithelial differentiation of CSCs[72]. Gupta *et al*[72] revealed that salinomycin decreases the proportion of $CD44^{high}/CD24^{low}$ breast cancer cells, whereas paclitaxel has opposing effects. Importantly, cells exposed to salinomycin were less capable of inducing tumors following injection into mice; salinomycin also slowed the growth of the animals' tumors through unknown mechanisms[71]. Salinomycin is thought to inhibit potassium-positive channel-regulated migration and interfere with EMT and metastasis[72]. Also induction of differentiation in colon CSCs by exposing these cells to Bone Morphogenetic Protein 4 (BMP4), which can initiate a differentiation program as well as mediate apoptosis, sensitizes CRC cells to the effects of 5-Fluorouracil (5-FU) or oxaliplatin *in vivo*, resulting in complete and long term regression of colon xenografts[73].

The potential toxic effect, that might occur from the impact on normal SCs, can be minimized by targeting molecules or pathways that are preferentially active in CSCs[73]. Monoclonal antibodies could be directed against cell surface molecules, such as CD133, CD44, or even drug transporters, resulting in reduction of tumor size, metastatic potential, and resistance to chemoradiotherap[74,75].

Advances in high-throughput technologies and bioinformatics will allow developing additional compounds targeting CSC signaling pathways. Currently there are two established targets for such therapies: epidermal growth factor receptor (EGFR), which belongs to the ErbB family of tyrosine kinase receptors and is abnormally activated in many tumors[76], and vascular endothelial growth factor (VEGF), which is known to promote formation of new vessels by inducing growth and differentiation of endothelial cells[77,78]. Several clinical trials have demonstrated that introduction of targeted therapies with monoclonal antibodies against EGFR (cetuximab) and VEGF (bevacizumab) in addition to 5-FU, resulted in a significant survival increase in patients with advanced disease[79].

Another rational target includes blockage of various self-renewal pathways, including Wnt, Notch, PTEN, and Hedgehog[80]. Small-molecules that inhibit the Wnt pathway and γ-secretases that inhibit the Notch pathway have been recently identified as novel approaches to CRC[73]. The Wnt/β-catenin pathway has been implicated in the maintenance of the intestinal crypt stem cell phenotype and Wnt signaling dysregulation through either loss of APC function or oncogenic β-catenin mutations has been shown to cause the majority of sporadic cancer cases[81]. Disruption of Tcf/β-catenin complexes by selected small-molecule antagonists has been shown to antagonize cellular effects of β-catenin and to result in inhibition of cellular proliferation in colon cancer cells[82]. Similarly, the Notch signaling pathway has been reported to be overexpressed in colon CSCs, where it was found to play a role in colon CSC viability, tumorigenicity, and self-renewal[83-87]. Van Es *et al*[87] have demonstrated that blocking the Notch cascade with a gamma-secretase inhibitor induced goblet cell differentiation in adenomas in mice carrying a mutation of the APC tumor suppressor gene and subsequent tumor growth arrest. Moreover, Hoey *et al*[88] have demonstrated that by inhibiting delta-like 4 ligand (DLL4), an important component of the Notch pathway, with human monoclonal antibody 21M18 in colon carcinoma xenografts, the tumor growth as well as the CSC frequency, was decreased compared to control. Interestingly, even though treatment of the xenografts with irinotecan, a chemotherapeutic often used in colon cancer, slowed down tumor growth, and the clonogenicity was increased. Combination treatment of irinotecan with anti-hDLL4 reduced again the tumor growth and stem cell frequency, at even higher levels than the anti-DLL4 treatment alone. This indicates that inhibiting Notch signaling reduces CSC frequencies and sensitizes tumor cells for irinotecan treatment.

It has recently been observed that the inhibition of

the interleukin (IL)-4 pathway with an anti-IL-4 antibody or an IL-4 receptor antagonist in CD133+ colorectal CSCs augmented the antitumor effects of conventional chemotherapeutics[89-90]. Indeed, colon carcinomas produce IL-4 that functions in an autocrine manner, promoting antiapoptotic pathways in these tumors. Inhibiting IL-4 by blocking antibodies sensitizes the cells for killing by 5-FU and oxaliplatin IL-4[89-90].

Reversing chemoresistance and radioresistance represents a promising proposal. This can be achieved through interference with a plethora of cellular components, including inactivation of drug transporters and DNA checkpoint kinases, depletion of reactive oxygen species scavengers, and inhibition of signal transduction pathways.

Interestingly, little is as of yet known with regard to the metabolism of CSC population, leaving an exciting avenue unstudied in the dawn of the emerging field of metabolomics. The Warburg effect, the premise of which is that cancer cells restrict use of fatty-acid oxidation in favor of glycolysis as an ATP energy source, can also be harnessed to create novel broad-spectrum anticancer agents[91-94]. A recent study by Akao *et al*[95] provided initial evidence of metabolic changes in therapy-resistant cell populations by demonstrating significant overexpression of a metabolic "master-regulator" Sirt1 in DLD-1 5-FU-resistant cells.

Altogether, these data illustrate the therapeutic utility of the cancer stem cell concept, which, by enabling specific examination of more aggressive cancer-initiating and cancer propagating subpopulations, provides the tools for discovery of novel mechanisms of cancer therapeutic resistance (Table 1).

CONCLUSION

Understanding the details of CSCs' biology is a primary goal in basic oncology research but would also pave the way for a better clarification of CRC progression. The translation implication of such information is clearly deducible. In particular, the combination of previously known and new markers defining CSC specificity, could lead to the development of a better oriented anticancer therapy, possibly targeting CSCs.

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ORIGINAL ARTICLE

A silybin-phospholipids complex counteracts rat fatty liver degeneration and mitochondrial oxidative changes

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Abstract

AIM: To investigate the effectiveness of antioxidant compounds in modulating mitochondrial oxidative alterations and lipids accumulation in fatty hepatocytes.

METHODS: Silybin-phospholipid complex containing vitamin E (Realsil®) was daily administered by gavage (one pouch diluted in 3 mL of water and containing 15 mg vitamin E and 47 mg silybin complexed with phospholipids) to rats fed a choline-deprived (CD) or a high fat diet [20% fat, containing 71% total calories as fat, 11% as carbohydrate, and 18% as protein, high fat diet (HFD)] for 30 d and 60 d, respectively. The control group was fed a normal semi-purified diet containing adequate levels of choline (35% total calories as fat, 47% as carbohydrate, and 18% as protein). Circulating and hepatic redox active and nitrogen regulating molecules (thioredoxin, glutathione, glutathione peroxidase), NO metabolites (nitrosothiols, nitrotyrosine), lipid peroxides [malondialdehyde-thiobarbituric (MDA-TBA)], and pro-inflammatory keratins (K-18) were measured on days 0, 7, 14, 30, and 60. Mitochondrial respiratory chain proteins and the extent of hepatic fatty infiltration were evaluated.

RESULTS: Both diet regimens produced liver steatosis (50% and 25% of liver slices with CD and HFD, respectively) with no signs of necro-inflammation: fat infiltration ranged from large droplets at day 14 to disseminated and confluent vacuoles resulting in microvesicular steatosis at day 30 (CD) and day 60 (HFD). In plasma, thioredoxin and nitrosothiols were not significantly changed, while MDA-TBA, nitrotyrosine (from 6 ± 1 nmol/L to 14 ± 3 nmol/L day 30 CD, $P < 0.001$, and 12 ± 2 nmol/L day 60 HFD, $P < 0.001$), and K-18 (from 198 ± 20 to 289 ± 21 U/L day 30 CD, $P < 0.001$, and 242 ± 23 U/L day 60 HFD, $P < 0.001$) levels increased significantly with ongoing steatosis. In the liver, glutathione was decreased (from 34.0 ± 1.3 to 25.3 ± 1.2 nmol/mg prot day 30 CD, $P < 0.001$, and 22.4 ± 2.4 nmol/mg prot day 60 HFD, $P < 0.001$), while thioredoxin and glutathione peroxidase were initially increased and then decreased. Nitrosothiols were constantly increased. MDA-TBA levels were five-fold increased from 9.1 ± 1.2 nmol/g to 75.6 ± 5.4 nmol/g on day 30, $P < 0.001$ (CD) and doubled with HFD on day 60. Realsil

administration significantly lowered the extent of fat infiltration, maintained liver glutathione levels during the first half period, and halved its decrease during the second half. Also, Realsil modulated thioredoxin changes and the production of NO derivatives and significantly lowered MDA-TBA levels both in liver (from 73.6 ± 5.4 to 57.2 ± 6.3 nmol/g day 30 CD, $P < 0.01$ and from 27.3 ± 2.1 nmol/g to 20.5 ± 2.2 nmol/g day 60 HFD, $P < 0.01$) and in plasma. Changes in mitochondrial respiratory complexes were also attenuated by Realsil in HFD rats with a major protective effect on Complex Ⅱ subunit CII-30.

CONCLUSION: Realsil administration effectively contrasts hepatocyte fat deposition, NO derivatives formation, and mitochondrial alterations, allowing the liver to maintain a better glutathione and thioredoxin antioxidant activity.

Key words: Fatty liver; Glutathione; Lipid peroxidation; Nitrosothiols; Nitrotyrosine; Thioredoxin

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INTRODUCTION

Simple steatosis of the liver without inflammation is considered a rather benign condition, although it represents a favoring substrate for the potentially damaging effects of a second hit (*i.e.*, ischemia-reperfusion, starvation)[1]. However, severe fatty degeneration represents a leading factor of hepatocyte dysfunction (mitochondrial respiration, microsomal metabolism, biliary secretion)[2] and is associated with excess delivery of nitrosative and oxidative stress molecules[3], thus potentially rendering the liver a major source of systemic alterations in patients with metabolic syndrome[4]. Also, while several adaptive metabolic mechanisms have been described during the early phase of fatty infiltration[1,5] including expression of intracellular sensors and signaling molecules for lipid metabolism and oxidative stress pathways[6,7], the threshold above which fat infiltration becomes dangerous is not clear, so far. Indeed, it has been observed that transient hepatocellular triglycerides accumulation is essential for normal liver regeneration[8] and represents a mechanism of liver protection from lipotoxicity. Buffering free fatty acids might, therefore, prevent the formation of liver steatosis[9]. By contrast, several observations suggest that ongoing fatty degeneration indeed exposes hepatocytes to higher risk of oxidative damages[10].

Experimental rat models of liver steatosis are characterized by accumulation of triglycerides, decreased mitochondrial function, and increased activity of microsomal enzymes[2]. The altered functions of these subcellular organelles favor the enhanced production of reactive oxygen species (ROS) and NO derivatives with consequent morphological and functional modifications of crucial structures, thus rendering fatty hepatocytes particularly susceptible to additional injuring factors[3].

A number of natural or chemical compounds are able to counteract the damages induced by oxidative and nitrosative stress and therefore are claimed to have antioxidant properties. Vitamin E efforts a remarkable protection against lipid oxidation[11] and, if vehicolated within a phospholipid complex together with silybin, a *silybum marianum* extract, protects against pro-fibrotic oxidative injury[12]. Less is known about the effectiveness of such a compound to block or modulate ROS/NO production and their pro-oxidant effects. Also, it would be of interest to know if the administration of such an antioxidant complex may contribute to break off the intracellular mechanisms leading to a progressive accumulation of neutral lipids in fatty hepatocytes and in particular, mitochondrial dysfunction.

Therefore, this study aimed to evaluate the effect of a silybin-phospholipid complex containing vitamin E (Realsil®) on hepatocyte fatty degeneration and nitrosative/oxidative stress in two different rat dietary models [choline deficiency (CD) and high fat diet (HFD)] of fatty liver. Both diets induce fatty degeneration without major inflammation and fibrosis, representing therefore ideal models for assessing changes associated with simple steatosis without the metabolic consequences depending on inflammation. The results of this study contribute to clarify both pathophysiologic mechanisms of damage in fatty hepatocytes and of pharmacological protection.

MATERIALS AND METHODS

Male Wistar rats (b.w. 250-270 g, Harlan, S. Pietro al Natisone, Italy) were kept under controlled conditions of temperature and humidity and on a 12 h dark/light cycle. Animals were divided into five groups: rats fed a CD diet (Dyets, Bethlem, PA); rats fed a CD diet plus daily administration of Realsil by gavage (one pouch diluted in 3 mL of water and containing 15 mg vitamin E and 47 mg silybin complexed with phospholipids); rats fed a HFD (20% fat content), containing 71% total calories as fat, 11% as carbohydrate, and 18% as protein (Altromin Rieper, Vandoies, Italy); rats fed a HFD supplemented by daily administration of Realsil by gavage; control group fed a normal semi-purified diet containing adequate levels of choline, 35% total calories as fat, 47% as carbohydrate, and 18% as protein. The control diet, CD, and HFD were nutritionally adequate, calorically equivalent (1 kcal/mL), and contained equal amounts of fat as olive and safflower oil with excess corn oil added to the HFD.

Following an overnight fast, five rats per group were

Table 1 Characteristics and fat infiltration of rats fed a choline-deficient diet or a high fat diet with or without Realsil or a normal choline-supplemented diet (controls)

Characteristics	Controls day 30	CD day 30	CD + Realsil day 30	HFD day 30	HFD + Realsil day 30	Controls day 60	HFD day 60	HFD + Realsil day 30
Body weight (g)	305 ± 8	311 ± 12	304 ± 11	302 ± 10	308 ± 12	355 ± 11	364 ± 13	351 ± 12
Liver weight (g)	12.7 ± 0.5	19.7 ± 1.3[a]	16.7 ± 0.7[a]	15.7 ± 1.7[a]	13.1 ± 1.3	12.7 ± 0.5	18.8 ± 1.1[a]	15.1 ± 0.6[a,c]
ALT (IU/L)	30 ± 8	92 ± 14[a]	68 ± 12[a,c]	42 ± 10[a]	31 ± 7[c]	30 ± 8	69 ± 11[a]	52 ± 8[a,c]
% area	3 ± 2	311 ± 12	304 ± 11	16 ± 4	8 ± 3	4 ± 1	20 ± 4	15 ± 3[a,c]
No. of droplets	11 ± 5	19.7 ± 1.3[a]	16.7 ± 0.7[a]	570 ± 47[a]	356 ± 23	12 ± 6	389 ± 31[a]	519 ± 26[a,c]
Mean droplet diameter	1.1 ± 0.3	9.2 ± 1.4[a]	6.8 ± 1.2[a,c]	4.2 ± 1.0[a]	3.2 ± 0.7[c]	0.9 ± 0.5	4.4 ± 1.1[a]	3.4 ± 0.9[a,c]

Data represent percentage of fat in the whole area, number of fat droplets and mean diameter of the droplets. Results are mean ± SD of $n = 5$ different slides per rat per group at each time point. [a]$P < 0.05$ *vs* control rats; [c]$P < 0.05$ *vs* choline-deficient diet (CD) or high fat diet (HFD) only. ALT: Alanine transaminase.

anesthetized with xylazine/ketamine (5.45 mg/36.4 mg/kg *im*) and then sacrificed by decapitation on days 0, 7, 14, and 30 for the CD and 0, 14, 30 and 60 for the HFD groups. Blood was collected into heparinized tubes and centrifuged at 4000 × *g* for 5 min to obtain serum. Livers were removed immediately and homogenized in an ice-cold potassium-phosphate buffer containing 5 mmol/L EDTA (pH 7.4). General and liver parameters are reported in Table 1.

The protocol was conducted according to the Guide Principles for the care and use of laboratory animals and was approved by the local committee for animal experimentation.

Biochemical determinations

Total glutathione (GSH) determination was performed by precipitating tissue homogenates with 15% sulfosalicylic acid and processing the supernatant by the oxidized glutathione recycling procedure[13]. Protein thiols were measured with an Elmann's procedure modification[14]. The hepatic and serum levels of thiobarbituric acid malondialdehyde complex (MDA-TBA) were first separated by high-performance liquid chromatography using an analytical column Spherisorb ODS 5 μmol/L (250 mm × 4.6 mm) eluted with 60% (v/v) potassium phosphate buffer 50 mmol/L, pH 6.8 and 40% (v/v) methanol at a flow rate of 1 mL/min. Next, spectrophotometric detection of the MDA-TBA adducts occurred at 532 nm[15]. Glutathione Peroxidase (GPx) activity was assessed by use of the method described by Flohè *et al*[16]. Calculations were made with 1 unit enzyme considered as the amount consuming 1.15 μmol of nicotinamide adenine dinucleotide phosphate reduced (NADPH) in 1 min at 37 ℃ (pH 7.0). Thioredoxin levels were quantitated by a standardized ELISA method in serum and liver homogenates. Procedure followed the manufacturer's instructions (Histo-line Laboratories S.r.l., Milan, Italy); samples were located in micro-wells previously coated with a polyclonal antibody (LF-PA0002) and successively with a primary (LF-MA0077) and a secondary HRP-conjugated anti-mouse antibody (81-6720). After the addition of a chromogenic system, the reaction was stopped and absorbance read at 492 nm. Nitrosothiols were measured according to the method of Cook *et al*[17] using a mixture of sulfanilamide/N-1-naphty-lethylendiamine dihydrochloride, neutral Griess as reagents. Nitrotyrosine in the serum was quantified using an ELISA Kit containing Streptavidin-peroxidase conjugate which reacts with the substrate tetramethylbenzidine as per manufacturer's instructions (HyCult Biotechnology b.v., UDEN, The Netherlands) with absorbance read at 450 nm. Keratin 18 fragments (K-18) level was quantified in serum using the ELISA Kit as per manufacturer's instructions (Cusabio Biotech Co., Ltd): samples were located in microwells previously coated with a human-specific antibody and successively with one substrate and a chromogenic system. The reaction was stopped and absorbance read at 450 nm. Protein concentration was measured by using a Bio-Rad kit for the assay of proteins (Bio-Rad GmbH, Munich, Germany).

Western blotting analysis

The expression of mitochondrial oxidative phosphorylation (OXPHOS) system components was assessed by using an antibody cocktail targeting specific subunits from complexes I (NADH-ubiquinone oxidoreductase), Ⅱ (succinate dehydrogenase), Ⅲ [ubiquinone-cytochrome c oxidoreductase (COX)], Ⅳ (cytochrome c oxidase), and V (ATP synthase). β-actin was used as a loading control. Frozen tissues were homogenized in a cold Ripa buffer (50 mmol/L Tris-HCl, pH 8.8; 150 mmol/L NaCl; 1% Igepal; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with a protease inhibitor cocktail (Sigma) and ruptured by 30 passages through a needle. Homogenized tissues were centrifuged at 14000 rpm (4 ℃, 10 min). The supernatant was collected and kept at -80 ℃ until used. Protein concentration of each sample was measured using BCA Protein Assay kit according to the manufacturer's protocol. After denaturation at 100 ℃ during 5 min in Laemmli buffer (BioRad), proteins (30 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 2% of milk in TBST (50 mmol/L Tris-HCl, pH 8; 154 mmol/L NaCl, and 0.1% Tween 20) for 1 h at room temperature, membranes were incubated overnight at 4 ℃ with antibodies against OXPHOS components (1:2000) and β-actin (1:2500). Membranes were incubated with secondary alkaline phosphatase-

conjugated antibodies: goat anti-mouse IgG (1:5000) for 1 h at room temperature. Membranes were incubated with ECF detection system (Amersham, GE Health-Care, Piscataway, NJ) and read with the Versa Doc imaging system (Bio-Rad, Barcelona, Spain).

Histology

Liver specimens were fixed in 10% neutral buffered formalin and paraffin embedded. Five sections of 4 μm thickness from each sample were cut and stained with toluidin blue-periodic acid-schiff stain. Histologic features were examined on five low-power fields per specimen, and a semi-quantitative estimation of the empty vacuoles (fat) was performed by measuring the fat percentage distribution on the surface areas.

Chemicals

Total OXPHOS Rodent Antibody Cocktail was purchased from Mitosciences Inc. (Cambridge, MA). Secondary alkaline phosphatase-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Cambridgeshire, United Kingdom). All other chemicals used were of the highest purity available and were purchased from Sigma-Aldrich Chemical Co. (Barcelona, Spain or Milan, Italy).

Statistical analysis

All data are expressed as mean ± SD. The Mann-Whitney rank sum test was used to compare groups. For multiple comparisons, the ANOVA on ranks analysis of variance followed by Dunn's method was used. The nonparametric Spearman rank order correlation was used to relate biochemical parameters. $P < 0.05$ defined significance.

RESULTS

Effect of CD and HFD on general parameters and liver histology

Feeding CD or HFD was associated with a progressive and significant increase of liver weight but not of body weight at days 30 and 60, respectively, as compared with control rats (Table 1). In rats with fatty liver, ALT levels increased progressively. At days 30 and 60, both CD and HFD rats showed a two-to-threefold increase.

Rats on CD and HFD showed remarkable liver steatosis (Figure 1 and Table 1). There was a progressive hepatic fat infiltration ranging from large droplets at day 14 to disseminated and confluent vacuoles resulting in microvesicular steatosis at day 30 (CD) and day 60 (HFD). Single cell necrosis was rarely noted. No evidence for inflammation and/or fibrosis was present.

Effect of CD on oxidative and nitrosative stress parameters

In the liver, CD was associated with a higher content of GSH, thioredoxin, and nitrosothiols, while GPx activity was higher at day 7 and then lower at day 30 (Table 2). MDA-TBA levels were five-fold higher from 9.1 ± 1.2 nmol/g to 75.6 ± 5.4 nmol/g at day 30 (Figure 2). Serum MDA-TBA and nitrosothiols levels as well as nitrotyrosine and K-18 (Table 2) were higher in CD rats.

Effect of HFD on oxidative and nitrosative stress parameters

In the liver, HFD determined a progressive decrease in the content of GSH, thioredoxin and nitrosothiols (Table 3). GPx activity was initially unchanged, then increased and next decreased (Table 3); MDA-TBA were doubled at day 60 (Figure 2). Serum levels of MDA-TBA, K-18, nitrotyrosine and nitrosothiols were significantly higher in HFD rats (Figure 3).

Effect of HFD on mitochondrial oxidative phosphorylation complexes

Samples from liver, heart and skeletal muscle were compared regarding the content in subunits of the mitochondrial respiratory chain. Western blotting yielded bands to all OXPHOS subunits studied, although the band corresponding to complex Ⅲ was very difficult to detect in the three tissues and hence was not quantified by densitometry. Band density for each protein was normalized for the corresponding β-actin band.

In the liver (Figure 4), the HFD caused an increase in the amount of the Complex Ⅰ subunit NDUFB8 (at day 60) and Complex Ⅳ subunit Ⅰ, COX Ⅰ (at day 30) and a decrease of Complex Ⅱ subunit 30kDa, CII-30 (days 14 and 60) and ATP synthase subunit α, CV-α (day 14). Minor changes in the heart and skeletal muscle proteins were observed (data not shown).

Effect of Realsil on general parameters, liver histology, stress markers, and mitochondrial proteins

Realsil administration was associated with a lower increase of liver weight and a less pronounced increase of serum transaminase levels in rats (Table 1). As shown (Figure 1 and Table 2), Realsil resulted in a lower extent of fat infiltration in both CD and HFD rats. In CD rats, Realsil determined an improvement in the number and diameter of fat droplets. In HFD rats, Realsil was associated with a lower diameter of fat droplets; the number of droplets per histological section was lower at day 30 but higher at day 60, as a likely consequence of a less confluent phenomenon.

Realsil was protective against CD and HFD induced oxidative and nitrosative changes both in the liver and in plasma. The improvement in such parameters was more evident for CD rats. In particular, the decrease of liver GSH and nitrosothiols was kept to 50% compared with untreated rats, while the changes in MDA-TBA, thioredoxin, and GPx were less marked although significant (Table 2). The same parameters were less affected by Realsil in HFD rats (Table 3 and Figure 3). In fact, the hepatic concentrations of GSH, MDA-TBA, GPx, and nitrosothiols did not differ significantly compared to untreated HFD rats. Only thioredoxin levels were maintained to a higher level by Realsil administration. In these

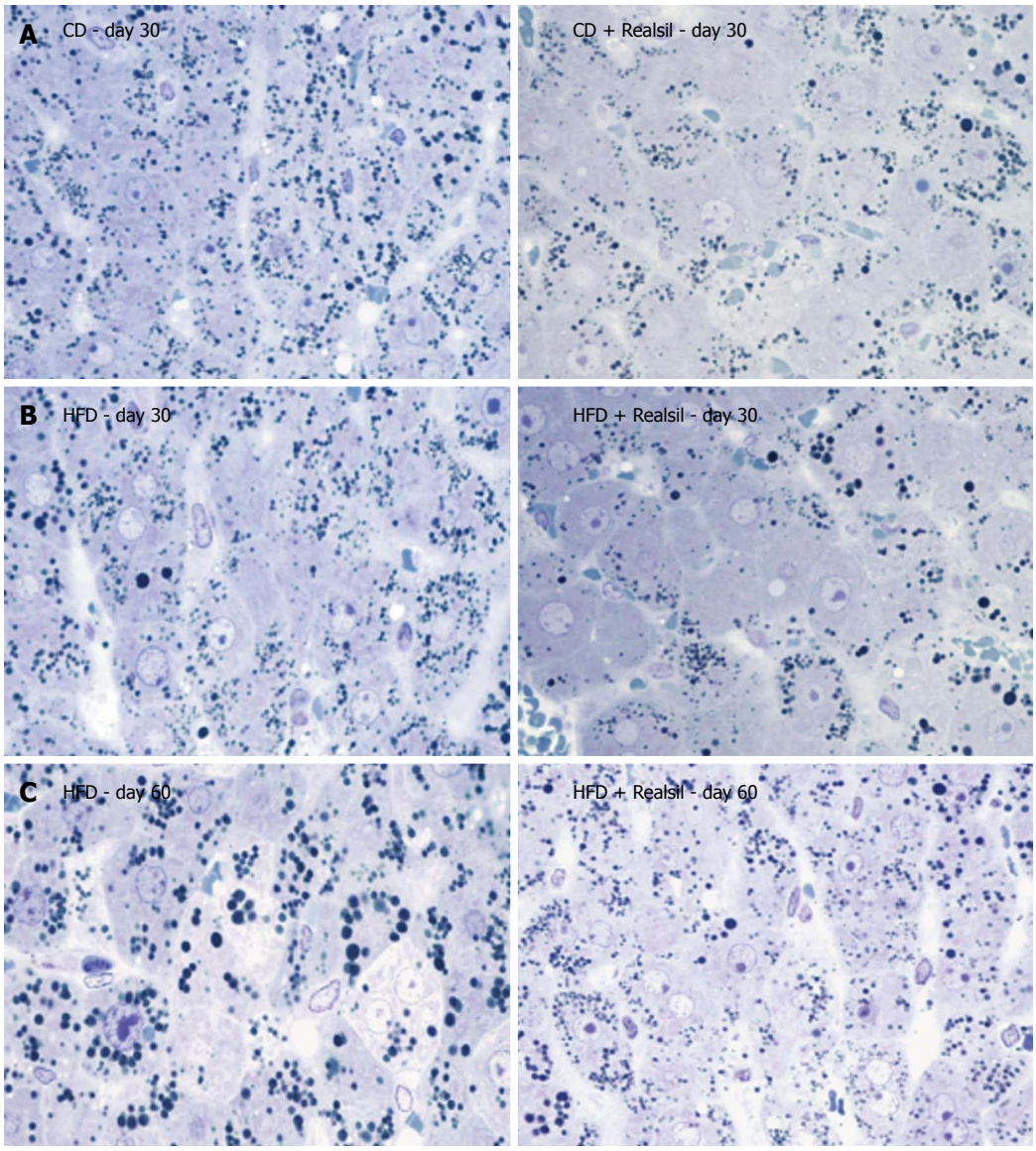

Figure 1 Light micrographs of rat liver stained with toluidin blue-periodic acid-schiff stain. A: Liver from rat fed a choline deficient diet (day 30); B: Liver from rat fed a high fat diet (day 30); C: Liver from rat fed a high fat diet (day 60). Right column without and left column with administration of Realsil (Magnification: 200 ×). CD: Choline deficiency; HFD: High fat diet.

rats, Realsil halved the variations of serum MDA-TBA and nitrotyrosine levels, while it was less effective on K-18 (222 ± 15 IU/L *vs* 242 ± 23 IU/L at day 60 in treated and untreated HFD rats, respectively) and nitrosothiols (72 ± 10 nmol/L *vs* 81 ± 9 nmol/L at day 60 in treated and untreated HFD rats, respectively) levels.

In HFD animals receiving also Realsil, several changes occurred in liver mitochondria (Figure 4). At day 14, administration of Realsil resulted in a significant increase in the protein levels of NDUFB8 subunit, which were slightly decreased (although not statistically significant) by the diet alone. On the other hand, Realsil in HFD animals further decreased the amount of CII-30 (day 14), NDUFB8 (day 30), and COX I (day 60). At day 60, Realsil had protective effect on the Complex Ⅱ subunit CII-30 by increasing the amount of the protein that was decreased by the diet alone. Interestingly, in the muscle, at day 60 Realsil reversed the effect induced by HFD in CII-30 subunit and in COX I subunit and, at day 30, decreased CV-α subunit protein amount.

DISCUSSION

Obesity is a health problem in developed countries[18-21] and is related to insulin resistance, dyslipidemia, type 2 diabetes, hypertension, and liver steatosis (NAFLD)[22]. Simple steatosis is considered a benign condition with low risk of evolution, while its inflammatory form (NASH) is considered a risk factor for liver cirrhosis.

Mechanisms governing hepatocellular fat deposition involve metabolic pathways partly depending on the up-regulation of peroxisome proliferator-activated receptor and on the consequent activation of adipocyte differentiation programs[23]. The relationship between

Table 2 Concentrations of total glutathione, thioredoxin, nitrosothiols, and activity of glutathione peroxidase, malondialdehyde-thiobarbituric acid reactive substances, nitrosothiols, nitrotyrosine, and keratin-18 in the liver of rats

		Day 7	Day 14	Day 30
Glutathione	S	34.3 ± 3.1	33.2 ± 1.4	25.3 ± 1.2[a]
34.0 ± 1.3 nmol/mg prt	R	35.1 ± 1.2	33.7 ± 1.0	30.9 ± 1.1[a,c]
Thioredoxin	S	14.2 ± 1.0[a]	16.3 ± 2.4[a]	5.2 ± 0.7
5.6 ± 0.9 nmol/mg prt	R	11.3 ± 1.0[a]	14.4 ± 1.0[a]	6.2 ± 0.7[c]
Nitrosothiols	S	32.9 ± 3.5[a]	30.0 ± 2.2[a]	13.0 ± 1.2[a]
16.8 ± 4.7 pmol/mg prt	R	35.4 ± 2.7[a]	27.3 ± 2.2[a,c]	15.8 ± 1.5[a,c]
MDA-TBA	S	17 ± 4[a]	39 ± 8[a]	92 ± 10[a]
12 ± 3 nmol/L	R	12 ± 3	19 ± 4[a,c]	41 ± 9[a,c]
Nitrosothiols	S	73 ± 15	79 ± 12[a]	88 ± 10[a]
62 ± 10 nmol/L	R	67 ± 10	69 ± 10	74 ± 11[a,c]
Nitrotyrosine	S	6 ± 2	10 ± 3[a]	14 ± 3[a]
6 ± 1 nmol/L	R	5 ± 1	5 ± 2[c]	10 ± 2[a,c]
K-18	S	222 ± 21	233 ± 16[a]	289 ± 20[a]
198 ± 20 U/L	R	210 ± 10	213 ± 13[c]	240 ± 17[a,c]
GPx activity	S	9.3 ± 1.2[a]	21.1 ± 3.7[a]	13.7 ± 1.8[a]
4.5 ± 0.4 nmol NADH/min/mg prt	R	6.3 ± 0.9[a,b]	20.8 ± 1.4[a]	16.6 ± 2.6[a,b]

The liver of rats fed a choline-deficient (CD) diet without (S)/with (R) Realsil or a normal choline-supplemented diet (controls). Values from controls are reported in the first column under the parameter. Data are mean ± SD of n = 5 rats per group at each time point. [a]$P < 0.05$ *vs* control rats; [c]$P < 0.05$ *vs* rats on CD diet without Realsil at the same time point. GPx: Glutathione peroxidase; MDA-TBA: Malondialdehyde-thiobarbituric acid reactive substances; K-18: Keratin-18; prt: Protein.

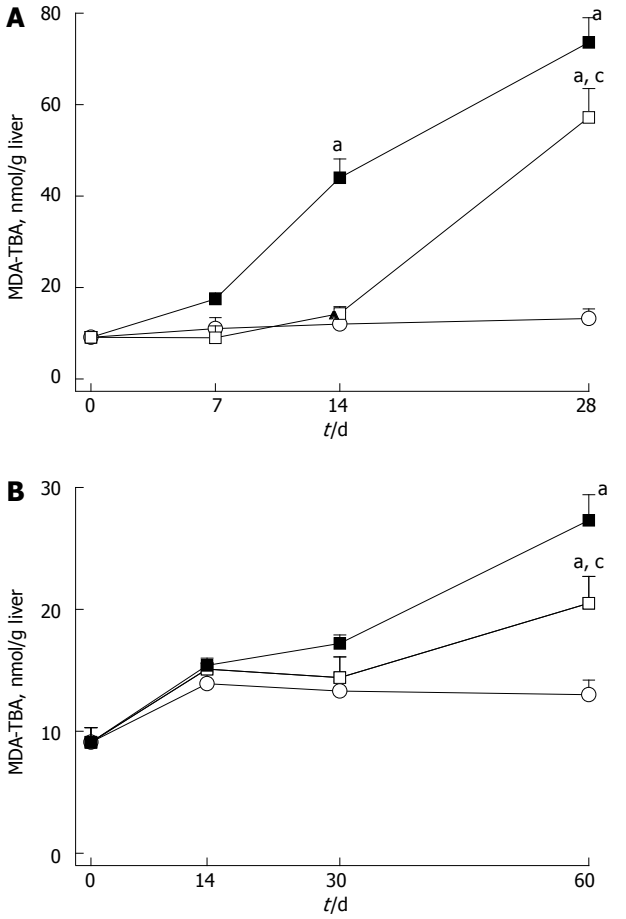

Figure 2 Concentrations of malondialdehyde-thiobarbituric acid reactive substances. A: Liver of rat fed a choline-deficient diet; B: Liver of rat fed a high fat diet. Closed square: Without; open square: With Realsil administration; open circle: Control rats fed a standard chow-diet with adequate content of choline. Data are mean ± SD of n = 5 rats per group at each time point. [a]$P < 0.05$ *vs* control rats; [c]$P < 0.05$ *vs* untreated rats at the same time points. MDA-TBA: Malondialdehyde-thiobarbituric.

intracellular metabolic processes and systemic changes occurring in patients with fatty liver are still under debate. Also, the effect of modulating compounds and antioxidant molecules on such lipid effectors and on NO metabolic changes has been poorly investigated. Mitochondria, coordinators of energy metabolism, are actively involved in such processes[18,21]. In NASH patients, mitochondria show morphological alterations and functional impairment[7,24]. Ultrastructural modifications were also observed in rats fed a steatogenic diet (HFD)[25]. Mitochondria are the most relevant source of ROS in most cells and especially in fatty hepatocytes[26,27]. ROS alter the activity of JNK enzymes, disturb insulin signaling, and enhance potassium transport across the inner mitochondrial membrane[28] leading to mitochondrial uncoupling and triggering as well adaptive response[29-31].

In a recent study[3], we observed that hepatocytes react to fat deposition by a very early increase of GSH and thioredoxin both in the cytosol and in the mitochondria to likely prevent lipid and protein oxidation. Prolongation of steatogenic diet, however, led to major mitochondrial redox changes, *i.e.*, increased MDA-TBA, progressive decrease of GSH and thioredoxin, and increase of mixed disulfides, nitrates, and nitrosothiols, all consistent with both oxidative damage and increased NO synthesis. In fatty livers, NO synthase (iNOS) is induced by enhanced inflow of gut-derived toxins[32] and tumor necrosis factor-α expression[33] and generates hyper reactive species such as NO with accumulation of nitrotyrosine[33]. This likely reflects an increased peroxynitrite formation and suggests potential NO participation to liver injury[34]. Thioredoxin, a redox-active protein induced by oxidative stress[35], is actively involved in NO regulation through nitrosothiols cleavage[36,37]. Nitrosothiols, formed by conjugation of NO with free thiols, oppose peroxynitrite formation and act as intracellular messenger controlling cellular and mitochondrial functions[38,39].

In the fatty liver, intracellular redox changes and protein nitrosation may represent a major factor stimulating the progression from simple steatosis to NASH[40]. Also, a critical role for such hepatic variations in the pathogenesis of systemic chronic inflammatory conditions has been recently proposed[41,42]. In our study, we show that liver steatosis is associated with high levels of circulating NO derivatives (nitrosothiols and nitrotyrosine) and with high levels of K-18, the major keratin expressed in the liver and one of the most prominent substrates of caspases during hepatocytes apoptotic death[43]. These alterations were closely dependent on the changes occurring in the liver in which GSH content and thioredoxin activity declined with ongoing steatosis. Also, the close

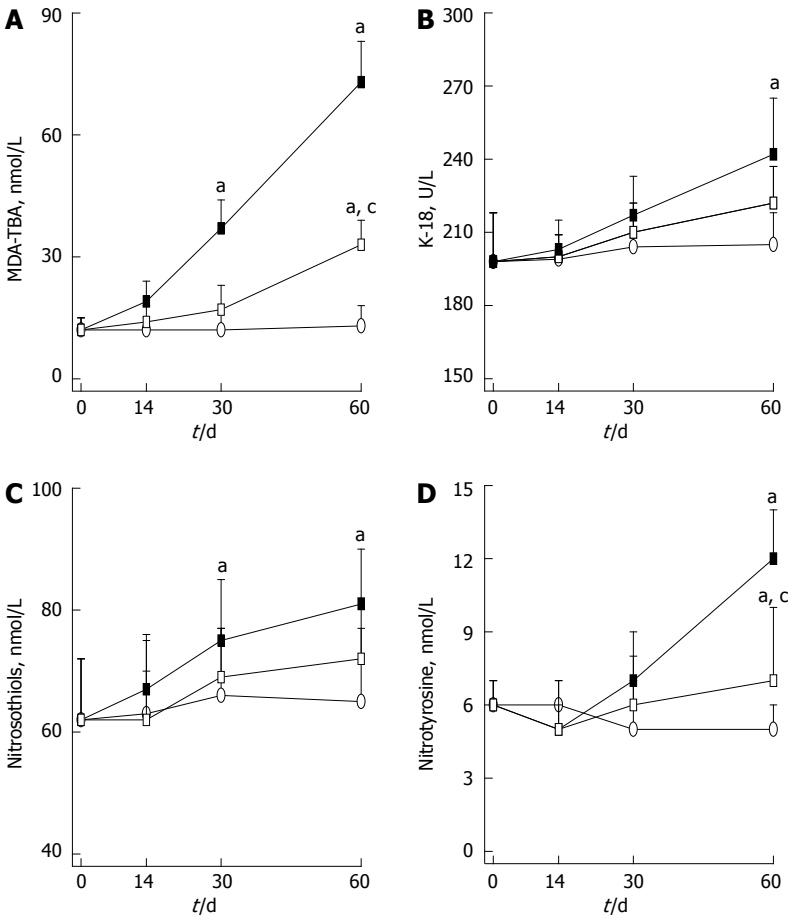

Figure 3 Plasma concentration of redox active and nitrogen regulating molecules. A: Malondialdehyde-thiobarbituric acid reactive substances (MDA-TBA); B: Keratine 18 (K-18); C: Nitrosothiols; D: Nitrotyrosine. Rats were fed a high fat diet without (closed square) or with (open square) Realsil administration; control rats fed a standard chow-diet (open circle). Data are mean ± SD of n = 5 rats per group at each time point. $^{a}P < 0.05$ *vs* control rats; $^{c}P < 0.05$ *vs* untreated rats at the same time points.

Table 3 Concentrations of total glutathione, thioredoxin, nitrosothiols, and activity of glutathione peroxidase in the liver of rat

		Day 14	Day 30	Day 60
Glutathione	S	23.7 ± 1.9[a]	20.8 ± 2[a]	22.4 ± 2.4[a]
33.4 ± 0.8 nmol/mg prt	R	22.3 ± 1.8[a]	23.9 ± 1.7[a]	23 ± 1.6[a]
Thioredoxin	S	9.2 ± 0.9[a]	8.9 ± 0.8[a]	4.3 ± 1.0
5.4 ± 0.5 nmol/mg prt	R	7.6 ± 0.7[a,c]	7.8 ± 0.7[a]	7.4 ± 1.7[a,c]
Nitrosothiols	S	18.1 ± 1.5	19.6 ± 2.5	10 ± 2.3[a]
20.4 ± 3.1 pmol/mg prt	R	14.4 ± 1.7[a,c]	16.1 ± 2.3[a]	14.3 ± 1.9[a,c]
GPx	S	4.7 ± 0.6[a]	6.5 ± 1.3[a]	4.6 ± 1.6
3.9 ± 0.3 nmol NADH/ min/mg prt	R	3.4 ± 0.5[c]	4.3 ± 1.0[c]	4.1 ± 1.0

The liver of rats fed a high fat diet (HFD) diet without (S)/with (R) Realsil or a standard control diet (controls). Values from controls are reported in the first column under the parameter. Data are mean ± SD of n = 5 rats per group at each time point. $^{a}P < 0.05$ *vs* control rats; $^{c}P < 0.05$ *vs* rats on HFD diet without Realsil at the same time point. GPx: Glutathione peroxidase; NADH: Nicotinamide adenine dinucleotide; prt: Protein.

relation observed between the circulatory NO derivatives and K-18 levels clearly links NO with the hepatic inflammatory processes occurring under marked liver steatosis. Previous studies have demonstrated elevation of these molecules in NAFLD[44] as a consequence of the increased apoptotic rate due to hepatic inflammation[45].

In the present work, the HFD promoted alterations in mitochondrial complexes, although not deep enough to result in bioenergetic changes, as reported for other diet models including CD[46,47]. Interestingly, the limited array of alterations observed in the heart and skeletal muscle points to the conclusion that this model selectively affects the liver.

In this scenario, the administration of antioxidant molecules able to contrast oxidative and nitrosative phenomena improved most of the investigated stress parameters both in the liver and in serum. In fact, in our study the administration of Realsil was effective in reducing the extent of fatty infiltration of the liver and in modulating the changes in mitochondrial function and oxidative and nitrosative stress both in the liver and in the systemic circulation. This would suggest that Realsil is effective in contrasting the metabolic alterations resulting in excess fat deposition and at the same time in counteracting the increased formation of ROS and NO species.

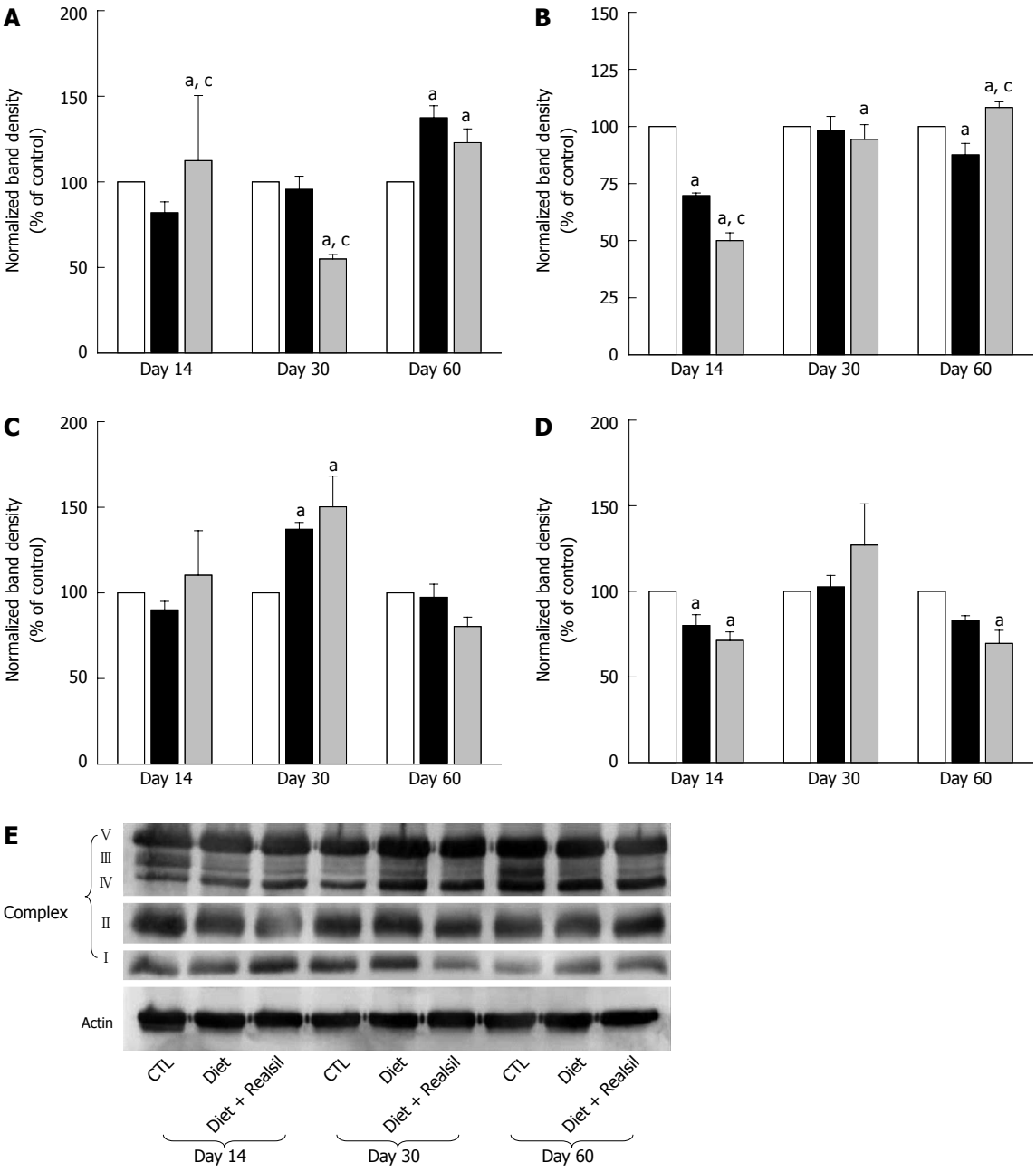

Figure 4 Western blotting analysis of the components of liver mitochondrial oxidative phosphorylation system in rats receiving high fat diet with (grey bar) or without (black bar) Realsil and in control (white bar) rats fed a standard chow-diet. Band density for the target protein normalized for the corresponding β-actin for A: Respiratory complex Ⅰ; B: Respiratory complex Ⅱ; C: Respiratory complex Ⅳ; D: Respiratory complex Ⅴ; E: Picture representative of Western blotting analysis. Data are means ± SE of n = 3-4 independent experiments. $^{a}P < 0.05$ *vs* control rats; $^{c}P < 0.05$ *vs* untreated rats at the same time points.

Silybin, the major constituent of milk thistle extract, affords hepatoprotection *in vitro* and *in vivo*[48,49] by inhibiting the production of pro-inflammatory and pro-fibrogenic factors[12,50]. The conjugation with phospholipids greatly increases its intestinal absorption and the systemic bioavailability[51]. However, the mechanisms of the hepatocyte protection have not been completely defined. Some hypotheses point to a potential antioxidant cytoprotective effect of Realsil by including the modulation of protein oxidation/denitrosation and the maintenance of membrane lipid composition and function[52]. To demonstrate these effects, we investigated the protective effects of Realsil on oxidative/nitrosative changes both in the liver and in circulation.

Indeed, although the subtle mechanisms regulating the protein nitrosation/denitrosation process have not been completely identified, it has been shown that thioredoxin-deficient cells denitrosate nitrosothiols less efficiently[37] and that nitrosative stress is critically important in promoting *S*-nitrosylation and nitration of various mitochondrial proteins, leading to mitochondrial dysfunction, decreased energy supply, and increased hepatic injury[53]. With ongoing steatosis, the decrement in hepatic thioredoxin and GSH levels we observed may be due

also to a down-regulation process associated with excess deposition of fat and toxic molecules and may indirectly contribute to the progressive appearance of other oxidative changes.

In our study, Realsil was able to counteract most of the oxidative biochemical alterations during the early phases of steatosis while it resulted less effective later (30 and 60 d) when the extent of fat infiltration was massive. The protection promoted by Realsil is certainly exerted at different levels and particularly at mitochondrial level[54]. The changes (both morphological and biochemical) observed in the liver of rats receiving Realsil were also evident at systemic level and this more explicitly relates systemic with hepatic changes in animals with fatty liver.

COMMENTS

Background

Fatty degeneration represents a leading factor of hepatocyte dysfunction and is associated with excess delivery of stress molecules thus rendering the liver a major source of systemic alterations in patients with metabolic syndrome. Adaptive metabolic mechanisms have been described during the early phase of fatty infiltration including expression of intracellular sensors and signaling molecules for lipid metabolism and oxidative stress pathways. Vitamin E efforts a remarkable protection against lipid oxidation and, if vehicoled within a phospholipids complex together with silybin, a *silybum marianum* extract, protects against pro-fibrotic oxidative injury.

Research frontiers

The threshold above which fat infiltration becomes dangerous is not clear, so far. Several observations suggest that ongoing fatty degeneration indeed exposes hepatocytes to higher risk of oxidative damages.

Innovations and breakthroughs

Little is known about the effectiveness of vitamin E-silybin-phospholipid complex in blocking or modulating reactive oxygen species/NO production and their oxidant effects. This study also gives answer to the question whether administration of an antioxidant complex is able to break off the intracellular mechanisms leading to a progressive accumulation of neutral lipids in fatty hepatocytes and mitochondrial dysfunction.

Applications

Realsil was able to counteract most of the oxidative hepatic changes during the early phases of steatosis while it resulted less effective later when the extent of fat infiltration was massive. The changes observed in the rat fatty liver were also evident at systemic level pointing to a relationship between systemic and hepatic alterations.

Terminology

Liver steatosis occurs when the amount of neutral fat exceeds 5% of organ weight. Realsil is a compound constituted by vitamin E and silybin complexed with phospholipids. Silybin is a *silybum marianum* extract; it is known to protect against pro-fibrotic oxidative injury.

Peer review

In this descriptive study, the authors show that Realsil is able to reduce liver injury in two different animal models of steatosis. Realsil seems to protect by decreasing toxic free radicals species as highly delivered by fatty hepatocytes.

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ORIGINAL ARTICLE

Incidence and mortality of acute and chronic pancreatitis in the Netherlands: A nationwide record-linked cohort study for the years 1995-2005

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Abstract

AIM: To analyze trends in incidence and mortality of acute pancreatitis (AP) and chronic pancreatitis (CP) in the Netherlands and for international standard populations.

METHODS: A nationwide cohort is identified through record linkage of hospital data for AP and CP, accumulated from three nationwide Dutch registries: the hospital discharge register, the population register, and the death certificate register. Sex- and age-group specific incidence rates of AP and CP are defined for the period 2000-2005 and mortality rates of AP and CP for the period 1995-2005. Additionally, incidence and mortality rates over time are reported for Dutch and international (European and World Health Organization) standard populations.

RESULTS: Incidence of AP per 100000 persons per year increased between 2000 and 2005 from 13.2 (95%CI: 12.6-13.8) to 14.7 (95%CI: 14.1-15.3). Incidence of AP for males increased from 13.8 (95%CI: 12.9-14.7) to 15.2 (95%CI: 14.3-16.1), for females from 12.7 (95%CI: 11.9-13.5) to 14.2 (95%CI: 13.4-15.1). Irregular patterns over time emerged for CP. Overall mean incidence per 100000 persons per year was 1.77, for males 2.16, and for females 1.4. Mortality for AP fluctuated during 1995-2005 between 6.9 and 11.7 per million persons per year and was almost similar for males and females. Concerning CP, mortality for males fluctuated between 1.1 (95%CI: 0.6-2.3) and 4.0 (95%CI: 2.8-5.8), for females between 0.7 (95%CI: 0.3-1.6) and 2.0 (95%CI: 1.2-3.2). Incidence and mortality of AP and CP increased markedly with age. Standardized rates were lowest for World Health Organization standard population.

CONCLUSION: Incidence of AP steadily increased while incidence of CP fluctuated. Mortality for both AP and CP remained fairly stable. Patient burden and health care costs probably will increase because of an ageing Dutch population.

Key words: Acute pancreatitis; Chronic pancreatitis; Epidemiology; Incidence; Mortality

Core tip: Large scale epidemiological studies reporting time trends of incidence and mortality of chronic pancreatitis (CP) are strikingly scarce compared to the also limited epidemiological studies on acute pancreatitis (AP). Reported are the Dutch incidence rates of AP and CP for the period 2000-2005, and mortality rates of AP and CP for the period 1995-2005. The incidence rates of AP steadily increased while the incidence of CP fluctuated. Population mortality for both AP and CP remained fairly stable. Both incidence and mortality rates increased markedly by age. So, especially in ageing populations, it is to be expected that patient burden and health care costs will increase.

Spanier BWM, Bruno MJ, Dijkgraaf MGW. Incidence and mortality of acute and chronic pancreatitis in the Netherlands: A nationwide record-linked cohort study for the years 1995-2005. *World J Gastroenterol* 2013; 19(20): 3018-3026 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3018.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3018

INTRODUCTION

Over the last decades, the incidence and number of hospital admissions of both acute pancreatitis (AP)[1-10] and chronic pancreatitis (CP) have consistently increased in the Western countries[11-14]. Increasing alcohol intake, more gallstone-related pancreatitis, increased pancreatic enzyme testing and improvements of diagnostic tests and interventional techniques have all been suggested as possible explanations[4,5,6,8,15-17].

The disease spectrum of AP ranges from mild and self-limiting (approximately 85%) to a life-threatening illness resulting in significant morbidity and mortality[18-21]. At onset, AP regularly results in hospitalization[22,23]. Generally, in the Western countries the case fatality proportion of AP decreased over time, but the overall population mortality did not change[2,16].

CP is characterized by ongoing or recurrent episodes of abdominal pain accompanied by progressive pancreatic exocrine and endocrine insufficiency. Hospitalization is required in case of an exacerbation to control pain (*e.g.*, opioid medication, endoscopic duct drainage, pancreaticojejunostomy and/or resection) and for the treatment of complications such as pseudocysts[15,16,24,25]. The overall survival for CP patients is reduced compared with the general population. Most notably because of the impact of non-pancreatic effects of excess alcohol consumption and/or smoking, independent of CP itself[15,16].

Epidemiological studies from the Netherlands for AP are scarce and this is even more the case for CP[2,11,22]. Eland *et al*[2] reported the latest incidence rates and mortality of AP from 1985 to 1995, both based on hospital discharge data. Our group reported about the trend in hospital admissions in the Netherlands for AP and CP[11]. For both groups, the hospital admissions have increased substantially from 1992 to 2004.

In this study we report the incidence rates of AP and CP for the period 2000-2005 and mortality rates of AP and CP for the period 1995-2005 following linkage of three distinct nation-wide Dutch registries. Additionally, data on incidence and mortality rates over time are reported for Dutch and international standard populations.

MATERIALS AND METHODS

Case finding

Cases of AP and CP were identified by linkage of three distinct nation-wide Dutch registries: the hospital discharge register [HDR: formerly also known as the National Information System on Hospital Care (NISHC)], the population register (PR), and the death certificate register (DCR). The HDR contains discharge data from academic and general hospitals in the Netherlands (http://www.dutchhospitaldata.nl/). Since 1992 almost all (> 97%) Dutch hospitals are linked to the HDR with 99% coverage of all hospital admissions. For each hospital discharge the dates of admission and discharge, type of admission, and diagnoses at discharge (primary and secondary) are recorded along with anonymous patient characteristics like: sex, date of birth, postal code of the living address, and country of origin. Hospital discharge diagnoses in the HDR are coded according to the International Classification of Diseases, 9th revision, Clinical Modification (ICD-9CM). We retrospectively retrieved all hospital admissions (> 1 d of hospital stay) for the period 1 January 1995-31 December 2005 from the HDR with acute (code 577.0) or chronic (code 577.1) pancreatitis as the primary discharge diagnoses.

A single patient with multiple admissions will have several records in the HDR. Because of its anonymous nature however, only partially identifiable information is provided at the record level. For the accurate count of pancreatitis cases, it is necessary to identify different admissions, potentially in different hospitals, by the same patient. To identify these unique cases in the HDR the records were linked to the PR which is maintained by Statistics Netherlands of the Dutch Ministry of Economy Affairs. The PR contains continuously updated demographic data on all citizens residing in the Netherlands like name, date of birth, sex, nationality, living address, dates of immigration and/or emigration, and date of death.

HDR records can be linked to the PR by a combination of three identifiers: sex, date of birth, and (the numeric part of) the postal code at the time of admission. Linkage fails if several citizens are of the same sex, are born on the same date, and live in the same postal code area at the time at least one of them is admitted ("administrative siblings"). Also, linkage fails if any key data are lacking in the HDR or in the PR.

Statistics Netherlands enables the unique linkage of about 86% of all yearly hospital admissions to the PR, suggesting that counts at the person level should be multiplied by 100/86 to derive national estimates. However, the probability of unique linkage is not equal across subgroups in the general population and over time. For instance, persons in high-density population areas, persons born in countries with inaccurate birth dates, and moving persons have a lower probability of unique linkage. To compensate for linkage failure, stratum-specific multipliers were calculated for sex, 5-yearly age cohorts, and country of origin (available from the corresponding author upon request). While doing so, we took into account that our aim was to estimate the incidence rather than the prevalence of AP and CP.

From case finding to incident cases

We defined an incident case during any specific year as

a person with an admission for AP or CP during that year which is not preceded by another admission of the same person with the same discharge diagnosis during at least five years prior to the specified year. To identify incident cases, it is mandatory that all admissions during the observation period have been identified for each person. Hence, a person known in the PR should be always uniquely identifiable during the observation period 1995-2005 based on the linkage keys sex, age, and the postal code.

Incidence rates and standardized incidence rates

Sex- and age- specific incidence rates for the years 2000-2005 were defined as the yearly number of new pancreatitis cases per 100000 members of the Dutch reference population alive during the year, excluding immigrated or emigrated citizens and after adjustment for stratum-specific linkage failure between the HDR and the PR[26]. The yearly incidence rates were standardized with reference to the age and sex distribution of the Dutch reference population in 2000 to identify the possible impact of a Dutch ageing population on the observed trends in incidence rates over time. Standardizations were also performed with reference to the age distribution (assuming evenly distributed male and females) of the European and World Health Organization (WHO) standard populations to enable direct comparisons among trends in incidence and mortality rates across countries that differ by age (and sex) distributions of their respective populations[27].

Mortality rates and standardized mortality rates

Deaths associated with AP and CP were identified by linkage of the PR and the DCR which is also maintained by Statistics Netherlands.

General practitioners and hospitals are obliged to complete a death certificate for each deceased person, and notify the primary and up to three secondary causes of death. Causes of death are coded according to the International Classification of Diseases coding system with acute and chronic pancreatic related death coded as 577.0 and 577.1 respectively for the year 1995 (ICD-9CM) or K850 and K860/K861 respectively for the years 1996 and later (ICD-10CM). The register is nearly complete (99.7%) with regard to persons deceased in the Netherlands, meaning that deaths after emigration are lacking. For their completeness and reliability, only the primary causes of death were analyzed. The mortality rate was expressed per 1000000 general population members excluding the ones that emigrated any time between 1995 and 2005. Again, yearly mortality rates were recalculated with reference to the age and sex distribution of the Dutch reference population in 2000 as well as with reference to the age distribution of the European and WHO standard populations.

Statistical analysis

We assumed the AP and CP incidence and mortality rates to follow Poisson distributions when calculating 95%CI. The Statistical Package for the Social Sciences versions 14.0 and 18.0 (SPSS, Chicago, IL, United States) were used for statistical analysis.

RESULTS

In 1995-2005, 18819221 citizens were known in the population register with 49.75% being male. Slightly under 10.4 percent immigrated or emigrated, leaving 16866819 patients in the Dutch reference population for the incidence estimates, 12163581 of whom were always uniquely identifiable during 1995-2005. Hence, the average multiplier for the incidence estimates amounted to 1.387.

Incidence rates acute pancreatitis per 100000 persons per year

The overall incidence rate of AP increased during the 2000-2005 period from 13.2 (95%CI: 12.6-13.8) in 2000 to 14.7 (95%CI: 14.1-15.3) in 2005. The incidence rate for males rose from 13.8 (95%CI: 12.9-14.7) to 15.2 (95%CI: 14.3-16.1), for females from 12.7 (95%CI: 11.9-13.5) to 14.2 (95%CI: 13.4-15.1), reflecting yearly increases of 1.6% and 1.9% respectively (Figure 1A).

Incidence rates ranged from below 1 in the younger age groups (< age 15 years) to as high as 50.7 in 2005 for the patients of 85 years and above. Figure 1B shows the incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). The incidence rate in the 50-74 age group was highest in 2004 (26.1; 95%CI: 24.6-27.7); in the eldest age group the incidence rate was increased in 2005 (46.5; 95%CI: 42.5-50.9) compared to the preceding years.

The mean yearly incidence rates of AP by 5-yearly age groups and by sex peaked at the ages of 80-84 for both, males (55.4; 95%CI: 42.8-71.6) and females (41.9; 95%CI: 33.8-52.0). The mean yearly incidence rate was higher for females (7.1; 95%CI: 4.7-10.7) than males (3.4; 95%CI: 1.9-6.0) for persons in their early twenties. However, between the ages of 44 and 84, the opposite was observed with 30%-63% higher mean yearly incidence rates for males than for females.

Figure 1C and D (males and females) show the original as well as the Dutch 2000, the European and the WHO standardized incidence rates of AP. Both figures show lowest incidence rates for the WHO standard population (range males: 10.4-11.6; range females: 8.6-10.1), followed by the European standard population (range males: 12.5-13.9; range females: 10.0-11.7), and the Dutch standard population (range males: 13.8-15.3; range females: 12.1-14.0). The figures show that the discrepancy between the original incidence rate and the standardized rates increases over time for males, indicating a small effect of an ageing Dutch population.

Incidence rates chronic pancreatitis per 100000 persons per year

The overall incidence rate of CP during the 2000-2005 period averaged 1.77, fluctuating between 1.52 (95%CI: 1.32-1.74) in 2001 and 1.98 (95%CI: 1.76-2.22) in 2004. Figure 2A shows the incidence rates of male and female

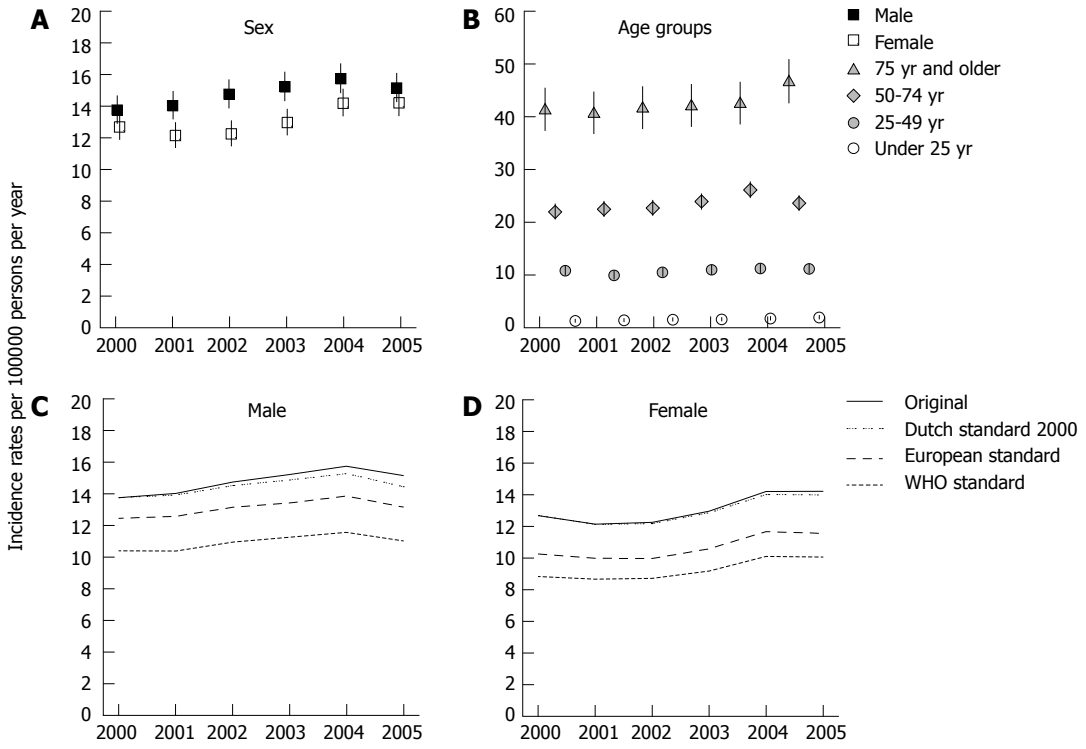

Figure 1 Incidence rates acute pancreatitis per 100000 persons per year. A: The incidence rates of male and female acute pancreatitis (AP) cases; B: The incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized incidence rates of AP.

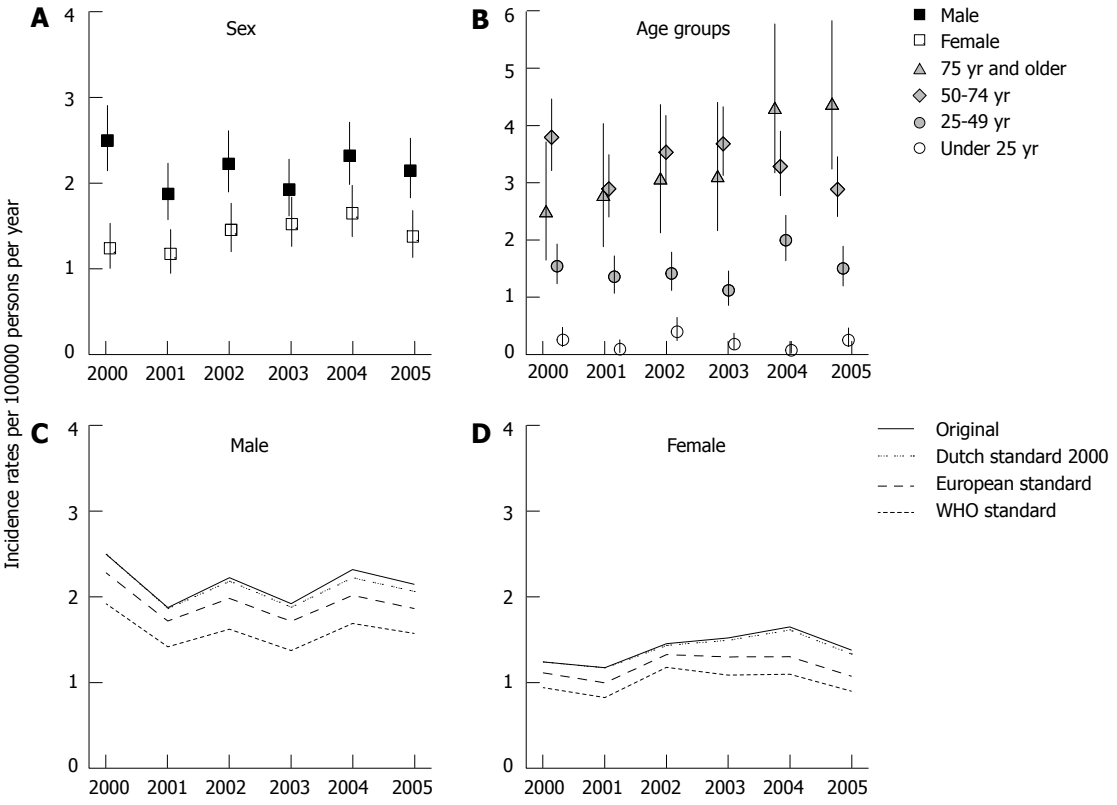

Figure 2 Incidence rates chronic pancreatitis per 100000 persons per year. A: The incidence rates of male and female chronic pancreatitis (CP) cases; B: The incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized incidence rates of CP.

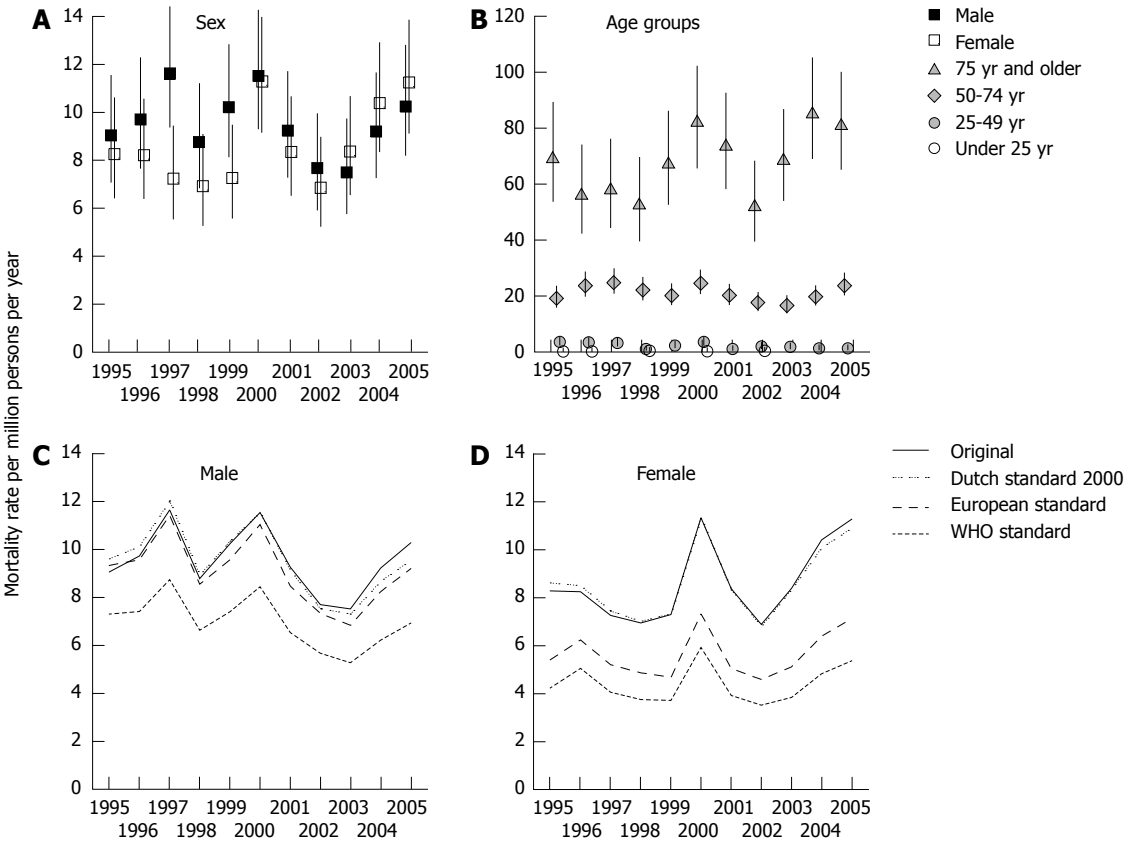

Figure 3 Mortality rates acute pancreatitis per million persons per year. A: The male and female mortality rates of acute pancreatitis (AP) for 1995-2005; B: The mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized mortality rates of AP.

CP cases. The incidence rates for males averaged 2.16, 50% higher than the incidence rates for females (mean of 1.4). The incidence rates for males fluctuated over time with lower rates in the years 2001 and 2003 (both 1.9; 95%CI: 1.6-2.3) compared with the year 2000 (2.5; 95%CI: 2.1-2.9). Among females, the incidence rates during the years 2003 (1.5; 95%CI: 1.3-1.8) and 2004 (1.6; 95%CI: 1.4-2.0) were higher than during the year 2001 (1.2; 95%CI: 0.9-1.5).

Incidence rates ranged from below 0.5 in the younger age groups (< age 20 years) to as high as 5.2 (95%CI: 3.5-7.8) in 2005 for the patients between 75 and 79 years of age. Figure 2B shows the incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). While the incidence rate fluctuated in the 50-74 years age group between 3.8 (95%CI: 3.2-4.5) in 2000 and 2.9 (95%CI: 2.4-3.5) in 2001 and 2005, the incidence rate in the age group of 75 and older increased steadily by 75%, from 2.5 (95%CI: 1.7-3.7) in 2000 to 4.4 (95%CI: 3.2-5.8) in 2005.

The mean yearly incidence rates of CP for males between 65 and 74 years (65-69 years: 6.2, 95%CI: 3.9-9.8; 70-74 years: 5.1, 95%CI: 3.0-8.9) more than doubled the rates for females (65-69 years: 2.3, 95%CI: 1.2-4.7; 70-74 years: 2.4, 95%CI: 1.2-5.0).

Figure 2C and D (males and females) show the original as well as the Dutch 2000, the European and the WHO standardized incidence rates of CP. The incidence rates for the WHO standard population ranged from 1.4 to 1.9 for males and from 0.8 to 1.2 for females. For the European standard population, the ranges were 1.7-2.3 and 1.0-1.3 for males and females respectively. The ranges for the Dutch standard population in 2000 (range males: 1.9-2.5; range females: 1.2-1.6) were nearly identical to the non-standardized data.

Mortality rates acute pancreatitis per million persons per year

Between 1995 and 2005, 1524492 persons died in the Netherlands. In 2264 cases AP was notified as a cause of death. Of these, 65.6% or 1484 cases (0.97 pro mille of all deceased persons) died with AP as the primary cause of death, including 764 males and 720 females.

Figure 3A shows the male and female mortality rates of AP for 1995-2005. The mortality rates fluctuated between 6.9 and 11.7 were similar for males and females except for the years 1997 and 1999 when more males than females died (1997: 11.7; 95%CI: 9.4-14.5 *vs* 7.26; 95%CI: 5.56-9.48); 1999: 10.3; 95%CI: 8.2-12.9 *vs* 7.3; 95%CI 5.61-9.51). Figure 3B shows the mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). The mortality rate for patients younger than 50 years of age stayed below 3.6 (95%CI: 2.3-5.6) and for the 50-74 years age group below 18.6 (95%CI: 14.8-23.5), whereas the mortality rate more than tripled up to 85.3 (95%CI:

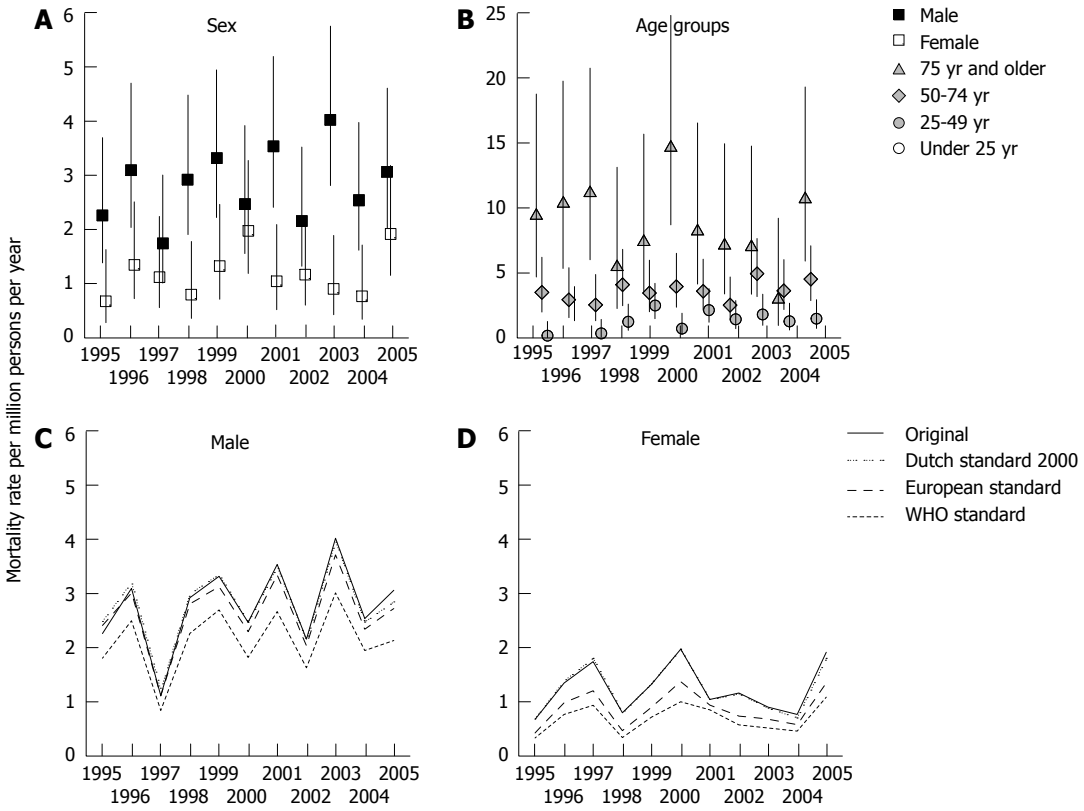

Figure 4 Mortality rates chronic pancreatitis per million persons per year. A: The male and female mortality rates of chronic pancreatitis (CP) for 1995-2005; B: The mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized mortality rates of CP for 1995-2005.

69.1-105.4) for the oldest age group.

Figure 3C and D (males and females) show the original as well as the Dutch 2000, the European, and the WHO standardized mortality rates of acute pancreatitis. An ageing effect for males is present with in 1995 a 6.1% higher and in 2005 a 7.1% lower Dutch standardized rate compared to the original rate. On average, the mortality rates for the European and Dutch standard populations are 31.6%, respectively 36.8% higher than the WHO standard. Although the overall pattern is irregular, a gradual decline over time in mortality rates for males can be noted.

Among the females a smaller ageing effects emerges with in 1995 a 4% higher and in 2005 a 3.4% lower Dutch standardized rate compared to the non-standardized rate. On average, the mortality rates for the European and WHO standard populations are 52.7%, respectively 96.8% below the Dutch standard.

Mortality rates chronic pancreatitis per million persons per year

Between 1995 and 2005 745 patients died in the Netherlands with CP notified as a cause of death. Of these, 43.9% or 327 cases (0.21 pro mille of all deceased Dutch patients in the same period) died with CP as the primary cause of death, including 223 males and 104 females.

Figure 4A shows the male and female mortality rates of CP for 1995-2005. The mortality rates for males fluctuated between 1.1 (95%CI: 0.6-2.3) in 1997 and 4.0 (95%CI: 2.8-5.8) in 2003, for females between 0.7 (95%CI: 0.3-1.6) in 1995 and 2.0 (95%CI: 1.2-3.2) in 2000. Except for 1997, 2000, 2002 and 2005 the male mortality rate exceeded the female mortality rate.

Figure 4B shows the mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). The mortality rate for patients younger than 50 years of age stayed below 2.3 (95%CI: 1.3-4.0) and for the 50-74 age group below 4.5 (95%CI: 2.9-7.1), whereas for the oldest age group the mortality rate went up to as high as 14.7 (95%CI: 8.7-24.9).

Figure 4C and D (males and females) show the original as well as the Dutch 2000, the European, and the WHO standardized mortality rates of CP for 1995-2005. Again, an ageing effect for males is present with in 1995 a 9.7% higher and in 2005 a 7.1% lower Dutch standardized rate compared to the original rate. On average, the European and WHO standardized rates are 5.9% and 32.4% lower than the Dutch rate. No clear ageing effect is observed for females. On average, the European and WHO standardized rates are 42% and 83.5% lower than the Dutch rate.

DISCUSSION

We performed a nationwide record-linked study to analyze the time trends of the incidence and mortality rates of AP and of CP in the Netherlands. We show that

between 2000-2005 the incidence of AP per 100000 persons per year increased over time for both, males (from 13.8 to 15.2) and females (from 12.7 to 14.2). Relatively stable patterns over time emerged for the incidence rate of AP per 100000 persons per year by different age groups. The steady increase of the incidence of AP over time corresponds to the results of a former retrospective study performed in the Netherlands between 1985-1995[2]. However, the similar growth pattern in our study was observed at a lower level. Eland *et al*[2] observed incidence rates for the year 1995 of 17.0 males and 14.8 females per 100000 person-years. If we take for granted that no decrease in incidence rates took place during the unobserved years 1996-1999, then several decisions concerning study design may have contributed to the difference in incidence rate level. Eland *et al*[2] retrieved primary as well as secondary discharge diagnoses from the HDR, whereas we only included AP as a primary discharge diagnosis in order to reduce the risk of misclassification of cases. Further, we excluded single-day admissions for AP. This seems reasonable, because patients with a first attack of AP usually get admitted for several days[2,9]. Moreover, by identifying unique cases following the linkage of two nationwide registries, double counting of cases is a circumvented issue in our study. Considering these reasons for differences in level despite a similar growth pattern, the former reported incidence rates in the Netherlands may be somewhat overestimated.

Other recent population-based studies in Western countries too report increasing incidence rates of AP[3,4,6,7,10,28-31]. The studies - although somewhat heterogeneous by design - indicate that the reported incidence rates of AP in the Netherlands are low and even far lower compared to reported rates from several Scandinavian countries and the United States[16]. The observed differences in incidence rates between these geographical locations are not clearly understood and presumably reflects differences in risk factor prevalence. It has been suggested that the increase of the incidence of AP in the Western countries could be explained by an increase in alcohol intake[16]. According to the registry data of Statistics Netherlands the general self-reported alcohol consumption slightly decreased in the period 2000-2005 (www.cbs.nl). Therefore, the incidence increase of acute pancreatitis seems not to be explained by a change in alcohol consumption, at least not in the Netherlands. We observed that the incidence rates of AP increased considerably with age. This is in accordance with observations elsewhere[2-4,7,29].

The incidence rates of CP in the Netherlands have now been reported for the first time. In contrast to AP, irregular patterns over time emerged for the incidence rates of CP per 100000 persons per year for males (mean: 2.16) and females (mean: 1.4). The incidence rate increased with age, with a top in 2005 at 4.4 in the age group of 75 or older. Recent large scale epidemiological studies reporting the time trends of incidence of CP are strikingly scarce[15,16]. The latest reported incidence rates of CP vary between 5.9 and 7.9 per 100000 persons[11,31,32]. In comparison to those studies, our observed incidence rates of CP are somewhat lower. This may in part be explained by our study limitations (see below). In accordance to other studies, we show that CP is predominantly a disease of males[11-13,15,16,31]. The average incidence rate for males is 50% higher than for females. This is even more pronounced in the older age groups.

The mortality rate for AP per million persons per year fluctuated between 6.9 to 11.7 during the 1995-2005 period. Others too reported fairly stable overall annual population mortality rates[2,10,13]. The mortality rate increased rapidly for patients of 50 years of age and above, which too is in accordance with observations elsewhere[2,4,10,29]. Advanced age may be an independent risk factor for severe AP[33]. Mortality rates were almost similar for males and females.

Concerning CP, the mortality rate per million persons per year for both males and females fluctuated within a stable bandwidth. Tinto *et al*[13] also reported that between 1979 and 1999 the age-standardized mortality rate broadly remained unchanged in England. Merely, the mortality rate for males exceeded the female rate. The mortality rate for persons of 75 years of age and older increased most promptly. Generally, the survival rate for patients with CP is poor[15,16,34,35]. CP patients tend to die of other causes such as smoking related cancers, cardiovascular disease and alcoholic liver cirrhosis.

Both incidence and mortality rates over time are reported for Dutch and international standard populations. On average, the reported rates are the lowest for the WHO standard population, followed by the European and the Dutch standard populations, which clearly reflects their different age distributions. In addition, an ageing effect was observed during the study period, particularly for male incidence rates. The ageing of the Dutch population will most likely continue for another decade, for which reason an increase in patient burden and a rise in health care costs can be anticipated.

Our study has several potential limitations. We defined an incident case during any specific year as a person with an admission that was not preceded by another admission of the same person with the same discharge diagnosis during at least five years prior to the specified year. So, an admitted, incident case in 2000 had no previous admission during 1995-1999 (five years). Further, an admitted, incident case in 2002 had no previous admission in 1995-2001 (seven years). It would have been a contradiction to allow a person to be classified as an incident case in 2002, if he had already been admitted once before in 1996 for the same reason. This analytical approach however may have led to a downward pressure on the incidence rates in later years (near 2005) compared to the earlier years (near 2000). Hence, growth patterns may be slightly underestimated.

Another limitation is that we did not identify incident CP cases among the persons already identified as incident AP cases. Presumably, this resulted in only a minimal underestimation of the incidence rate of CP. A recent study showed that CP developed in alcoholic AP cases with a

cumulative incidence of just 13% in 10 years[36]. Only, in patients with a recurrent alcoholic AP, the incidence of CP at 2 years after initial relapse was 38%. Unfortunately, we do not have sufficient data about the etiology of AP and CP in our study population.

Another issue concerns the reliability of the reported incidence rates which depends heavily on the HDR as a reliable source for hospital discharge data concerning AP and CP.

Eland *et al*[2] performed, as a part of a retrospective study in which incidence rates of AP in the Netherlands were assessed, a restricted validation analysis. They concluded that the overreporting due to miscoding and the underreporting due to non-coding were comparable and that observed incidence rates seem to reflect true rates.

Previously, we retrospectively analyzed the reliability of hospital discharge data of in total 483 admissions for both AP and CP collected in the HDR. We observed a substantial miscoding and non-coding of discharge diagnoses of AP and CP on the level of individual hospital admissions, ultimately leading to a limited underestimation at group level of the total number of AP and CP diagnoses of 15.8% and 6% respectively[37].

Furthermore, there is a potential underestimation of the incidence rates due to non-referral of AP and CP patients. For the present study, we retrieved the AP and CP hospital admissions (> 1 d of hospital stay) from the HDR. Underestimation due to non-referral is probably limited for AP, because in the Netherlands almost all AP patients are admitted to a hospital for more than one day[22]. Whether or not this also holds for CP patients in the Netherlands is unknown. Generally, some CP patients, especially in the early stage of the disease, are only treated in an outpatient clinic setting and treated in a single-day hospital admission. So, by excluding the single-day admissions in our study, the underestimation will be of greater importance for CP compared to the AP. Historical volume data from the forerunner of the HDR (the earlier mentioned NISHC) on the use of hospital resources by CP patients suggest that approximately 6% of all admissions are single-day admissions. Considering that individual CP patients frequently need multiple single-day admissions, once under such treatment, it is likely that at the person count level, the degree of underestimation is even less than 6%.

In conclusion, we observed an increase in the incidence rate of AP and a fluctuating incidence rate of CP between 2000 and 2005 in the Netherlands. On average, the mortality rate for both AP and CP remained fairly stable between 1995-2005. Both incidence and mortality rates increase markedly by age and are lower for international standard populations. Therefore, in light of the continuing ageing of the Dutch population, patient burden and health care costs will most probably increase.

COMMENTS

Background

Over the last decades, the incidence and number of hospital admissions of both acute pancreatitis (AP) and chronic pancreatitis (CP) have consistently increased in the Western countries. AP and CP are associated with significant morbidity and mortality and a substantial use of health care resources.

Research frontiers

Large scale epidemiological studies reporting time trends of incidence and mortality of CP are strikingly scarce compared to the also limited epidemiological studies on AP. Mostly, national registries are used in isolation for epidemiological studies on AP and CP. Joint application of these registries by record linkage at the level of the individual patients provides a unique opportunity for improving the accuracy of epidemiological data.

Innovations and breakthroughs

By following linkage of three distinct nationwide Dutch registries, the authors report in a more valid way the incidence and mortality rates of AP and CP (now for the first time) in the Netherlands. Standardizations were performed with reference to age distribution of the European and World Health Organization standard populations to enable direct comparisons among trends in incidence and mortality rates across countries that differ by age and sex distributions of their respective populations.

Applications

The incidence rates of AP steadily increased while the incidence of CP fluctuated. Population mortality for both AP and CP remained fairly stable. Both, incidence and mortality rates increased markedly with age. In ageing populations, it is to be expected that patient burden and health care costs will increase.

Terminology

The disease spectrum of AP ranges from mild and self-limiting to a life-threatening illness. CP is characterized by ongoing or recurrent episodes of abdominal pain accompanied by progressive pancreatic exocrine and endocrine insufficiency. Main etiological factors are gallstones and alcohol abuse.

Peer review

This is a descriptive study of the trends in incidence and mortality of AP and CP from three linked nationwide Dutch registries for the years 1995-2005. The databases included the hospital discharge registry, the population registry, and the death certificate registry. The authors discuss the limitations of each database and the methods to link them. Given these limitations, major findings included the observation that the incidence of AP increased in both males and females, but the mortality rates remained stable. The incidence and mortality of AP and CP increased with age, which has implications for future incidence in an aging population.

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ORIGINAL ARTICLE

Matrix metalloproteinase-9 in the initial injury after hepatectomy in mice

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Abstract

AIM: To investigate the role of matrix metalloproteinase (MMP)-9 in the pathogenesis of postoperative liver failure (PLF) after extended hepatectomy (EH).

METHODS: An insufficient volume of the remnant liver (RL) results in higher morbidity and mortality, and a murine model with 80%-hepatectomy was used. All investigations were performed 6 h after EH. Mice were first divided into two groups based on the postoperative course (*i.e.*, the PLF caused or did not), and MMP-9 expression was measured by Western blotting. The source of MMP-9 was then determined by immunohistological stainings. Tissue inhibitor of metalloproteinase (TIMP)-1 is the endogenous inhibitor of MMP-9, and MMP-9 behavior was assessed by the experiments in wild-type, MMP-9(-/-) and TIMP-1(-/-) mice by Western blotting and gelatin zymography. The behavior of neutrophils was also assessed by immunohistological stainings. An anti-MMP-9 monoclonal antibody and a broad-spectrum MMP inhibitor were used to examine the role of MMP-9.

RESULTS: Symptomatic mice showed more severe PLF (histopathological assessments: 2.97 ± 0.92 *vs* 0.11 ± 0.08, $P < 0.05$) and a higher expression of MMP-9 (71085 ± 18274 *vs* 192856 ± 22263, $P < 0.01$). Non-native leukocytes appeared to be the main source of MMP-9, because MMP-9 expression corresponding with CD11b positive-cell was observed in the findings of immunohistological stainings. In the histopathological findings, the PLF was improved in MMP-9(-/-) mice (1.65% ± 0.23% *vs* 0.65% ± 0.19%, $P < 0.01$) and it was worse in TIMP-1(-/-) mice (1.65% ± 0.23% *vs* 1.78% ± 0.31%, $P < 0.01$). Moreover, neutrophil migration was disturbed in MMP-9(-/-) mice in the immunohistological stainings. Two methods of MMP-9 inhibition revealed reduced PLF, and neutrophil migration was strongly disturbed in MMP-9-blocked mice in the histopathological assessments (9.6 ± 1.9 *vs* 4.2 ± 1.2, $P < 0.05$, and 9.9 ± 1.5 *vs* 5.7 ± 1.1, $P < 0.05$).

CONCLUSION: MMP-9 is important for the process of PLF. The initial injury is associated with MMP-9 derived from neutrophils, and MMP-9 blockade reduces PLF. MMP-9 may be a potential target to prevent PLF after

EH and to overcome an insufficient RL.

Key words: Matrix metalloproteinase; Shear stress; Sinusoidal injury; Hepatectomy; Portal hypertension

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INTRODUCTION

Liver resection is considered the standard treatment for primary malignant tumors and liver metastases. Currently, advanced surgical techniques for hepatectomy, development of preoperative evaluation and improvements in intensive postoperative care have resulted in a decline in perioperative morbidity and mortality. However, postoperative liver failure (PLF) still occurs despite these developments. Extended hepatectomy (EH) has the advantage of high curability, but increases morbidity and mortality compared with more limited resections[1]. The volume of the remnant liver (RL) is correlated with perioperative morbidity and mortality[1]. PLF is also related to the patient's condition[2]. The rationale for PLF is the overall recovery course after EH. Prognosis of PLF in insufficient RL after EH is poor[1,2].

The mechanism of progressive PLF after EH is not fully understood. The main pathway of failure of liver regeneration is still controversial, and it is unclear how excessive apoptosis or progressive necrosis is attributable to insufficient RL after EH. Matrix metalloproteinases (MMPs) are a family of zinc-containing neutral proteases that are capable of degrading the extracellular matrix and basement membrane. Among MMPs, MMP-9, also known as gelatinase B, is well characterized[3-5]. Expression of MMP-9 is reported to be associated with several pathophysiological conditions, such as rheumatoid arthritis[6], atherosclerosis[6], encephalopathy[7] and tumor invasion[8,9]. Recent studies have demonstrated that MMP-9 plays a pivotal role in ischemia/reperfusion injury in the liver transplantation field[4,10]. We hypothesized that MMP-9 plays a role in the pathogenesis of PLF after EH accompanied by postoperative shear stress due to portal hypertension and insufficient RL, and we previously reported the results of our preliminary study[11]. In this study, we examined the initial pathway of necrosis in the PLF process and MMP-9 expression in the early phase after EH by using an experimental mice model. In addition, we evaluated the efficacy of MMP-9 blockade on initial injury in the liver by deleting MMP-9 in the mouse, using a monoclonal antibody and a broad spectrum MMP inhibitor.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice [wild-type (WT), 10-14 wk old] purchased from Jackson Laboratory (Bar Harbor, ME), were housed in a conventional mouse room with a 12-h light/dark cycle, food, and water. MMP-9(-/-) mice, which were a gift from Dr. Roberts Senior (Washington University, St. Louis, MO), and tissue inhibitor of metalloproteinase (TIMP)-1(-/-) mice, which were purchased from Jackson Laboratory were used. Both knock-out strains had the background of C57/BL/6, and all mice were bred in our secure animal facility. All experimental protocols were approved by the ethical committee of the Mayo Clinic (Protocol No. IACUC 24907) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Eighty percent-partial hepatectomy in mice

Surgical procedures for the murine hepatectomy model are well established, and our method has been described in detail elsewhere[12]. For 80%-partial hepatectomy (PH), the left posterior, left and right anterior, and right posterior lobes were resected. In brief, under general anesthesia using isoflurane, liver lobes were mobilized after laparotomy. A hemostatic clip (Teleflex Medical, Triangle Park, NC) was applied across the pedicle at the base of the liver lobes instead of ligation, and liver lobes were cut distal to the applied clip. Only laparotomy was performed as the sham control group. Before abdominal closure, 2 mL of warm saline was administered intraperitoneally. Cephalexin (30 mg/kg) and buprenorphine (0.1 mg/kg) were given subcutaneously. To preserve the same quality throughout the series, all procedures for presented data were performed only by Ohashi N. To confirm the presented data, Hori T repeated this study including 90%-PH. Postoperatively, mice were housed under a controlled temperature, humidity, and light with free access to food and water.

First, we performed 90%-PH in 50 WT mice, and all mice died at the early postoperative period. In this study, we employed 80%-PH as the relevant clinical model. Ninety-percent-PH showed greater damage in the preliminary study, but we considered that 10%-RL was clinically irrelevant. Paradoxically, some survivors without any symptoms were observed after 80%-PH.

We then performed 80%-PH in 38 WT mice and sham surgery in 12 WT mice, to evaluate the postoperative course. In the following experiments, 80%-PH was performed with 20 age-matched WT mice, 19 MMP-9(-/-) mice and 20 TIMP-1(-/-) mice.

Focal and/or patchy necrosis is an important finding after PH[13-16], and progressive necrosis is found from the early postoperative period after EH[13,14,16]. In this study, all of the mice were sacrificed at 6 h after 80%-PH. The RL was harvested and divided for histological analysis (formalin and frozen fixation) and protein analysis (snap frozen at -80 ℃). Serum samples were collected and sep-

arated by using Microtainer tubes (BD, Franklin Lakes, NJ).

MMP-9 inhibition experiment using anti-MMP-9 monoclonal antibody and the broad-spectrum MMP inhibitor GM6001

The WT mice were treated with 3 mg/kg of anti-MMP-9 neutralizing monoclonal antibody (clone 6-6B, EMD, Gibbstown, NJ) by intravenous injection 1 h before 80%-PH (MMP-9 mAb group, n = 6). In the control mice, the same volume of non-immunized murine IgG of the same isotype (EMD, Gibbstown, NJ) was injected in the same manner (control IgG group, n = 6). In another experiment, a broad spectrum MMP-inhibitor, GM6001 (Millipore, Billerica, MA) (100 mg/kg), diluted in 10% dimethyl sulfoxide (DMSO) was administrated intraperitoneally 2 h before 80%-PH (GM6001 group, n = 10). Ten percent DMSO was injected in the control mice in the same manner as for GM6001 (vehicle group, n = 10).

Biochemical analysis

Serum levels of aspartate aminotransferase (AST) and alanine aminotran-saminase (ALT) were determined by a commercially available kinetic detection kit (Pointe Scientific, INC, Canton, MI), and total bilirubin (T-Bil) levels were determined by the QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems, Heyward, CA).

Western blotting analysis

Liver samples were homogenized in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylene diamine tetra-acetic acid (EDTA), 1 mmol/L ethylene glycol tetra-acetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, and protease and phosphatase inhibitors. Homogenates were centrifuged at 105000 *g* for 1 h at 4 ℃. Supernatants were collected and protein concentration was determined by BCA assay (Pierce, Rockford, IL). Forty micrograms of protein was separated *via* SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in TBS-T [20 mmol/L Tris (pH 7.4), 500 mmol/L NaCl, and 0.05% Tween-20] and probed using an antibody for MMP-9 (R and D, Minneapolis, MN), and then they were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) followed by enhanced chemi-luminescence (ECL) or ECL-plus reagent (Amersham Biosciences, Piscataway, NJ). Equal loading was confirmed by immunoblotting using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (IMGENEX, San Diego, CA) on the same membrane. Signals were quantified using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Gelatin zymography

The RL extracts were analyzed by gelatin zymography with affinity chromatography to characterize gelatinase activity. In brief, 400 μg of extract samples were incubated with 100 μL of Gelatin-Sepharose 4B (GE Healthcare) and equilibrated buffer containing 50 mmol/L Tris-HCL pH 7.5, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.02% Tween-20, and 10 mmol/L EDTA for 2 h at 4 ℃. After multiple washing, gelatin-Sepharose beads were resuspended in the same volume of 2X zymography sample buffer (Bio-Rad Laboratories, Hercules, CA) and loaded on 10% SDS-PAGE gels containing 1 mg/mL of gelatin (Bio-Rad Laboratories) After electrophoresis, the gel was washed with 2.5% Triton X-100 for renaturing twice for 30 min, and it was then incubated in development buffer (Bio-Rad Laboratories) for 20 h at 37 ℃. After incubation, the gel was fixed and stained with 0.5% Coomassie Blue R-250 (Bio-Rad Laboratories) for 1 h and destained with 10% acetic acid in 40%-methanol solution. Gelatinase zymography standards (Millipore, Billerica, MA) were used for the positive control.

Histology and immunohistochemical staining

Formalin-fixed liver specimens were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin (HE). Immunohistochemical (IHC) staining for CD11b (MAC-1) and CD68 was performed on frozen sections (5 μm), while paraffin sections were used for desmin, myeloperoxidase (MPO) and MMP-9 single staining. Antigen retrieval heating with citric acid (pH 6.0) was performed after deparaffinization with paraffin sections. For horseradish peroxidase based staining with 3,3' diaminobenzidine tetrahydrochloride (DAB) (DAKO, Carpinteria, CA), 0.3% H_2O_2 was added to quench endogenous peroxidase activity, and an ABC kit (Vector Laboratories Inc, Burlingame, CA) was used during the staining procedure according to the manufacturer's instructions. For dual staining, sections were blocked with 5% bovine serum albumin (BSA) in PBS. The antibodies for MMP-9 (AF909), intercellular adhesion molecule 1 (ICAM-1) (AF796), CD11b (clone M1/70) (R and D, Minneapolis, MN), CD68 (clone FA-11), desmin (Abcam, Cambridge, MA), and MPO (Ab-1) (Thermo scientific, Fremont, CA) were incubated for the primary reaction. Alexa Fluor 568 donkey anti-goat IgG (H + L), Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-rat antibody (Invitrogen, Carlsbad, CA) were incubated for the secondary reaction. In each experiment, BSA solutions without antibodies were applied for a negative control.

Histological analysis

The HE sections were digitally scanned with the Scanscope XT system and analyzed with Aperio Imagescope software (Aperio Technologies, Inc., Vista, CA). Histological areas were blindly counted and measured with three randomly selected fields approximately 3 mm^2 in each section. The positivity of DAB staining for MMP-9 was automatically calculated using the positive pixel count function with Imagescope software with 10 ran-

domly selected fields with the reference to the presence of necrosis, respectively. The value was indicated by the percentage of positive pixels out of the total pixels. The number of infiltrated granulocytes was evaluated in five randomly-captured 40 high power fields in each section with an Olympus BX50 fluorescence microscope (Olympus Optical, Tokyo, Japan).

In situ zymography

In situ gelatinolytic activity was performed on cryostat sections (20-μm thickness) using the EnzChek Gelatinase assay kit (Molecular Probes, Eugene, Oregon, United States). Sections were incubated with 20 μg/mL fluorescent conjugated gelatin (DQ gelatin) in reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.2 mmol/L sodium azide) for 2 h at 37 ℃. After being washed with PBS three times, they were fixed in 4% paraformaldehyde in PBS. We incubated sections with GM6001 (100 μmol/L) prior to DQ gelatin incubation as a negative control in each experiment. Sections were mounted with Vectashield (Vector Laboratories Inc, Burlingame, CA). Gelatinase activity was visualized using fluorescent microscopy (Olympus BX50, Japan).

Statistical analysis

Data are presented as the mean ± SE. Statistical comparisons were performed using ANOVA followed by the two-sample *t*-test with Bonferroni adjustment. A P value < 0.05 was considered statistically significant.

RESULTS

Necrosis is a pivotal event for PLF in the RL after 80%-PH

Murine behavior in hepatic failure has been well described[17,18]. Mice with intrahepatic-hemorrhage and necrosis were observed to be sick and inactive, whereas mice without such injury were asymptomatic and active. We classified these mice into groups based on these findings as follows below. Mice were divided into two groups; asymptomatic (Group Ⅰ) and symptomatic (Group Ⅱ).

We performed 80%-PH in 38 WT mice and sham surgery in 12 WT mice to compare the difference in the RL with or without PLF at several time points after 80%-PH. Among these mice, we randomly selected five asymptomatic mice which were active, nimble and reactive to stimulation, and six symptomatic mice which were sick and inactive, slow-moving and sluggish 6 h after 80%-PH. As shown in Figure 1A and B, multiple necrotic foci with microhemorrhage in the middle zone were observed in the symptomatic mice, while a small amount of necrosis was observed in the asymptomatic mice. These HE findings are consistent with a previous report[19]. We measured the area of necrosis as an objective marker of liver injury. The area of necrosis (2.97% ± 0.92% *vs* 0.11% ± 0.08%, $P < 0.05$) (Figure 1C) and the number of necrotic foci (3.60 ± 0.87/mm^2 *vs* 0.26 ± 0.17/mm^2, $P < 0.01$) in the RL were significantly larger in symptomatic mice than those in asymptomatic mice (Figure 1D). Serum levels of AST (785 ± 15 IU/L *vs* 452 ± 39 IU/L, $P < 0.01$) (Figure 1E), ALT (744 ± 135 IU/L *vs* 328 ± 77 IU/L, $P < 0.05$) (Figure 1F), and T-Bil (4.45 ± 0.63 mg/dL *vs* 1.41 ± 0.19 mg/dL, $P < 0.01$) (Figure 1G) were significantly higher in symptomatic mice than those in asymptomatic mice. These results clearly indicated that symptomatic mice were in the process of PLF as shown by the increase in AST and ALT levels and hyperbilirubinemia. In some mice, which failed to recover even 6 h after 80%-PH, multiple necroses might have been responsible for the process of PSL in this model. Enhanced necrosis was observed in the RL at the late phase (12 h or later, data not shown), and these preliminary data confirmed that the necrotic pathway is progressive. In addition, intrahepatic-hemorrhage was observed in most of the necrotic lesions, which suggested the possibility that the initial sinusoid endothelial cell injury leading to sinusoidal breakdown is attributable to necrosis in the RL. In our model, dehiscence of sinusoids and loss of continuity of endothelial cells were observed in ICAM-1 immunostaining, which was strongly positive in endothelial cells (Figure 1H).

We preformed retrospective analysis of perioperative factors including the resected liver weight/body weight and surgery time with 11 mice whose prognosis was predictable. Perioperative factors were not different between asymptomatic and symptomatic mice, but the resected liver weight/body weight ratio in the symptomatic mice tended to be larger than that in the asymptomatic mice (3.8% ± 0.1% *vs* 3.5% ± 0.1%, $P = 0.06$). It is possible that the extent of hepatectomy is an important factor for postoperative liver injury after EH.

Macro- and microscopically, we confirmed the preservation of vascularization in the RL, immediately after 80%-PH by sodium fluorescent perfusion via the portal vein and abdominal aorta, to exclude model specific effects including surgical issues. In addition, we observed similar histological findings after 80%-PH with the traditional suture ligation method[13] (data not shown). These results demonstrated that the process of PSL after EH was widely and histologically characterized by RL necrosis, and was probably initialized as early onset sinusoid breakdown following progressive necrosis of hepatocytes.

MMP-9 expression is enhanced in RL dysfunction 6 h after 80%-PH and is associated with liver necrosis

We compared MMP-9 expression in the RL between asymptomatic mice and symptomatic mice at 6 h. Enhanced MMP-9 expression was confirmed in symptomatic mice after EH compared with that in the asymptomatic mice after EH and sham surgery mice as shown by Western blotting analysis ($P < 0.01$) (Figure 2). IHC showed that MMP-9 expression was mainly observed in round-shaped cells in the liver parenchyma and necrotic areas, and was less common in stellate-shaped cells. A small amount of MMP-9 expression was observed in hepatocytes compared with the above-mentioned cells (Figure 3A and B). To evaluate the association of necrosis and MMP-9 in liver tissue, we compared the expression level of MMP-9 between 10 randomly selected fields (3 mm^2)

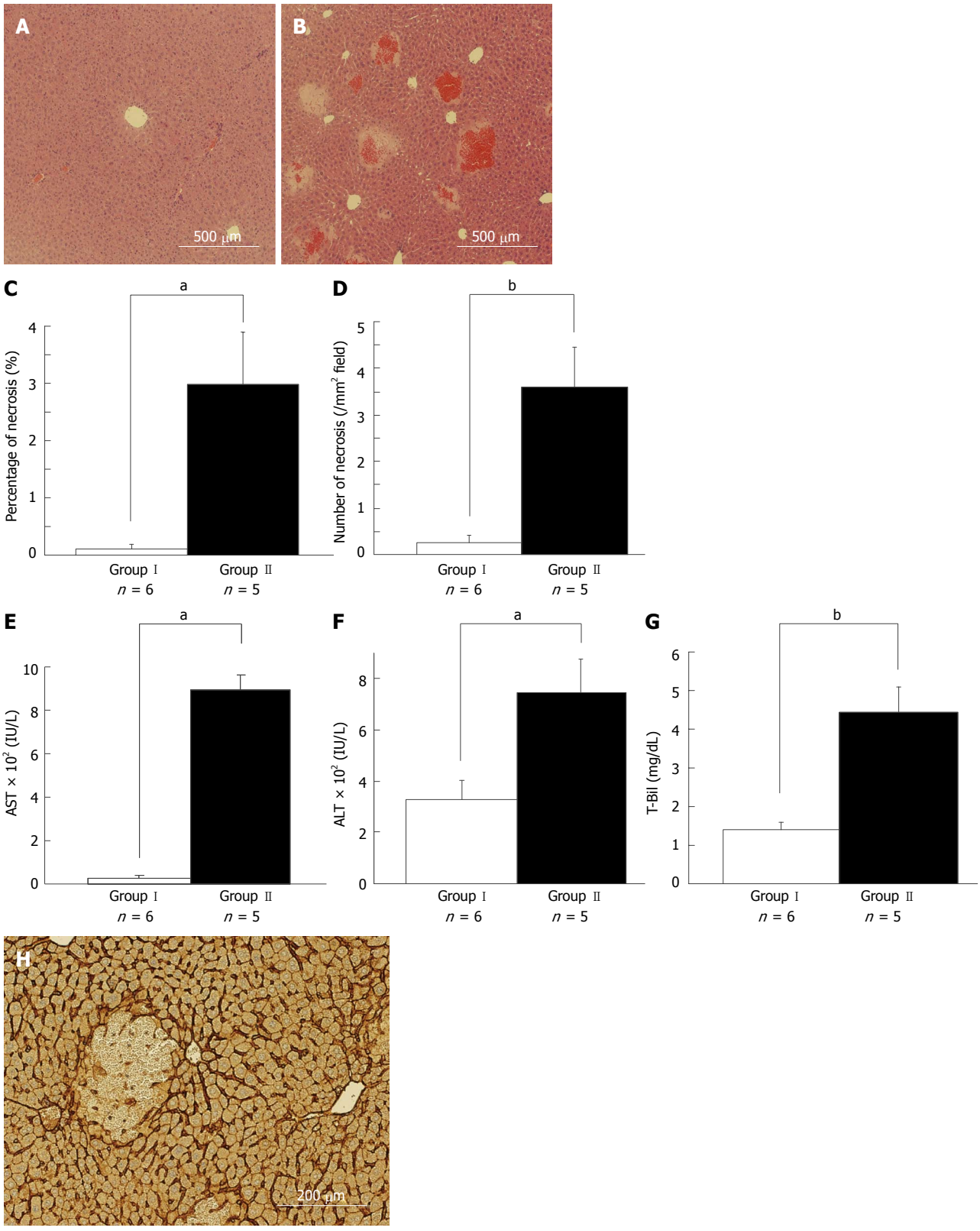


Figure 1 Necrosis is a pivotal event for postoperative liver failure in the remnant liver after 80%-partial hepatectomy. There were histological and hematological differences between asymptomatic and symptomatic mice suspected as having postoperative liver failure 6 h after 80%-partial hepatectomy (PH) (A, B). Representative images of the remnant liver (RL) 6 h after 80%-PH in asymptomatic mice (A) and symptomatic mice (B) are shown. Significant multiple necroses with microhemorrhage were observed in the middle zone in symptomatic mice (B), while no obvious liver damage was observed in asymptomatic mice (A). The percentage area of necrosis (C) and the number of necrosis in each mm^2 (D) in the RL were confirmed. Necrotic areas were significantly larger and more prevalent in symptomatic mice liver compared with asymptomatic mice. Serum levels of aspartate aminotransferase (AST) (E), alamine aminotransferase (ALT) (F) and total bilirubin (T-Bil) (G) 6 h after 80%-PH are shown. AST, ALT and T-Bil levels were significantly elevated in symptomatic mice compared with those in asymptomatic mice. Representative image of immunohistochemistry of intercellular adhesion molecule-1 (ICAM-1) in the RL 6 h after 80%-PH is shown (H). ICAM-1 expression was clearly observed, particularly in the sinusoid lining in areas without necrosis. We observed a breakdown in sinusoid structure with hemorrhage in necrotic areas after 80%-PH in mice, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* asymptomatic mice.

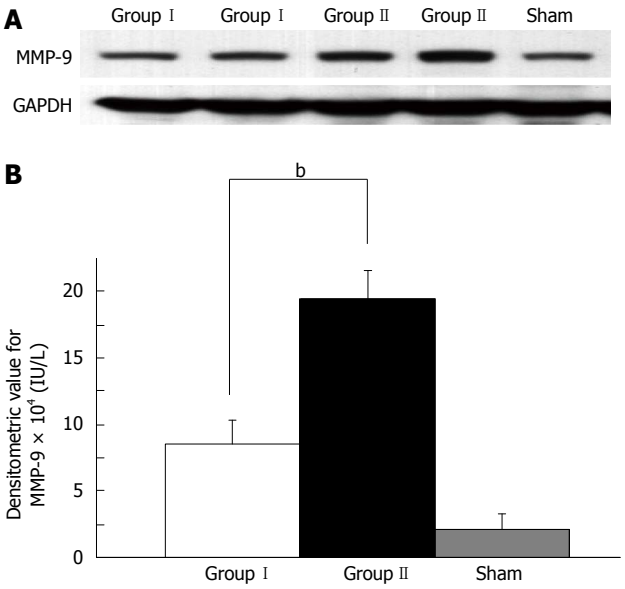


Figure 2 Western blotting analyses for matrix metalloproteinase-9 were performed in the remnant liver in asymptomatic mice and symptomatic mice after 80%-partial hepatectomy. Representative image (A) and histograms (B) of Western blotting analyses for matrix metalloproteinase-9 (MMP-9) are shown. MMP-9 protein expression in the liver was enhanced in symptomatic mice in the process of postoperative liver failure compared with that in asymptomatic mice after extended hepatectomy and sham-treated mice (^bP < 0.01 between Groups Ⅰ and Ⅱ).

of areas with necrosis and those without necrosis in IHC sections. Enhanced MMP-9 expression was observed in the areas including necrosis compared with those without necrosis ($P < 0.05$, Figure 3C). This result suggested that MMP-9 expression is associated with necrosis in the RL after EH.

Main source of MMP-9 is CD11b-positive nonnative leukocytes in the RL 6 h after 80%-PH

To determine the source of MMP-9, we performed dual immunofluorescent analysis with MMP-9 and various cell marker antibodies in the liver. CD68 and desmin were used as specific markers for Kupffer cells and hepatic stellate cells in immunostaining, respectively. MMP-9 expression was not observed in CD68-positive Kupffer cells, while MMP-9 expression was partially confirmed in desmin-positive hepatic stellate cells (Figure 4A-F). In addition, we clearly detected MMP-9 expression corresponding with CD11b positive-cells, which are generally expressed in nonnative leukocytes in the liver including monocytes, granulocytes and macrophages (Figure 4G-I). These results suggested that MMP-9 was expressed in the RL 6 h after 80%-PH, and it appeared to be mainly produced by leukocytes that had migrated from the outside of the liver, similar to that described in a liver ischemia/reperfusion injury model[20].

MMP-9 deletion ameliorates initial injury in the RL 6 h after 80%-PH

TIMP-1 is an endogenous inhibitor of MMP-9. We performed 80%-PH in 20 WT, 19 MMP-9(-/-) and 20

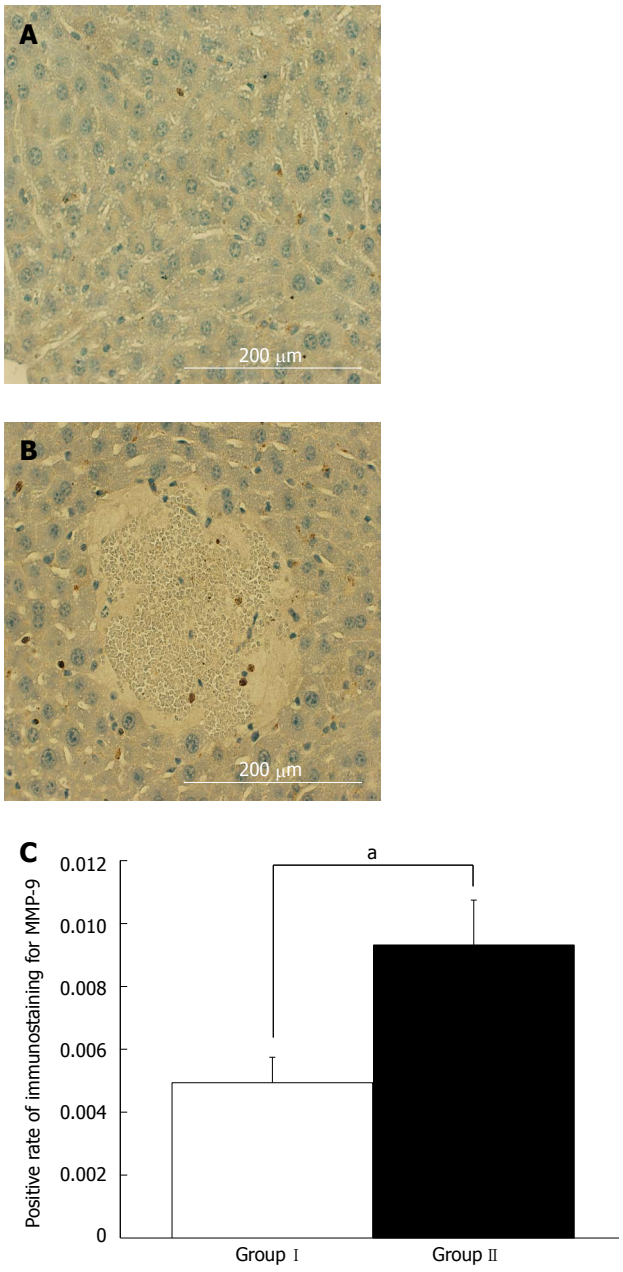


Figure 3 Immunohistochemical analysis for matrix metalloproteinase-9 in the remnant liver in symptomatic mice after 80%-partial hepatectomy was performed. A: Representative images of matrix metalloproteinase-9 (MMP-9) staining in an area without necrosis; B: A necrotic area in the same sample are shown. Histograms of the results of distribution analysis show MMP-9 expression; C: Enhanced expression was observed in the areas close to necrosis compared with areas without necrosis in damaged RL (^aP < 0.05 between Groups Ⅰ and Ⅱ).

TIMP-1(-/-) mice to compare the difference in the RL 6 h after 80%-PH. Among these mice, we randomly selected samples from 15 WT, 15 MMP-9(-/-) and 14 TIMP-1(-/-) mice. To evaluate the effects of MMP-9 on the initial injury in the RL, we compared the area (percentage) of necrosis and the number of necrotic foci in the RL among these samples. There were significantly fewer and smaller necrotic lesions in MMP-9(-/-) mice compared with WT mice and TIMP-1(-/-) mice (area, WT: 1.65% ± 0.23%; MMP-9(-/-): 0.65% ± 0.19%;

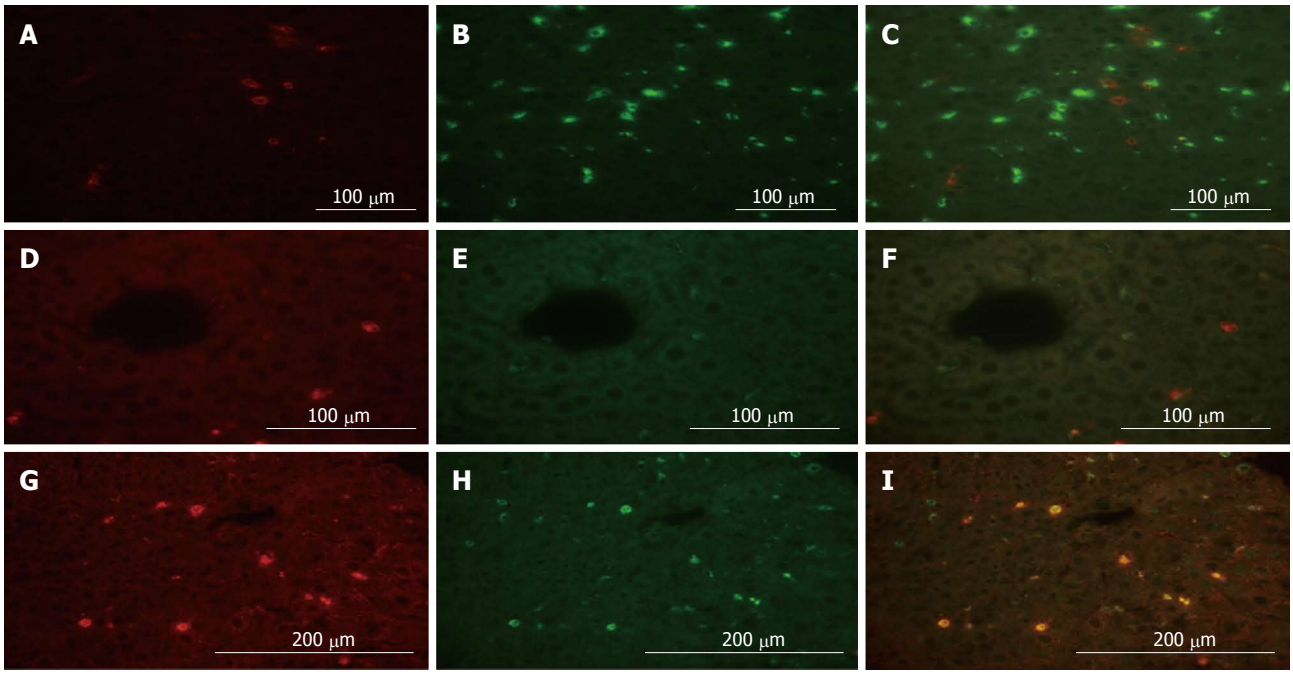


Figure 4 Localization analysis of matrix metalloproteinase-9 by dual immune-fluorescence in the remnant liver after 80%-partial hepatectomy is shown. A: Fluorescent microscopy images of matrix metalloproteinase-9 (MMP-9) labeled in red (Alexa Fluor 568); B: CD68 labeled in green (Alexa Fluor 488); C: A merged image of MMP-9 and CD68 staining are shown; D: MMP-9 labeled in red (Alexa Fluor 568); E: Desmin labeled in green (Alexa Fluor 488); and F: A merged image of MMP-9 and desmin are shown; G: MMP-9 labeled in red (Alexa Fluor 568); H: CD11b labeled in green (Alexa Fluor 488); I: A merged image of MMP-9 and CD11b are shown. Protein expression of MMP-9 was mainly localized in CD11b-positive cells and desmin-positive cells to some degree.

TIMP-1(-/-): 1.78% ± 0.31%; MMP-9(-/-) *vs* WT: $P < 0.01$; MMP-9(-/-) *vs* TIMP-1(-/-): $P < 0.01$), (number of necrotic foci, WT: 1.34 ± 0.20/mm^2; MMP-9(-/-): 0.68 ± 0.16/mm^2; TIMP-1(-/-): 1.50 ± 0.30/mm^2; MMP-9(-/-) *vs* WT: $P < 0.05$; MMP-9(-/-) *vs* TIMP-1(-/-): $P < 0.05$) (Figure 5). There was no significant difference in necrosis between WT and TIMP-1(-/-) mice. The levels of AST (WT: 520 ± 69 IU/L; MMP-9(-/-): 482 ± 76 IU/L; TIMP-1(-/-): 636 ± 58 IU/L) and ALT (WT: 544 ± 73 IU/L; MMP-9(-/-): 466 ± 92 IU/L; TIMP-1(-/-): 566 ± 54 IU/L) were lowest in MMP-9(-/-) mice, but this was not statistically significant among the groups. Additionally, T-Bil levels showed a similar tendency (WT: 2.72 ± 0.36 mg/dL; MMP-9(-/-): 2.23 ± 0.27 mg/dL; TIMP-1(-/-): 2.76 ± 0.28 mg/dL). In Western blotting and gelatin zymography analysis, MMP-9 deletion of protein expression and activity in MMP-9(-/-) mice were confirmed. Protein expression of MMP-9 in WT mice was similar to that in TIMP-1(-/-) mice (Figure 6A). In gelatin zymography analysis, a distinct pro-form of MMP-9 and a slightly active form of MMP-9 were detected in WT and TIMP-1(-/-) mice (Figure 6B). No difference in MMP-9 activity was detected in the genotypes. Since there was no change in MMP bands among the groups because of the principle of protein separation by SDS-PAGE in zymography, we performed *in situ* gelatin zymography to examine the gelatinolytic activity *in situ*. We observed reduced gelatinolytic activity in MMP-9(-/-) mice and enhanced activity in TIMP-1(-/-) mice compared with that in WT mice (Figure 6C-E). MMP-9 deletion, which suppresses gelatinolytic activity, was associated with the amelioration of formation of necrosis and necrotic progression in the RL after 80%-PH. However, TIMP-1 deletion, which induces the enhancement of gelatinolytic activity, did not exacerbate initial injury of the RL after EH. These results suggested that the involvement of TIMP-1 in inhibition of MMP activity appeared to be limited to PLF 6 h after EH. We investigated survival after 80%-PH was performed in WT, MMP-9(-/-) and TIMP-1(-/-) mice, but a significant survival benefit was not observed by MMP-9 deletion ($P \geqslant 0.05$).

MMP-9 deletion inhibits the accumulation of neutrophils in the RL 6 h after 80%-PH

To evaluate the effect of MMP-9 deletion on cell kinetics, we investigated whether neutrophil migration after 80%-PH has an effect on the RL in WT, MMP-9(-/-) and TIMP-1(-/-) mice. We performed immunofluorescence staining for myeloperoxidase (MPO) to investigate this issue. As shown in Figure 7A, MPO-positive neutrophils could be easily recognized by characteristic staining patterns in cytoplasmic azurophilic granules, and MPO was also positive in liver tissue macrophages (Kupffer cells)[21]. Because of the difficulty in distinguishing between infiltrated neutrophils in the liver parenchyma and those that were adhered to the sinusoidal wall, accumulated neutrophils were counted in liver tissue. We observed significantly fewer neutrophils in MMP-9(-/-) mice (3.3 ± 0.4) than in WT (7.6 ± 1.3) and TIMP-1(-/-) mice (6.1 ± 1.0; each value per 40 high-power field; WT *vs* MMP-9(-/-), $P < 0.01$; MMP-9(-/-) *vs* TIMP-1(-/-), $P < 0.05$) (Figure 7B-E). There were no differences in ICAM-1 expression

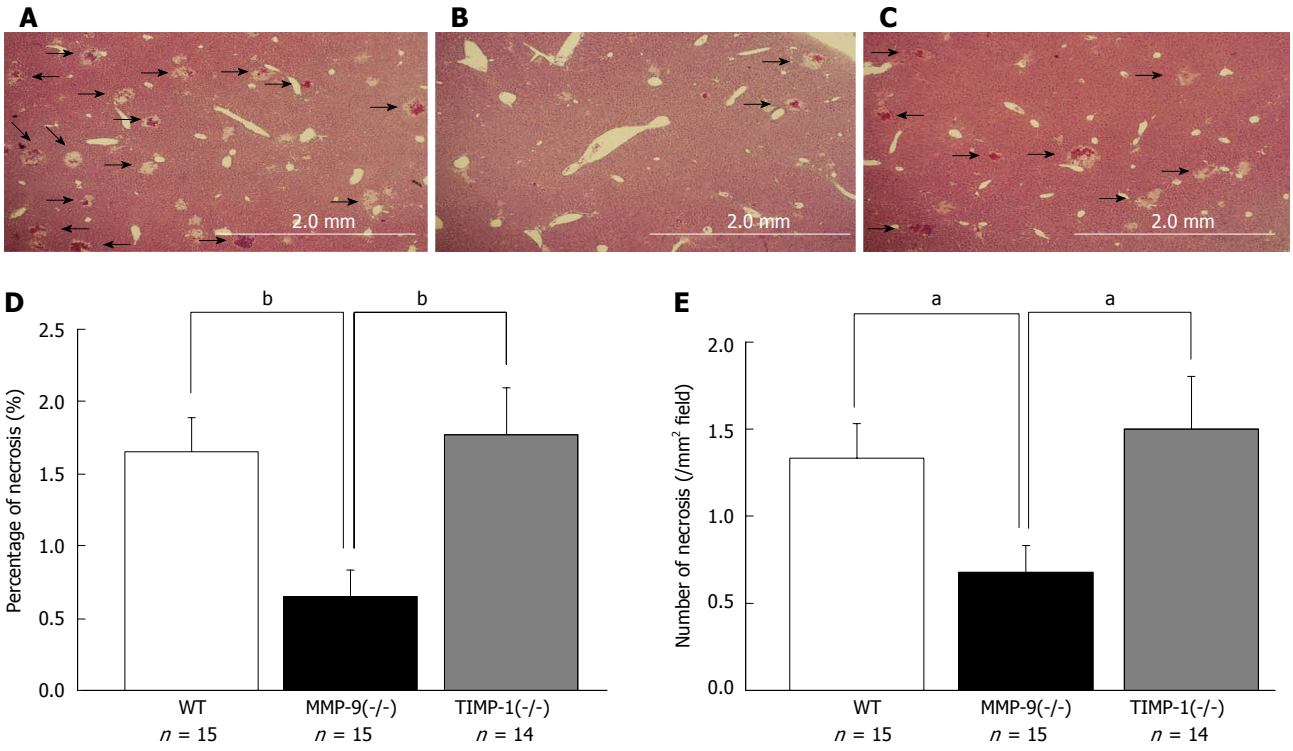


Figure 5 Histological analysis between wild-type, matrix metalloproteinase-9(-/-), and tissue inhibitor of metalloproteinase-1(-/-) mice 6 h after 80%-partial hepatectomy were performed. Representative images of the remnant liver (RL) 6 h after 80%-PH in wild-type (WT) (A), matrix metalloproteinase-9 (MMP-9)(-/-) (B), and tissue inhibitor of metalloproteinase-1 (TIMP-1)(-/-) mice (C) are shown. Samples that included necrosis were selected for this figure. The percentage (D) and the number of necrotic foci per mm^2 (E) of the necrotic area in the RL are shown. We observed significantly smaller and less necrotic foci in MMP-9(-/-) mice compared with WT and TIMP-1(-/-) mice ($^aP < 0.05$, $^bP < 0.01$ *vs* WT and TIMP-1).

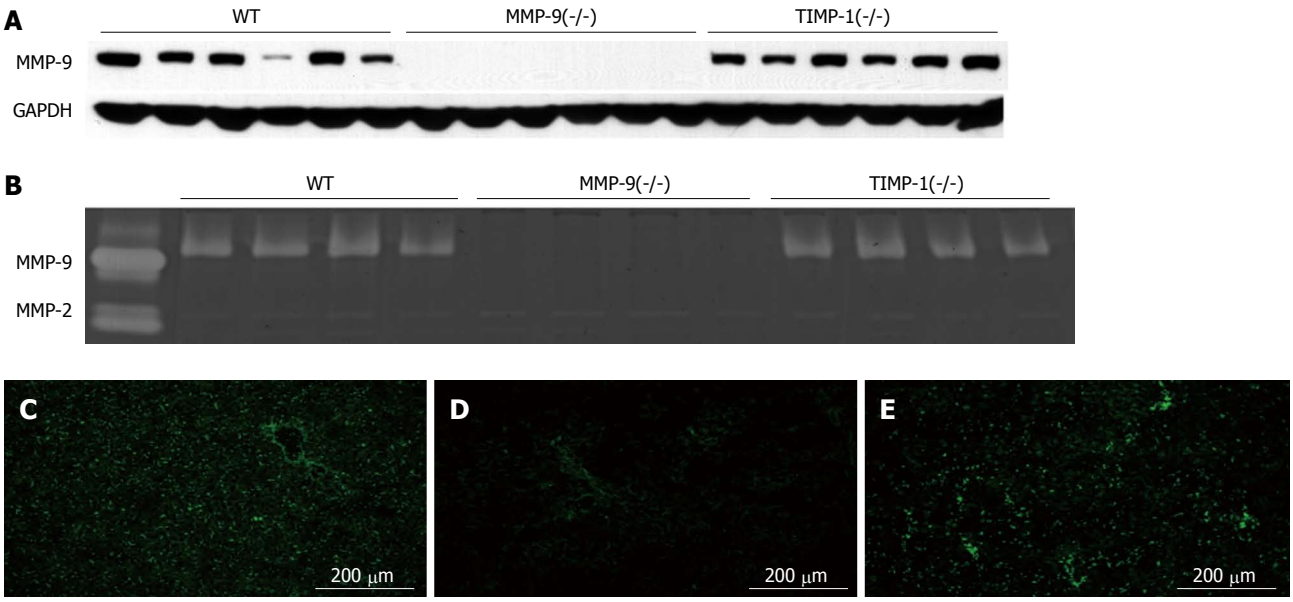


Figure 6 Matrix metalloproteinase-9 activity in the remnant liver with wild-type, matrix metalloproteinase-9(-/-), and tissue inhibitor of metalloproteinase-1(-/-) mice 6 h after 80%-partial hepatectomy is shown. A: Western blotting analysis for matrix metalloproteinase-9 (MMP-9) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for a loading control was performed. The expression of MMP-9 in wild-type (WT) and tissue inhibitor of metalloproteinase-1 (TIMP-1)(-/-) mice and deletion in MMP-9(-/-) mice were observed at the protein level; B: Gelatin zymography analysis is shown; C-E: Pro-form MMP-9 activity and a slightly active form of MMP-9 were detected in WT and TIMP-1(-/-) mice. The activity of MMP-2 was the same depending on its genotype. *In situ* gelatin zymography analysis using DQ gelatin for gelatinolytic activity in the liver tissue is shown. A reduction in gelatinolytic activity in MMP-9(-/-) mice and enhancement in TIMP-1(-/-) mice are observed.

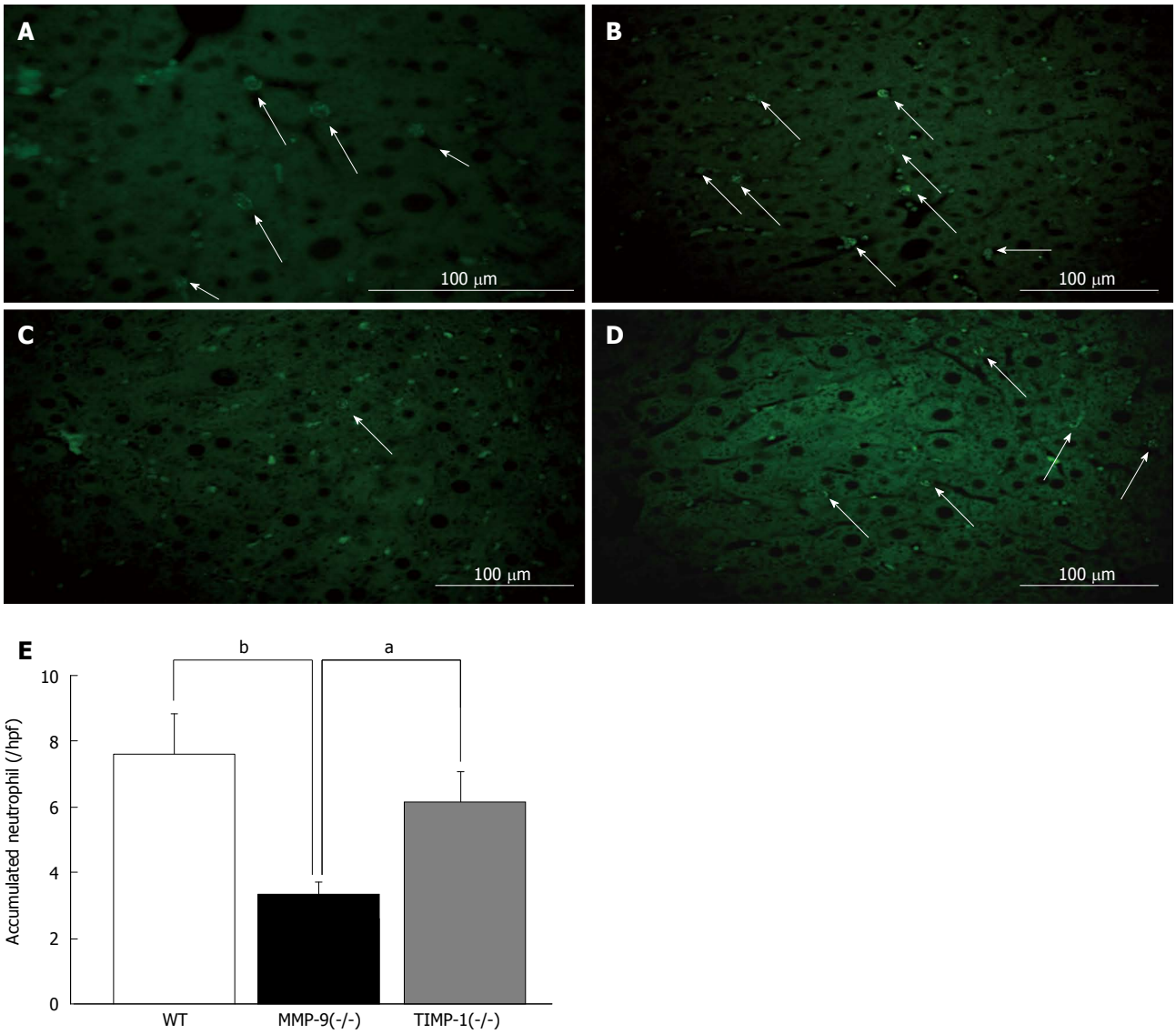


Figure 7 Immunohistochemistry analysis for accumulated neutrophils with myeloperoxidase staining between wild-type, matrix metalloproteinase-9(-/-) and tissue inhibitor of metalloprote-inase-1(-/-) mice 6 h after 80%-partial hepatectomy was performed. Myeloperoxidase (MPO) staining labeled in green (Alexa Fluor 488) is shown (A). Cytoplasmic azurophilic granules were characteristically stained in neutrophils. Representative images of MPO staining on a remnant liver (RL) section in green in wild-type (WT) (B), matrix metalloproteinase-9 (MMP-9)(-/-) (C), tissue inhibitor of metalloproteinase-1 (TIMP-1)(-/-) mice (D) are shown. A histogram of the number of accumulated neutrophils in the liver is shown. We observed significantly fewer neutrophils in the RL in MMP-9(-/-) mice than in WT and TIMP-1(-/-) mice ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* WT and TIMP-1) (E).

among WT, MMP-9(-/-) and TIMP-1(-/-) mice in Western blotting and IHC analysis (data not shown). These results suggested that MMP-9 deletion was associated with a decrease in migrated neutrophils in the liver, which was related to an amelioration of the initial injury in the RL after EH, and probably an infiltrating process seemed to be influenced. Therefore, MMP-9 might play a supportive role for infiltration of neutrophils into the RL at the early phase after EH.

Inhibition of MMP-9 by a monoclonal antibody of MMP-9 and the broad-spectrum MMP inhibitor GM6001 ameliorate initial injury of the liver 6 h after 80%-PH

Investigation of inhibition of MMP-9 *in vivo* was performed by two different methods using a blocking monoclonal antibody and a broad-spectrum MMP inhibitor (GM6001). Each treatment clearly improved survival curves after 80%-PH ($P < 0.01$). In the RL, there were significantly smaller and fewer necrotic lesions in the monoclonal antibody-injected mice (mAb group) than in the control IgG-injected mice (control IgG group) (area: 0.17% ± 0.15% *vs* 1.81% ± 0.66%, $P < 0.05$, Figure 8A; number of foci: 0.23 ± 0.16/mm^2 *vs* 1.23 ± 0.44/mm^2, $P < 0.05$, Figure 8B). Serum levels of AST, ALT and T-Bil were lower in the monoclonal antibody-injected mice than those in the control IgG-injected mice, but this not statistically significant ($P \geqslant 0.05$). We confirmed suppression of gelatinolytic activity in the mAb group by *in situ* gelatin zymography compared with that in the control IgG group (Figure 8C and D). There were significantly

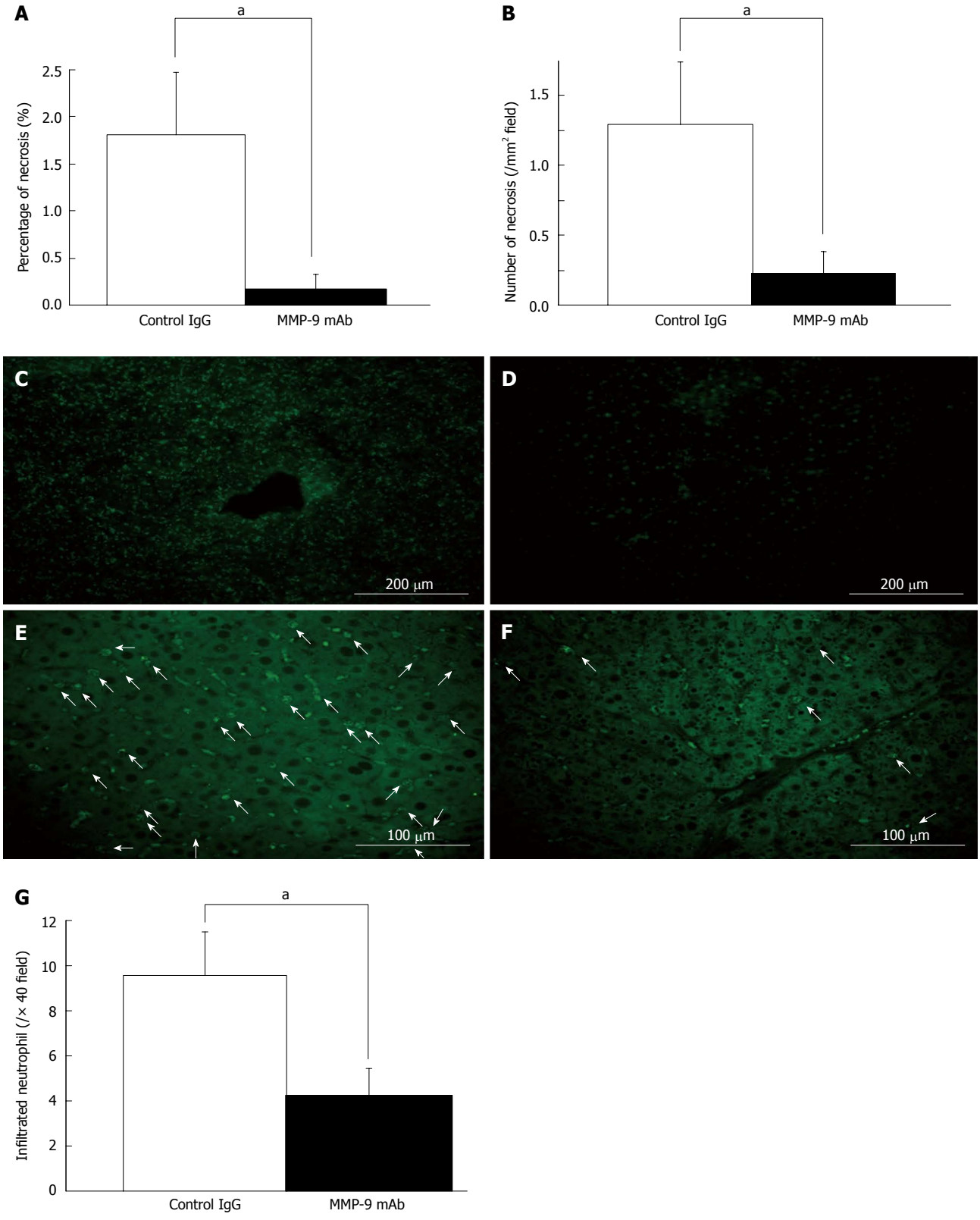
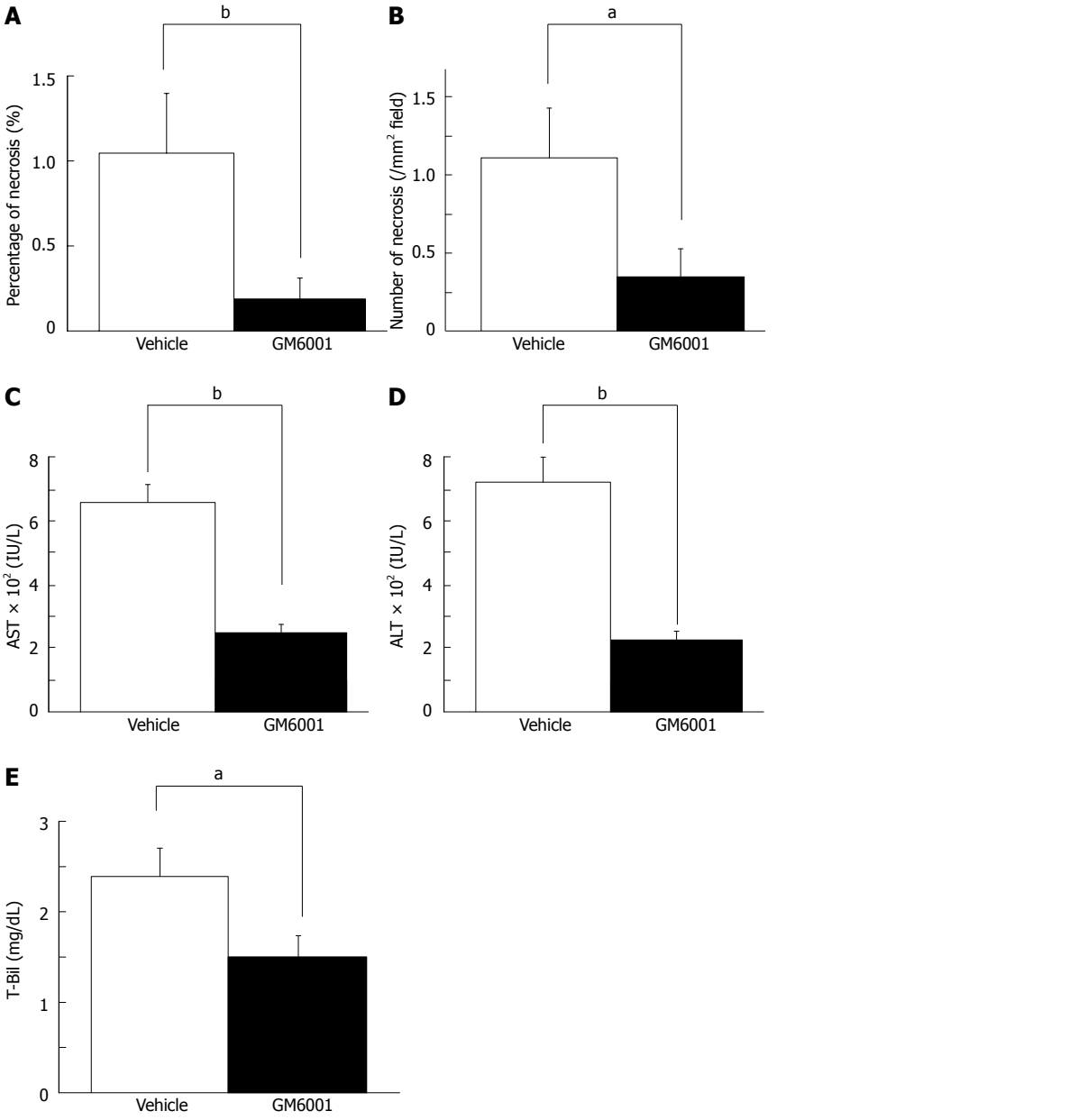


Figure 8 Inhibition of matrix metalloproteinase-9 by a monoclonal antibody of matrix metalloproteinase-9. We investigated the outcome of inhibition of matrix metalloproteinase-9 (MMP-9) by monoclonal antibody (mAb) in 80%-partial hepatectomy (PH) in mice, and performed histological analysis of liver necrosis 6 h after 80%-PH (A, B). Liver damage was significantly reduced in the group with mAb compared with that in the control IgG group. Serum levels of aspartate aminotransferase, alamine aminotransferase and total bilirubin were lower in the mAb-treated mice, but this was not significant. Representative images of *in situ* gelatin zymography analysis with DQ gelatin revealed that gelatinolytic activity was suppressed in the mAb-treated mice (D) compared with that in control IgG-treated mice (C). Representative images of myeloperoxidase staining on remnant liver (RL) sections in control IgG-treated mice (E) and mAb-treated mice (F) are shown. A histogram of the number of accumulated neutrophils in the RL 6 h after 80%-PH shows that there are significantly fewer neutrophils in mAb-treated mice than in IgG-treated controls (G) ($^aP < 0.05$ *vs* IgG group).


A
b
Percentage of necrosis (%)
1.5
1.0
0.5
0
Vehicle
GM6001
B
a
Number of necrosis (/mm2 field)
1.5
1.0
0.5
0
Vehicle
GM6001
C
b
AST × 10^2 (IU/L)
8
6
4
2
0
Vehicle
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D
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ALT × 10^2 (IU/L)
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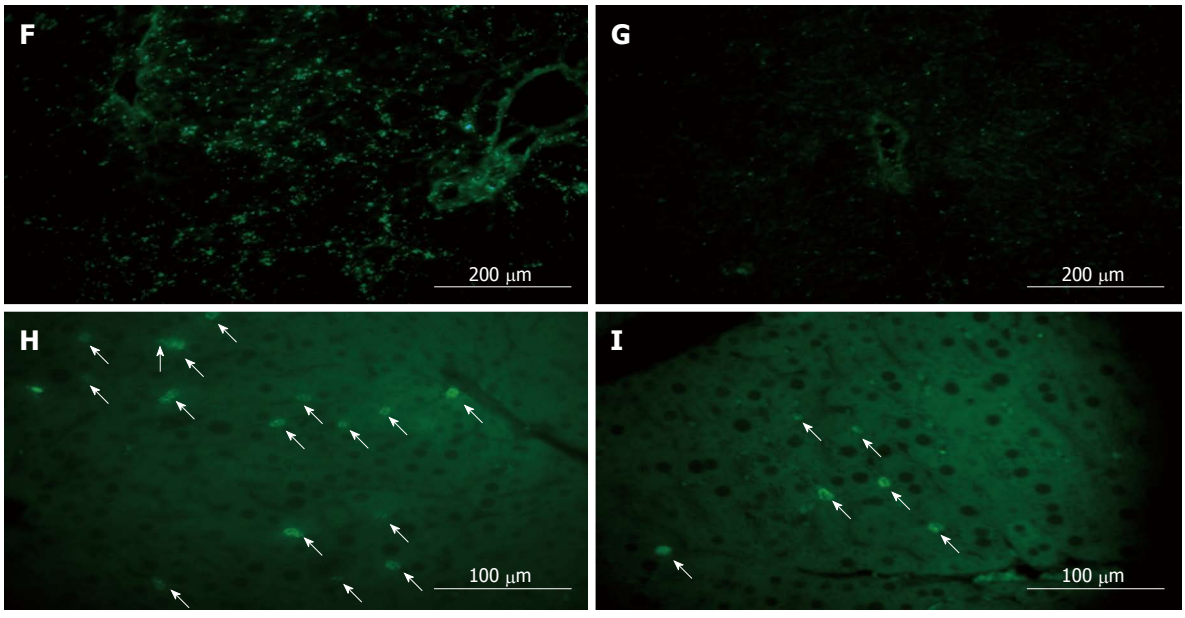
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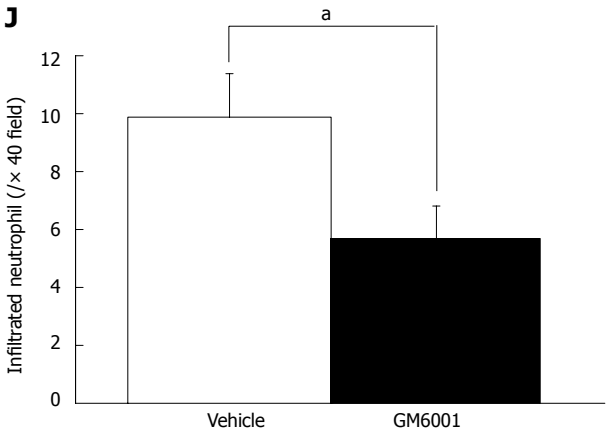

Figure 9 Broad-spectrum matrix metalloproteinase-9 inhibitor GM6001 ameliorate initial injury of the liver 6 h after 80%-partial hepatectomy. We inhibited matrix metalloproteinase-9 by GM6001 in 80%-partial hepatectomy (PH) mice, and determined liver necrosis 6 h after 80%-PH. Liver damage was significantly reduced in the GM6001-treated mice compared with that in the vehicle-treated group (A, B). Serum levels of aspartate aminotransferase (AST) (C), alamine aminotransferase (ALT) (D) and total bilirubin (T-Bil) (E) were significantly lower in the GM6001-treated mice than those in the vehicle-treated mice. Representative images of *in situ* gelatin zymography analysis with DQ gelatin are shown. Gelatinolytic activity was suppressed in the GM6001-treated mice (G) compared with that in the vehicle-treated mice (F). Representative images of myeloperoxidase staining on remnant liver (RL) sections in vehicle-treated mice (H) and GM6001-treated mice (I) are shown. A histogram of the number of accumulated neutrophils in the RL after extended hepatectomy shows that there are significantly fewer neutrophils in the GM6001-treated mice than in the vehicle-treated mice (J) ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* vehicle-treated mice).

fewer accumulated neutrophils in the mAb group than in the control IgG group (mAb: 4.2 ± 1.2 *vs* control IgG: 9.6 ± 1.9, each per 40 high-power field, $P < 0.05$) (Figure 8E-G).

Similarly, the inhibition study by GM6001 showed a significant suppression in the area of liver necrosis (1.04% ± 0.36% *vs* 0.19% ± 0.13%, $P < 0.01$, Figure 9A) and the number of foci compared with the vehicle group (1.12 ± 0.31/mm^2 *vs* 0.35 ± 0.19/mm^2, $P < 0.05$, Figure 9B). The levels of AST (660 ± 66 IU/L *vs* 243 ± 28 IU/L, $P < 0.01$, Figure 9C), ALT (702 ± 88 IU/L *vs* 212 ± 33 IU/L, $P < 0.01$, Figure 9D) and T-Bil (1.49 ± 0.25 mg/dL *vs* 2.39 ± 0.30 mg/dL, $P < 0.05$, Figure 9E) were significantly lower in the GM6001 group than those in the vehicle group. *In situ* gelatin zymography analysis indicated inhibition of gelatinolytic activity in the GM6001 group (Figure 9F-I). Additionally, the accumulation of neutrophils was significantly suppressed in the GM6001 group compared with the vehicle group (9.9 ± 1.5 per 40 high-power field *vs* 5.7 ± 1.1 per 40 high-power field, $P < 0.05$) (Figure 9J). These results suggested that therapy with exogenous MMP-9 inhibition has the potential to reduce the initial injury of RL after EH.

DISCUSSION

The mechanism of PLF is not fully understood, because of the involvement of various clinical perioperative factors, such as pre-existing liver disease, infection, transfusion, and insufficient volume of the RL. Murine and rat models have been used to determine the mechanisms for many years. Jin *et al*[15] showed necrosis of hepatocytes by oxidative injury after 87%-PH in mice. Catardegirmen *et al*[22] reported that blockage of the receptor for advanced glycation end products (RAGE) improves survival after 85%-PH and preserves liver parenchyma with the absence of necrosis in 85%-PH. Yoshida *et al*[23] reported the efficiency of a caspase inhibitor for survival after 95%-PH in rats by attenuating apoptosis of hepatocytes. Both necrosis and apoptosis pathways appear to be involved in PLF process after EH. We previously performed marginal hepatectomy to evaluate the difference between predictive liver failure and normal recovery at the early phase. Use of hemoclips and a surgical microscope enabled us to complete the surgical procedure within 10 min and to stabilize the outcomes after surgery in our model[12]. In this previous study, a quick procedure appeared to reduce the opportunity for an infection or any complications[12]. In our preliminary study, histological changes from 80%-PH[12] were slightly improved compared with a conventional model by Higgins *et al*[24]. Our marginal PH model could be helpful for evaluation of the direct effects of insufficient volume of the RL after EH, from the viewpoint of the deletions of irrelevant factors.

With regard to the mechanism of liver failure, only a few studies have focused on the initial changes in the RL after EH. Panis *et al*[16] reported the presence of centrilobular necrosis, which varied in the severity in different areas of sections as well as a fatty change and microvascular steatosis 24 h after 85%-PH in the rat. Jin *et al*[15] reported severe sinusoidal narrowing as early as 6 h, and swelling, fatty degeneration and nuclear condensation of hepatocytes by 48 h after 87%-PH in asymptomatic mice[15]. In small-for-size grafts used in transplantation, a vacuolar cytoplasm or considerable mitochondrial swelling in hepatocytes, irregular large gaps between sinusoidal lining cells and collapse of the space of Disse have been observed[25]. Our study clearly showed multiple necroses with intrahepatic-hemorrhage in the RL, and biochemical results also revealed PLF at the early phase after 80%-PH in symptomatic mice. In-

terestingly, these histological changes were similar to the findings of a lipopolysaccharide/galactosamine-induced acute liver failure model[26]. Initial extravasation of red blood cells, sinusoidal congestion, destruction of parenchymal architecture and subsequent total destruction of micro-architecture with hemorrhage were reported 4 to 8 h after a challenge of galactosamine and lipopolysaccharide[26]. We also observed dehiscence of sinusoids 6 h after 80%-PH. The mechanism of sinusoidal cell injury is not fully understood. Many efforts after PH, such as hyperperfusion and shear stress due to portal hypertension, appear to have the potential to physically damage sinusoidal endothelial cells (SECs), and intensive decompression of the portal vein has recently been proposed as an intra-operative strategy for liver transplantation with small-for-size grafts[27]. We observed distention of the portal vein during 80%-PH. An increase in portal flow indicates an increased exposure of bacterial products or toxic agents from the gut for an insufficient RL after EH, and this condition may affect SECs. The development of SEC injury followed by parenchymal cell injury is considered as an important mechanism in monocrotaline-induced veno-occlusive disease or acetaminophen hepatotoxicity[28-30], because SEC injury leads to hepatic microcirculatory dysfunction and hemorrhage[28,29]. SEC gap formation after treatment with galactosamine/endotoxin in mice has been reported to affect neutrophil extravasation and hemorrhage of liver parenchyma, and activated MMP is involved in SEC gap formation[31]. MMPs have been shown to be involved in several liver injury mechanisms including the fulminant liver failure model[32], sinusoidal obstruction syndrome model[33] and hepatic ischemia/reperfusion injury model[20]. Olle *et al*[34] have reported the importance of MMP-9 associated with tumor necrosis factor, hepatocyte growth factor, vascular endothelial growth factor and the apoptosis pathway for liver regeneration. Mohammed *et al*[35] showed that TIMP-1 affects the hepatocyte cell cycle in the liver regeneration process. These findings indicate MMPs' negative and positive potential in liver disease. A RL with considerable damage requires emergent regeneration for survival after EH. The role of MMPs appears to be more complex.

We demonstrated that MMP-9 expression was enhanced in the damaged liver associated with necrosis, and the main source of MMP-9 was CD11b-positive nonnative leukocytes and hepatic stellate cells 6 h after 80%-PH. Our results appear to be consistent with the ischemia/reperfusion injury model and acute liver failure model[26]. Kim *et al*[36] showed initial MMP-9 expression at 3 h in only periportal hepatocytes after 70%-PH in the rat, and positive staining gradually spread for up to 48 h. In our 80%-PH model, there was little expression of MMP-9 in hepatocytes 6 h after surgery, and our results suggested that a severe situation after 80%-PH impairs the regular process for normal regeneration observed after 70%-PH[13,36]. Yan *et al*[26] showed bimodal alteration of MMP-9 mRNA in the acute liver failure model. We observed a change in bimodal activity (the second peak was after 12 h) by zymography after 80%-PH (data not shown). Taken together, these results suggest that there are two different modalities for MMP-9 production. Although we previously reported that MMP-9 plays an important role in the development of parenchymal hemorrhage and necrosis in the small remnant liver after massive hepatectomy and that successful MMP-9 inhibition attenuates the formation of hemorrhage and necrosis and might be a potential therapy to ameliorate liver injury[11], the origin of MMP-9 production was unclear. We speculate that it is necessary to separately consider the roles of MMP-9 produced from leukocytes at the early phase and from hepatocytes at the late phase of EH.

In the current study, we focused on the initial MMP-9 expression and hypothesized that it plays a role in liver injury in the RL after EH. To examine the involvement of MMP-9 in initial liver injury after EH, we evaluated the effect of inhibition of MMP-9 using three different methods *in vivo* including MMP-9-deficient mice, an inhibitory monoclonal antibody and a broad spectrum inhibitor. In all of the MMP-9 inhibitory experiments, suppression of the initial injury was clearly observed with the inhibition of MMP-9. Our results suggested that MMP-9 plays a pivotal role in liver injury, especially in the formation of necrosis, likely sinusoidal injury after 80%-PH. Perioperative factors in each experiment were not different between each experimental group such as the resected volume, the surgical time and intra-operative blood loss. In the experiments with genetic deficient mice, we examined whether TIMP-1 deficiency had any effects on the theoretical enhancement of MMP-9 activity on liver injury simultaneously. We observed the reverse effects in TIMP-1 deficient mice compared with MMP-9-deficient mice with an increased gelatinolytic activity. No exacerbation of liver injury in the TIMP-1-deleted condition suggested insufficiency of TIMP-1 to regulate MMP-9 activity after EH. Mohammed *et al*[35] showed that TIMP-1 was slightly increased 6 to 18 h after 70%-PH in the rat. We did not assess TIMP-1 expression in the current study. However, it is possible that TIMP-1 could be used as a therapeutic target to reduce MMP-9 levels related to liver injury.

In the current study, we did not observe any survival benefits of MMP-9 inhibition in MMP-9(-/-) mice, although the other two methods of inhibition of MMP-9 showed improvement of survival. MMP-9 was shown to be important in liver regeneration in PH by Olle *et al*[34]. Additionally, the possibility of impairment of the host defense by MMP-9 deficiency was reported in a sepsis model by Renckens *et al*[37]. Excessive MMP-9 activity induced by shear stress after PH may have the potential to induce initial liver injury. Application of MMP-9 blockade should be precisely controlled.

In this study, we evaluated the accumulation of neutrophils in the RL after 80%-PH. Areas of necrosis were correlated with the number of accumulated neutrophils. The infiltration of polymorphonuclear neutrophils (PMNs) has been reported in several liver diseases[38-41] and even after EH[42]. We could not identify infiltrated

neutrophils as a central effecter on liver injury after 80%-PH, but a possible explanation may be provided by an association between neutrophils and SEC destruction. In addition, recent studies have shown that MMP-9 derived from neutrophils affects several pathophysiological conditions such as blood brain barrier breakdown after an ischemic stroke and gut barrier dysfunction in an acute pancreatitis model[43,44]. Keck *et al*[45] showed that PMN-derived MMP-9 contributes to the accumulation of PMNs in the inflammation site and it initiates damage to the basement membrane. Although our results are consistent with the previous work described above, the mechanism of liver damage or regeneration is complicate. Periportal infiltration of inflammatory cells will increase the liver damage, but this infiltration is paradoxically necessary to trigger the liver regeneration[46-48]. Though the balance of damage and regeneration is still unclear, we speculated that the MMP-9 produced from neutrophil may be important factor for a successful liver regeneration after EH. Further study is needed to elucidate the involvement of MMP-9 associated with neutrophils in liver injury after EH with an insufficient RL.

In summary, our data showed that the pathway of liver necrosis related to initial sinusoidal endothelial cell injury is responsible for the PLF process after EH with an insufficient RL. The initial injury is associated with MMP-9 derived from neutrophils, and blockade of MMP-9 reduces initial liver injury in an *in vivo* model. Our results suggest that MMP-9 may be a potential target for preventive care or further challenging for EH.

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COMMENTS

Background

Postoperative liver failure (PLF) after extended hepatectomy (EH) can cause an insufficient volume of the remnant liver (RL), resulting in higher morbidity and mortality.

Research frontiers

The mechanism of liver damage or regeneration is complicate. Periportal infiltration of inflammatory cells will increase the liver damage, but this infiltration is paradoxically necessary to trigger the liver regeneration. This PLF is progressive from the early postoperative period, but the mechanism is unknown. Authors hypothesized that matrix metalloproteinase (MMP)-9 plays a role in PLF pathogenesis.

Innovations and breakthroughs

Though the balance of damage and regeneration is still unclear in the RL after EH, the MMP-9 produced from neutrophil may be important factor for a successful liver regeneration after EH.

Applications

Their data showed that the pathway of liver necrosis related to initial sinusoidal endothelial cell injury is responsible for the PLF process after EH. The initial injury is associated with MMP-9 derived from neutrophils, and blockade of MMP-9 reduces initial liver injury.

Terminology

Their results suggest that MMP-9 may be a potential target for preventive care or further challenging for EH.

Peer review

In this manuscript, authors attempt to investigate that the effect of MMP9 in the initial injury after hepatectomy in mice. The work is of potential interest to the readership of the *World Journal of Gastroenterology*.

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BRIEF ARTICLE

MGMT and *MLH1* methylation in *Helicobacter pylori*-infected children and adults

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Abstract

AIM: To evaluate the association between *Helicobacter pylori* (*H. pylori*) infection and *MLH1* and *MGMT* methylation and its relationship with microsatellite instability (MSI).

METHODS: The methylation status of the *MLH1* and *MGMT* promoter region was analysed by methylation specific methylation-polymerase chain reaction (MSP-PCR) in gastric biopsy samples from uninfected or *H. pylori*-infected children (n = 50), from adults with chronic gastritis (n = 97) and from adults with gastric cancer (n = 92). *MLH1* and *MGMT* mRNA expression were measured by real-time PCR and normalised to a constitutive gene (β actin). MSI analysis was performed by screening MSI markers at 4 loci (Bat-25, Bat-26, D17S250 and D2S123) with PCR; PCR products were analysed by single strand conformation polymorphism followed by silver staining. Statistical analyses were performed with either the χ^2 test with Yates continuity correction or Fisher's exact test, and statistical significance for expression analysis was assessed using an unpaired Student's t-test.

RESULTS: Methylation was not detected in the promoter regions of *MLH1* and *MGMT* in gastric biopsy samples from children, regardless of *H. pylori* infection status. The *MGMT* promoter was methylated in 51% of chronic gastritis adult patients and was associated with *H. pylori* infection ($P < 0.05$); this region was methylated in 66% of gastric cancer patients, and the difference in the percentage of methylated samples between these patients and those from *H. pylori*-infected chronic gastritis patients was statistically significant ($P < 0.05$). *MLH1* methylation frequencies among *H. pylori*-infected and non-infected chronic gastritis adult patients were 13% and 7%, respectively. We observed methylation of the *MLH1* promoter (39%) and increased MSI levels (68%) in samples from gastric cancer patients in comparison to samples from *H. pylori*-infected adult chronic gastritis patients ($P < 0.001$ and $P < 0.01$, respectively). The frequency of promoter methylation for both genes was higher in gastric cancer samples than in *H. pylori*-positive chronic gastritis samples ($P < 0.05$). The levels of *MLH1* and *MGMT* mRNA were significantly reduced in chronic gastritis samples that were also hypermethylated ($P < 0.01$).

CONCLUSION: In summary, *MGMT* and *MLH1* methylation did not occur in earlier-stage *H. pylori* infections and thus might depend on the duration of infection.

Key words: *Helicobacter pylori*; Microsatellite instability; Promoter methylation; *MLH1*; *MGMT*; Gastric cancer

Core tip: Gastric carcinogenesis is a multistep process that is triggered by *Helicobacter pylori* (*H. pylori*) infection and characterised by multiple genetic and epigenetic alterations, including the DNA repair genes. To date, few advances have been made to determine the time duration required for to *H. pylori* infection to induce such epigenetics alteration and thus potentially induce gastric carcinogenesis. The results presented in this study indicate that the methylation of the *MGMT* and *MLH1* promoter regions might depend on the duration of infection because these methylation events were not observed in children.

Alvarez MC, Santos JC, Maniezzo N, Ladeira MS, da Silva ALC, Scaletsky ICA, Pedrazzoli Jr J, Ribeiro ML. *MGMT* and *MLH1* methylation in *Helicobacter pylori*-infected children and adults. *World J Gastroenterol* 2013; 19(20): 3043-3051 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3043.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3043

INTRODUCTION

Helicobacter pylori (*H. pylori*) is an important pathogen in the human stomach. The natural acquisition of *H. pylori* infection occurs mainly during childhood. Once established within the gastric mucosa, the infection persists for life if left untreated. The epidemiological evidence and the rare occurrence of peptic ulcers or gastric atrophy in children[1,2] suggest that *H. pylori*-related gastric mucosal damage might be progressive through childhood into adulthood. *H. pylori* infection elicits a host inflammatory response, including mucosal infiltration by polymorphonuclear leukocytes, macrophages, and T and B lymphocytes. The inflammatory response has a slow onset and becomes chronic after a long period of infection. Although the symptoms of chronic infection are not as severe as those of acute inflammation, the condition is persistent[3]. The activated inflammatory cells release reactive oxygen and nitrogen species that can induce DNA injury and cellular apoptosis[4]. The chronic colonisation of the stomach with *H. pylori* causes inflammation within the gastric mucosa and activates multiple oncogenic pathways[5].

The interaction of *H. pylori* with the surface mucosa results in the increased release of pro-inflammatory cytokines[6] that exacerbate the inflammatory response. The persistence of this immune response leads to chronic inflammation, one of the factors associated with DNA methylation. DNA methylation is one of the most important epigenetic modifications and primarily occurs on the cytosine residues of CpG dinucleotides, which are frequently clustered into CpG islands within the promoter regions of certain genes[7]. DNA methylation of these promoter regions inhibits transcription through chromatin structural changes that are mediated by the interactions of the methyl-cytosines with the protein complexes that recruit histone-modifying enzymes[8,9].

Globally, gastric cancer is the fourth most common type of cancer and the second leading cause of cancer death, and 930000 new cases of gastric cancer are projected per year. South Korea, Japan and Eastern Asia have the highest incidences of gastric cancer, followed by Eastern Europe and Latin America[10]. Since the discovery of *H. pylori* by Warren *et al* in 1982[11], many studies have demonstrated a strong association between *H. pylori* infection and the development of gastric cancer[12,13]. Moreover, in 1994, the International Agency for Research on Cancer recognised *H. pylori* as a definitive carcinogenic agent based on several epidemiological reports[14]. The primary mechanism by which *H. pylori* induces gastric cancer is thought to include the upregulation of several genes, including cytokines, oncogenes and growth factors, as well as the downregulation of tumour suppressor genes. These alterations in gene expression are believed to result from mutations and microsatellite instability[15]. Additionally, several studies have demonstrated a close association between *H. pylori* infection and aberrant CpG island methylation[16-18].

Many critical genes are silenced by DNA methylation during cancer development. Recent studies have shown that the silencing of certain DNA repair genes by DNA methylation might be related to the occurrence of tumorigenic mutations. The expression levels of *MLH1*, a mismatch repair gene, are frequently altered in gastric cancers, and changes in *MLH1* expression can promote tumour development[19]. Additionally, O^6-methylguanine DNA methyltransferase (MGMT) is a protein required for the repair of alkylated guanines in DNA that arise from exposures to environmental alkylation mutagens or through endogenous mechanisms. It has been reported that the loss of MGMT expression might increase the occurrence of genetic mutations and thus lead to gastric cancer development[20]. Additionally, Kitajima *et al*[21] reported that the loss of MGMT along with mismatch repair (MMR) proteins in gastric cancer patients is an important event in tumour progression.

Microsatellite instability (MSI) is one hallmark of DNA MMR deficiency that is observed in gastric carcinogenesis. Microsatellites are short DNA sequence repeats that are scattered throughout the human genome. Errors in the DNA MMR mechanisms of tumour cells can result in the expansion or contraction of these repeated sequences and thus MSI[22]. MSI occurs in nearly every case of gastric cancer that is associated with germline mutations of the MMR genes.

The inactivation of the MMR genes *MSH2* and

MLH1, either through a mutation or an epigenetic mechanism, is responsible for the development of MSI in gastric cancer. The aberrant loss of expression of either the MLH1 or MSH2 proteins has been observed in patients exhibiting gastric cancer with MSI. In particular, altered MLH1 expression is associated with self-gene inactivation through the hypermethylation of its promoter regions[23,24].

It is well established that *H. pylori* infection leads to chronic inflammation in the gastric mucosa, which is a condition associated with DNA methylation. Because such epigenetic alterations play an important role in the regulation of gene expression and the maintenance of DNA integrity and stability, the aim of this study was to analyse the effects of *H. pylori* infection on the methylation statuses of *MLH1* and *MGMT* and the relationship between the methylation of these promoters and microsatellite instability in *H. pylori*-infected or uninfected children as well as infected or uninfected adults with chronic gastritis or gastric cancer.

MATERIALS AND METHODS

Patients

This study was approved by the following institutions: the Ethical Committee of the Paulista Medical School, State University (UNESP), Botucatu, SP, Brazil; the Sao Francisco University, Braganca Paulista, SP, Brazil; the School of Medicine of the São Paulo University, SP, Brazil; and by the National Committee of Ethics in Research, Brasília, DF, Brazil. Informed consent to participate in the study was obtained from all patients.

This study included 239 patients, of which 50 were children between the ages of 2 and 18 years old (average age = 8 ± 4 years; 47% male, 53% female) who suffered from dyspepsia. Of the remaining patients, 97 suffered from chronic gastritis (average age = 35 ± 13 years; 33% male, 67% female), and 92 suffered from gastric cancer (average age = 60 ± 12 years; 82% male, 18% female). All of the patients were non-smokers, did not abuse alcohol and were not using prescription or recreational drugs.

Biopsy collection

Biopsies from patients with gastric complaints were obtained from the lesser curvature of the antrum (the distal region of the stomach) within 2 cm of the pyloric ring during endoscopies. For patients with gastric cancer, biopsies were obtained during gastric surgeries (to remove gastric carcinoma). One biopsy was used for a rapid urease test[25], 2 were used for histopathological evaluations, and 1 was used for bacterial genotyping by polymerase chain reaction (PCR) . *H. pylori* infection was considered present when positive results were obtained from all of the following tests: rapid urease test, histological analysis and gastric biopsy PCR. The patients were considered uninfected when negative results were obtained for all tests.

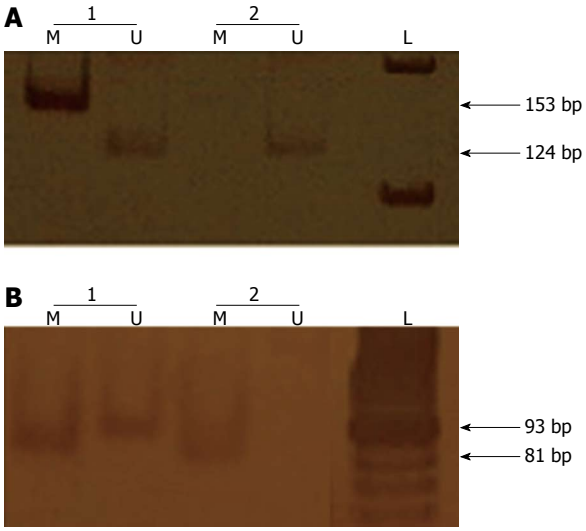

Figure 1 Example of the results from methylation-specific polymerase chain reaction analysis. A: *MLH1*; B: *MGMT*. Lane U: Amplified product with primers for unmethylated sequences; Lane M: Amplified product with primers for methylated sequences; L: Ladder.

Histopathology

Gastric mucosal samples were fixed in 10% formalin for 24 h, dehydrated in alcohol and xylene, and embedded in paraffin. Sequential 3-5-μm sections were cut and stained with haematoxylin-eosin for routine histology. Gastritis was classified according to Sydney's system[26], and the presence of *H. pylori* was determined by carbolfuchsin staining of the sections.

Bisulphite treatment and methylation-specific PCR

Bisulphite treatment was performed on 1 μg of DNA with the EpiTect Bisulfite kit (Qiagen, Valencia, CA, United States). Methylation-specific PCR (MSP-PCR) was performed with a primer set specific to the methylated or unmethylated sequences (M or U sets, respectively)[27]. The PCR reactions were performed in a final volume of 25 μL with approximately 200 ng of sodium bisulphite-treated DNA and 25 pmol of each primer. The PCR amplifications were performed for 40 cycles that each consisted of a denaturation step at 95 ℃ for 5 min, a primer-annealing step at 58 ℃ for 35 s and an extension step at 72 ℃ for 40 s, with a single final extension step at 72 ℃ for 7 min. The reaction products were separated by electrophoresis on 8% polyacrylamide gels and visualised by silver staining (Figure 1).

RNA extraction and real-time PCR

The gastric biopsies were collected, snap frozen, and stored at -80 ℃ in RNAlater® (Qiagen, Valencia, CA, United States). Total RNA was isolated with the RNeasy tissue kit® (Qiagen). The gastric cancer biopsies were microdissected prior to RNA extraction. The PixCell® IIe Laser Capture Microdissection (LCM) System (Arcturus Engineering, Mountain View, CA, United States)

Table 1 Primers used for real-time polymerase chain reaction

Gene	Primer	Sequence (5′-3′)
β-actin	Sense	ACACTGGCTCGTGTGACAAGG
	Antisense	CGGCTAATACACACTCCAAGGC
MGMT	Sense	CACCACACTGGACAGCCCTTT
	Antisense	CGAACTTGCCCAGGAGCTTTATTT
MLH1	Sense	CGGTTAACTACCCAATGCCTCAAC
	Antisense	TTCTCGACTAACAGCATTTCCAA

was used to obtain laser captures using an amplitude of 50 mW, a duration of 800 ms and a 7.5-mm beam. RNA from the capture lids (Arcturus) that contained the microdissected tissue was extracted with the PicoPure RNA isolation kit (Arcturus). Single-stranded cDNA was synthesised from the RNA using the high capacity cDNA archive kit® (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's protocol.

Quantitative PCR was performed on a 7300 real-time PCR system (Applied Biosystems) and the threshold cycle numbers were determined with the RQ Study software (Applied Biosystems). The reactions were run in triplicate and the threshold cycle numbers were averaged. The 50-μL reaction mixture was prepared as follows: 25 μL of Sybr Green® Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies, Alemeda, CA, United States), 10 mmol/L of each primer (Table 1) and 10 μL of cDNA (100 ng). The reactions was were performed with a preliminary UDG treatment for 2 min at 50 ℃ and denaturation for 2 min at 95 ℃, followed by 45 cycles of denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 15 s, and primer extension at 72 ℃ for 15 s. This treatment was followed by a melting-point analysis of the double-stranded amplicons that consisted of 40 cycles of 1 ℃ decrement (15 s each), beginning at 95 ℃. The first derivative of this plot, dF/dT, is the rate of change of fluorescence in the reaction. A significant change in fluorescence occurs at the melting point of the double-stranded PCR products. A plot of dF/dT versus temperature displays these changes as distinct peaks.

MLH1 and *MGMT* gene expression was measured and normalised to a constitutive gene (β-actin). The relative expression was calculated according to the formula $2^{(-\Delta\Delta Ct)}$[28], and the results are expressed as the average gene expression ± SD.

MSI analysis

All samples were analysed for 4 markers (BAT-25, BAT-26, D17S250 and D2S123) as recommended by the American National Cancer Institute (NCI) workshop on MSI[29].

PCR was performed in a final volume of 25 μL with approximately 200 ng of template genomic DNA and 25 pmol of each primer. PCR amplifications was performed for 35 cycles that consisted of a denaturation step at 95 ℃ for 30 s, a primer-annealing step at 55-58 ℃ for 30 s and an extension step at 72 ℃ for 30 min, followed by a final single extension step at 72 ℃ for 10 min.

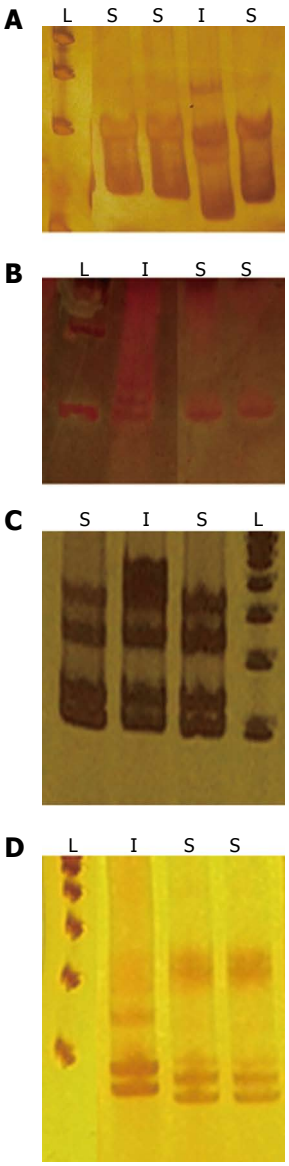

Figure 2 Example of the results from polymerase chain reaction-single strand conformation polymorphism analysis. A: BAT25; B: BAT26; C: D2S123; D: D17S250. Lane S: Stable; Lane I: Instable; L: Ladder 10 bp.

Single strand conformation polymorphism (SSCP) analysis was performed on each sample. Briefly, 12 μL of each PCR product were mixed with 12 μL of denaturing buffer (95% Formamide, 0.05% Bromophenol blue, and 0.05% xylenocianol), denatured at 95 ℃ for 5 min and separated by electrophoresis on a non-denaturing 7% polyacrylamide gel for 3 h at room temperature. The single strands of the PCR products were visualised as bands by silver staining.

MSI was defined as a shift in the mobility of the DNA band from either allele or by the appearance of a new band (Figure 2). The MSI status was also confirmed by direct sequencing (data not shown). High-level instability (MSI-H) was defined by the presence of more than one instability marker. Low-level instability (MSI-L) was defined as the presence of a single instability marker. Finally, if no instability markers were present, the sample

Table 2 Frequency of hypermethylation at the *MLH1* and *MGMT* promoter regions in chronic gastritis and gastric cancer patients *n* (%)

Subjects	*MLH1*		*MGMT*	
	Methylated	Unmethylated	Methylated	Unmethylated
Chronic gastritis				
Child *H. pylori* negative	-	28 (100%)	-	28 (100%)
Child *H. pylori* positive	-	22 (100%)	-	22 (100%)
Adults *H. pylori* negative	1 (7%)	13 (93%)	3 (21%)	11 (79%)
Adults *H. pylori* positive	11 (13%)	72 (87%)	42 (51%)[c]	41 (49%)
Gastric cancer	36 (39%)[a,b]	56 (61%)	61 (66%)[d,e]	31 (34%)

[a]$P < 0.05$ *vs Helicobacter pylori* (*H. pylori*) negative; [b]$P < 0.01$ *vs H. pylori* positive chronic gastritis; [c]$P < 0.05$ *vs H. pylori* negative; [e]$P < 0.05$ *vs H. pylori* positive chronic gastritis and [d]$P < 0.01$ *vs H. pylori* negative chronic gastritis group.

was classified as displaying microsatellite stability (MSS)[29].

Statistical analysis

The association between microsatellite instability and the methylation pattern was evaluated with either the χ^2 test with Yates continuity correction or Fisher's exact test. Statistical significance for expression analysis was assessed by an unpaired Student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

The presence of *H. pylori* infection was analysed in biopsy specimens from 50 paediatric patients and 189 adult patients who underwent endoscopies. Infections were detected in 22 of the 50 (44%) children, 83 of the 97 (86%) adults with chronic gastritis and all (100%) of the 92 adult gastric cancer patients.

Methylation was not detected in the promoter regions of *MLH1* and *MGMT* in the paediatric samples. The samples from the chronic gastritis patients showed hypermethylation in the *MHL1* gene promoter region in 11 of the 83 (13%) samples from the *H. pylori*-infected subjects and in 1 of the 14 (7%) samples from the uninfected subjects ($P > 0.05$; Table 2). Methylation of the *MHL1* promoter region was observed in the samples from 36 of the 92 (39%) gastric cancer patients. The differences observed in the percentages of methylated samples are statistically significant when the patients with gastric cancer are compared to those with chronic gastritis, regardless of *H. pylori* infection status ($P < 0.001$; $P < 0.05$, respectively; Table 2).

In patients with chronic gastritis, *MGMT* promoter region methylation was observed in the biopsies from 42 of the 83 (51%) *H. pylori*-positive patients and 3 of the 14 (21%) *H. pylori*-negative patients (Table 2); this difference was statistically significant ($P < 0.05$). In the gastric cancer patients, *MGMT* promoter region methylation was observed in the biopsies from 61 of the 92 (66%) patients. The difference in the percentages of samples with *MGMT* promoter region methylation between the gastric cancer patients and the non-infected chronic gastritis patients was statistically significant ($P < 0.01$). Additionally, there was a statistically significant difference between the percentages of methylated samples from the gastric cancer patients and the *H. pylori*-infected chronic gastritis patients ($P < 0.05$). Furthermore, the frequency of promoter methylation for both genes was higher in the gastric cancer samples than in the *H. pylori*-positive chronic gastritis samples; this difference was statistically significant ($P < 0.05$).

Table 3 Relative *MLH1* and *MGMT* mRNA expression levels

Subjects	*MLH1*		*MGMT*	
	Methylated	Unmethylated	Methylated	Unmethylated
Chronic gastritis				
Child *H. pylori* negative	-	0.25 ± 0.03	-	0.13 ± 0.08
Child *H. pylori* positive	-	0.27 ± 0.02	-	0.12 ± 0.01
Adults *H. pylori* negative	0.35 ± 0.01[b]	0.85 ± 0.07	0.40 ± 0.16[b]	0.99 ± 0.21
Adults *H. pylori* positive	0.88 ± 0.10[b]	1.29 ± 0.27	0.79 ± 0.19[b]	1.78 ± 0.47
Gastric cancer	0.27 ± 0.07	0.25 ± 0.08	0.49 ± 0.10	0.46 ± 0.10

MLH1 and *MGMT* mRNA values are expressed as the mean ± SD. [b]$P < 0.01$ *vs* unmethylated samples in the same subject category. *H. pylori*: *Helicobacter pylori*.

The *MLH1* and *MGMT* gene expression levels were measured and evaluated in the context of promoter methylation. The levels of both *MLH1* and *MGMT* mRNA were significantly reduced in the methylated samples compared to the unmethylated samples in both the *H. pylori*-positive and uninfected adult chronic gastritis samples (Table 3). Overall, gastric cancer patients and paediatric patients had low levels of *MLH1* and *MGMT* mRNA expression.

Biopsy samples from children with chronic gastritis were relatively stable for the tested microsatellite markers. Only 2 samples (both *H. pylori*-negative) were scored as MSI-H, and the remaining paediatric samples were scored as MSS (stable; Table 4). MSI was present in 51 of the 83 (61%) *H. pylori*-positive chronic gastritis samples from adults. Of these, 37 (73%) samples were scored as MSI-L and 14 (27%) samples as MSI-H. MSI was observed in 9 of the 14 (64%) uninfected patients with gastritis; 6 (67%) samples were scored as MSI-L and 3 (33%) as MSI-H. MSI was observed in 63 of the 92 (68%) gastric cancer patients; 38 (60%) were scored as MSI-L and 25 (40%) as MSI-H. No significant association was found between *H. pylori* infection and MSI in the samples from chronic gastritis adult patients. However, there was a significant difference ($P = 0.03$) in the percentages of samples with MSI for between the gastric cancer patients and the *H. pylori*-infected chronic gastritis patients.

Various authors have postulated that the methyla-

Table 4 Microsatellite instability status *n* (%)

Subjects	Microsatellite instability status			
	MSI-L	MSI-H	MSI	MSS
Chronic gastritis				
Child *H. pylori* negative	-	2 (7)	2 (7)	26 (93)
Child *H. pylori* positive	-	-	-	22 (100)
Adults *H. pylori* negative	6 (67)	3 (33)	9 (64)	5 (36)
Adults *H. pylori* positive	37 (73)	14 (27)	51 (61)	32 (39)
Gastric cancer	38 (60)	25 (40)	63 (68)[a]	29 (32)

MSI-H, the presence of more than one instability marker. MSI-L, the presence of a single instability marker. MSS, no instability markers were present. [a]$P < 0.05$ *vs Helicobacter pylori* (*H. pylori*)-positive chronic gastritis samples.

tion of the *MHL1* promoter region leads to the down-regulated expression of the *MHL1* gene, an effect that is strongly associated with MSI[23,24,30]. Therefore, we evaluated whether there was any association between the *MLH1* methylation status and MSI in our study population. The data presented in this study show a strong association between the *MLH1* methylation status and MSI in patients with gastric cancer ($P < 0.01$). However, we did not find any significant association between the *MLH1* methylation status and MSI in the samples from chronic gastritis adult patients, regardless of *H. pylori* infection status. Additionally, a multivariate analysis did not show that the *H. pylori infection* was associated with gene promoter methylation and MSI.

DISCUSSION

Genomic DNA methylation is one of the most important epigenetic modifications in eukaryotes. DNA methylation is essential for life, and alterations of the methylation process are often associated with carcinogenesis related to chronic inflammation or persistent infections of viruses or other pathogenic microorganisms. In this setting, we evaluated the effects of *H. pylori* infection on the methylation patterns of the *MLH1* and *MGMT* promoter regions as well as the MSI statuses of paediatric and adult patients.

Children are an interesting natural model for *H. pylori* infection studies not only because they are not usually exposed to gastric mucosal irritants such as alcohol, tobacco, and anti-inflammatory medications but also because the gastric mucosal changes in children might represent an earlier stage of the inflammatory response when compared to those in adult hosts, due to the shorter duration of *H. pylori* infections in children. To our knowledge, this is the first study to evaluate the methylation patterns of DNA repair genes in paediatric samples. Our data showed an absence of the methylation in the *MLH1* and *MGMT* promoter regions and that the mRNA levels of both genes were similar in infected and uninfected children. Although our data did not indicate an effect of *H. pylori* infection on the methylation patterns of two DNA repair genes, a recent study reported an association between *H. pylori* infection and the methylation patterns of 7 genes in paediatric samples; however, none of these genes were involved with DNA repair[31]. The methylation levels in these susceptible loci were increased in the adult samples when compared to the paediatric samples, suggesting that the altered methylation patterns might be related to the duration exposure to of *H. pylori*[31]. Accordingly, our data showed that the methylation rates were significantly higher in the samples from adults with chronic gastritis than in the samples from children with chronic gastritis.

Recently, it has been shown that prolonged bacterial infections lead to saturation of the repair capabilities of the host cells and thus to an ineffective and mutagenic DNA repair system[32]. Accordingly, it is believed that *H. pylori*-associated gastric mucosal damage might be progressive through childhood and into adulthood[1,2]. Taking this into account, our data suggest that *H. pylori* infection-mediated DNA methylation in adults may depend not only on the level of the inflammatory response but also on the persistence and duration of the infection.

Our data showed high levels of *MGMT* promoter region methylation in patients with *H. pylori*-positive chronic gastritis when compared to those with *H. pylori*-negative chronic gastritis, indicating that *MGMT* promoter methylation is significantly associated with *H. pylori* infection in chronic gastritis cases. Previous studies reported that *MGMT* CpG methylation is more frequent and extensive in *H. pylori*-infected versus uninfected patients[33]. Additionally, it has been suggested that the *MGMT* promoter methylation in *H. pylori*-infected patients is related to tumour progression[34,35].

Our data showed no differences in *MLH1* promoter region methylation between patients with *H. pylori*-positive chronic gastritis and those with *H. pylori*-negative chronic gastritis. However, the methylation levels were higher in patients with gastric cancer than in patients with chronic gastritis. Similarly, it has been reported that *MLH1* promoter methylation occurs late in intestinal metaplasia development[36]. Taken together, our data suggest that *MLH1* methylation occurs late in the progression of gastric carcinoma and that methylation depends partly on the persistence of the *H. pylori* infection.

The data presented herein also showed that the methylation of the promoter regions significantly reduced the mRNA levels of both *MGMT* and *MLH1* in patients with chronic gastritis, regardless of *H. pylori* infection status. Conversely, no differences in mRNA levels were observed in gastric cancer samples, regardless of methylation status; this is likely consequent to other epigenetic and genetic mechanisms. Recently, it was reported that *H. pylori* infections in patients with gastritis were associated with *MGMT* hypermethylation and reduced levels of *MGMT* mRNA in the gastric epithelium[33]. Similar results were reported for *MLH1* in gastric carcinoma samples[19]. Therefore, it is possible that the hypermethylation of the *MLH1* promoter region leads to the reduced expression of its protein product. This phenomenon could permit

the accumulation of mutations due to the lack of surveillance and repair that are consequent to this deficiency in the DNA repair system. Ultimately, the deficient DNA repair process can be detected by the appearance of microsatellite instability[37].

In this study, we screened a group of *H. pylori*-infected and uninfected paediatric patients as well as a group of adult patients who were divided as follows: *H. pylori*-infected chronic gastritis patients, uninfected chronic gastritis patients and gastric cancer patients (all of whom were infected with *H. pylori*). MSI instability was found to be a very rare event in the paediatric population. Our data showed that *H. pylori* infection was not associated with MSI among patients with chronic gastritis. The incidence of MSI in gastric cancer patients was 64%, which was consistent with results reported previously by others (incidences ranging from 58% to 76%)[38,39]. Additionally, several studies have reported the presence of MSI in patients with intestinal metaplasia and gastric cancer, suggesting that the development of MSI may be an early event in the multi-step progression of gastric carcinogenesis[40,41].

The presence of MSI-H in sporadic carcinomas has been significantly associated with the loss of *MLH1* expression[42,43]. This phenomenon was associated with the hypermethylation of the *MLH1* promoter, which is the underlying mechanism that causes MSI in gastric adenomas and early gastric cancers[24,44]. It is well known that *H. pylori* infection causes an increased rate of cell turnover in the gastric mucosa and thus overwhelms the DNA repair system. This process might allow for the accumulation of mutations that are consequent to *H. pylori* infection and other environmental risk factors[45].

Previously, we reported that *H. pylori* infection leads to decreased *MLH1* expression in patients with gastric cancer[46]. This result correlated with the high levels of MSI that were detected in these samples because the downregulation of *MLH1* can lead to DNA repair system failures. Moreover, when the methylation patterns were compared with the *MLH1* expression levels and the MSI levels in gastric cancer samples, we found higher methylation levels and a consequent downregulation of *MLH1* in samples that were characterised as MSI-H versus those characterised as MSI-L samples ($P < 0.03$). Similarly, Mizoshita *et al*[47] identified a strong association between the MSI phenotype and the loss of *MLH1* expression in advanced gastric cancers.

We did not find an association between *MGMT* promoter region methylation and MSI status. Furthermore, it was observed that the methylation of both the *MLH1* and *MGMT* promoter regions is a frequent event in gastric cancers. Similarly, Zou *et al*[34] reported an increase in *MGMT* methylation during the progression from intestinal metaplasia to early gastric carcinoma.

H. pylori infections are generally acquired during childhood and, if left untreated, will persist indefinitely. These infections lead to chronic inflammation, one of the factors associated with epigenetic alterations and possibly with the development of gastric cancer. The results presented in this study indicate that the methylation of the *MGMT* and *MLH1* promoter regions might be considered to be dependent on the duration of infection because similar methylation patterns had not been observed in children. Moreover, in gastric cancer patients it was shown that the *MLH1* expression levels, the hypermethylation pattern of the *MLH1* promoter region and the consequent increase in MSI frequency are all related events. The results of this study are in accordance with the results presented in previous studies; taken together, these findings provide a better understanding of gastric carcinogenesis in the Brazilian population. Although other authors had previously studied these genes in gastric cancers or in chronic gastritis samples from adult patients, this is the first study that included samples from children with chronic gastritis to represent the earlier stages of the inflammatory response, which supported the idea that the methylation of these genes might depend on the duration of the *H. pylori* infection, among other factors.

Another interesting point of this study is that it addresses a new concept described by Ogino *et al*[48], termed "Molecular Pathological Epidemiology", a new field of epidemiology based on the molecular classification of cancer, in which a known or suspected etiologic factor is examined in relation to a specific molecular change to gain insights into the carcinogenic mechanisms. It is well known that gastric carcinogenesis is a multifactorial process that results from the interactions of factors related to diet, environment, individual genetic susceptibility and *H. pylori* infection. In this manner, this study provides insights into the carcinogenic mechanisms that are induced by *H. pylori* infection, one of the etiologic factors involved in gastric carcinogenesis, and the induction of epigenetic alterations in the early stages of this process. The chronic gastric mucosal inflammation induced by *H. pylori* infection, which is characterised by mucosal infiltration by polymorphonuclear leukocytes, macrophages and T and B lymphocytes, leads to the release of reactive oxygen species (ROS) from activated inflammatory cells. ROS can induce DNA damage and the lack of a proper MMR repair system, which is partly due to the persistence of the organisms and the associated inflammation, and thus can lead to the accumulation of DNA mutations in gastric epithelial cells that contribute to gastric carcinogenesis. Additionally, recent studies have shown that specific types of inflammation that are characterised by the expression of specific inflammation-related genes, as well as increased cell proliferation, are necessary for methylation induction. In a previous study, the authors showed that *H. pylori*-induced inflammation was able to induce methylation, unlike alcohol or saturated NaCl-induced inflammation[49]. Despite all of the evidence for *H. pylori* infection-induced methylation, we cannot exclude the presence of potential confounding factors such as differences in individual host genetics, different bacterial strains and the local microenviroment, all of which might

affect an association study. Further studies of *H. pylori*-induced molecular pathogenesis will be useful to our understanding of gastric carcinogenesis.

COMMENTS

Background

Gastric carcinogenesis is a multistep process that is triggered by *Helicobacter pylori* (*H. pylori*) infection and characterised by multiple genetic and epigenetic alterations, including the DNA repair genes. The action of *H. pylori* through inflammatory mediators might play a key role in the epigenetic silencing of these genes.

Research frontiers

Impairment of the DNA MMR system is a known mechanism of carcinogenesis and tumour progression. *H. pylori* infection leads to chronic inflammation in the gastric mucosa, which is associated with DNA methylation; this epigenetic alteration plays an important role in the regulation of gene expression and the maintenance of DNA integrity and stability. To date, few advances have been made to determine the time duration required for to *H. pylori* infection to induce such epigenetics alteration and thus potentially induce gastric carcinogenesis.

Innovations and breakthroughs

To the knowledge, this is the first study to evaluate the methylation patterns of DNA repair genes in paediatric samples. Children can be considered interesting natural models for the study of *H. pylori* infection not only because they are not usually submitted to gastric mucosal irritants such as alcohol, tobacco, and anti-inflammatory medications but also because the gastric mucosal changes in children might represent an earlier stage of the inflammatory response compared to those in adult hosts, due to the shorter duration of *H. pylori* infections in children. The results presented in this study indicate that the methylation of the *MGMT* and *MLH1* promoter regions might depend on the duration of infection because these methylation events were not observed in children.

Applications

This study indicates that the impairment of the DNA MMR system through DNA promoter methylation is an infrequent event in the early stages of *H. pylori*-induced inflammation.

Peer review

This is overall an interesting study that analyses the relationship between *H. pylori* infection and molecular changes in the gastric mucosa, diseases, and cancers. In this respect, this is a very unique study in the field of MPE research in non-neoplastic diseases (gastritis).

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BRIEF ARTICLE

Neoadjuvant-intensified treatment for rectal cancer: Time to change?

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Abstract

AIM: To investigate whether neoadjuvant-intensified radiochemotherapy improved overall and disease-free survival in patients with locally advanced rectal cancer.

METHODS: Between January 2007 and December 2011, 80 patients with histologically confirmed rectal adenocarcinoma were enrolled. Tumors were clinically classified as either T3 or T4 and by the N stage based on the presence or absence of positive regional lymph nodes. Patients received intensified combined modality treatment, consisting of neoadjuvant radiation therapy (50.4-54.0 Gy) and infusional chemotherapy (oxaliplatin 50 mg/m^2) on the first day of each week, plus five daily continuous infusions of fluorouracil (200 mg/m^2 per die) from the first day of radiation therapy until radiotherapy completion. Patients received five or six cycles of oxaliplatin based on performance status, clinical lymph node involvement, and potential risk of a non-sphincter-conserving surgical procedure. Surgery was planned 7 to 9 wk after the end of radiochemotherapy treatment; adjuvant chemotherapy treatment was left to the oncologist's discretion and was recommended in patients with positive lymph nodes. After treatment, all patients were monitored every three months for the first year and every six months for the subsequent years.

RESULTS: Of the 80 patients enrolled, 75 patients completed the programmed neoadjuvant radiochemotherapy treatment. All patients received the radiotherapy prescribed total dose; five patients suspended chemotherapy indefinitely because of chemotherapy-related toxicity. At least five cycles of oxaliplatin were administered to 73 patients. Treatment was well tolerated with high compliance and a good level of toxicity. Most of the acute toxic effects observed were classified as grades 1-2. Proctitis grade 2 was the most common symptom (63.75%) and the earliest manifestation of acute toxicity. Acute toxicity grades 3-4 was reported in 30% of patients and grade 3 or 4 diarrhoea reported in just three patients (3.75%). Seventy-seven patients underwent surgery; low anterior resection was performed in 52 patients, Miles' surgery in 11 patients and total mesorectal excision in nine patients. Fifty patients showed tumor downsizing $\geqslant$ 50% pathological downstaging in 88.00% of tumors. Out of 75 patients surviving surgery, 67 patients (89.33%) had some form of downstaging after preoperative treatment. A pathological complete response was achieved in 23.75% of patients and a nearly pathologic complete response (stage ypT1ypN0) in six patients. An involvement of the radial margin was never present. During surgery, intra-abdominal metastases were found in only one patient (1.25%). Initially, 45 patients required an abdominoperineal resection due to a tumor distal margin $\leqslant$ 5 cm from the anal verge. Of these patients, only seven of them underwent Miles' surgery and sphincter preservation was guaranteed in 84.50% of patients in this subgroup. Fourteen patients received postoperative

chemotherapy. In the full analysis of enrolled cohort, eight of the 80 patients died, with seven deaths related to rectal cancer and one to unrelated causes. Local recurrences were observed in seven patients (8.75%) and distant metastases in 17 cases (21.25%). The five-year rate of overall survival rate was 90.91%. Using a median follow-up time of 28.5 mo, the cumulative incidence of local recurrences was 8.75%, and the overall survival and disease-free survival rates were 90.00% and 70.00%, respectively.

CONCLUSION: The results of this study suggest oxaliplatin chemotherapy has a beneficial effect on overall survival, likely due to an increase in local tumor control.

Key words: Rectal cancer; Neoadjuvant treatment; Intensified radiochemotherapy; Oxaliplatin; Fluorouracil

Core tip: Management of rectal cancer requires a multimodality treatment approach. The objective of this study was to determine whether neoadjuvant-intensified radiochemotherapy, using traditional radiation therapy in combination with oxaliplatin and 5-fluorouracil (5-FU), could improve the overall and disease-free survival rates in patients with locally advanced rectal cancer. Conventional chemotherapeutic strategies typically only use 5-FU infusion. The results from this study indicate that the addition of oxaliplatin to the chemotherapeutic regime enhances the 5-year overall survival rate, facilitates a high rate of sphincter preservation, and reduces the local recurrence rate relative to the traditional strategies previously reported in the literature. Furthermore, oxaliplatin addition was well tolerated by patients, demonstrating an acceptable level of toxicity.

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INTRODUCTION

Management of rectal cancer requires a multimodality treatment approach. Significant progress has been made in the conventional modalities of surgery, radiotherapy and chemotherapy, typically used to treat this type of cancer. The frequent spread of neoplastic cells to mesorectal nodes and the consequent increase in local recurrence has led to surgical standardization using total mesorectal excision (TME). With standard TME surgery, the incidence of local recurrence in lymph node metastasis-negative, pN0, tumors is reduced. However, the local recurrence rate is still higher than 20% in patients with lymph node positive, pN+, disease and chemotherapy has remained the standard adjuvant care. Technical progress in radiation techniques, identified in major accuracy planning, and further analysis of accurate timing in sequential multimodality therapy has created improvements in local control, toxicity (acute and chronic), and sphincter preservation[1-3].

Previous work by Sauer *et al*[4] from the German Rectal Cancer Study Group identified preoperative radiochemotherapy as the standard treatment for patients with stage cT3-4 and/or N+ tumors. Based on empiric data and on the efficacy demonstrated in stage Ⅲ colon cancer patients[5,6], the addition of a second chemotherapeutic agent in a neoadjuvant setting confirmed oxaliplatin (OXP) radiosensitizing properties both *in vitro* and *in vivo*[7].

The goal of this work was to determine whether neoadjuvant-intensified radiochemotherapy improved the overall and disease-free survival, which is typically only achieved with 5-fluorouracil (5-FU) treatment, in patients with locally advanced rectal cancer. In this study, tumor downstaging, pathological complete response (pCR), and negative radial (circumferential) margins of tumors were assessed as well as the overall and disease-free survival rates in a cohort of 80 patients, 51 of whom had already been evaluated as previously described[8]. The results indicate that oxaliplatin therapy, in addition to traditional radiation and 5-FU therapies, enhances the overall survival rate and reduces local recurrence in patients with rectal cancer.

MATERIALS AND METHODS

Eligibility criteria

Patients enrolled were positively diagnosed with rectal adenocarcinoma as shown by histological analysis. Tumors were within 12 cm from the anal margin and clinically classified as described below. The performance status (PS), age, normal blood parameters and normal renal function were also assessed. Patient exclusion criteria consisted of the presence of synchronous tumors, cardiovascular disease, history of neurological or psychiatric disorders, and previous pelvic radiotherapy. All patients were enrolled after providing informed consent.

Staging

The pre-treatment staging included obtaining the complete history and careful physical examination of the patient, digital rectal examination, rectocolonscopy, trans-rectal ultrasound and total body computerized tomography. Patient tumors were evaluated by ultrasound exam and tumor stage (T) was classified according to the American Joint Committee on Cancer tumor, nodes, metastasis (TNM) Staging System[9]. With the exception of one sample, all tumors were classified as either T3 or T4 and N+ if positive regional lymph nodes were detected without any distant metastases. In the case of an uncertain diagnosis, patients underwent abdominal-pelvic

magnetic resonance imaging. The evaluation of clinically positive lymph nodes (N) was performed by trans-rectal ultrasound and TC; lymph nodes ⩾ 1 cm were considered pathological.

Treatment

Radiotherapy: As the physical positioning of a patient must remain identical for both the initial localization of the tumor by computerised tomography (CT) scanning and during subsequent treatment, a planning CT scan was performed in the treatment position. Patients were treated in the prone position using a belly-board device to displace the small bowels out of the treatment field. A radio-opaque marker was placed on the anal verge. CT images were acquired from the level of L1 to 3 cm below the anal marker with 5 mm slice spacing. CT data were analyzed using Treatment Planning Software (Pinnacle®) for target volume definition and dose solutions. The planning target volume 1 (PTV1) encompassed the primary tumor, the mesorectal and posterior pelvic sub-regions, and the regional node. The presacral, obturator and internal iliac lymph nodes were monitored in all patients. The external iliac lymph nodes were monitored if clinically positive or in the case of T4 tumor. The inguinal lymph nodes were irradiated if there was major tumor extension to the internal and external anal sphincter. The superior field border was located at the bifurcation of the common iliac vessels (L5/S1 interspace); the inferior margin was 5 cm below the inferior edge of the tumor. The lateral extension was 2 cm outside of the pelvic bones. The posterior border was placed 1 cm behind the sacrum to include sacral hollows. The anterior limit was placed at the posterior margin (cT3) or anterior margin (cT4) of the symphysis.

The PTV2 included the tumor mass with a 2 cm 3-D margin. The organs at risk were bowel (Dmax < 55 Gy), bladder (V50 60%; V60 50%), femoral heads (V50 60%), and anal canal (Dmax < 55 Gy). Patients were set up daily, using sagittal and lateral tattoos and a laser to prevent lateral rotation. Electronic portal imaging was used to check treatment organization once a week from the start to the end of treatment; portal images were compared with digitally-reconstructed radiographs from the planning CT scan. Radiation therapy was delivered with a 3-D-conformational multiple field technique at a dose of 45 Gy (in 25 daily fractions of 1.8 Gy given in 5 wk) to the whole pelvis for PTV1 and 5.4-9.0 Gy (in 3-5 daily fractions of 1.8 Gy) to the tumor volume for PTV2 with 6-15 MV energy photons.

Chemotherapy: All patients received a central venous access (port-a-cath) for delivering chemotherapy. Chemotherapy consisted of a 2-h oxaliplatin infusion (50 mg/m^2) on the first day of each week of radiotherapy, and five daily continuous infusions of 5-FU (200 mg/m^2 per die). Patients received five or six cycles of oxaliplatin, dependent on PS, clinical lymph node involvement, and potential risk of a non-sphincter-conserving surgical procedure. Desamethasone (8 mg) and ondansetron (8 mg) were administered before the oxaliplatin infusion. Figure 1 shows the neoadjuvant treatment protocol.

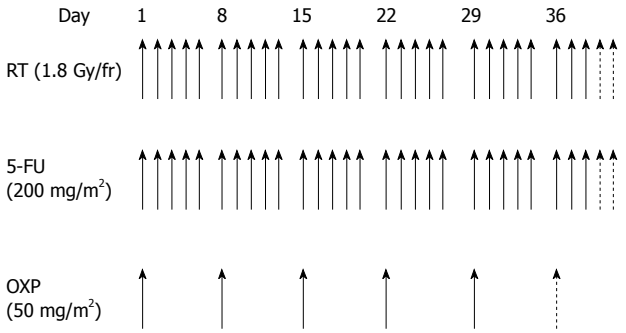

Figure 1 Neoadjuvant-intensified treatment protocol. Patients received 50.4 Gy (solid arrows) or 54.0 Gy (dashed arrows) of radiation therapy (RT), 28 d (solid arrows) or 30 d [dashed arrows of fluorouracil (5-FU) and five (solid arrows) or six (dashed arrow)] weekly cycles of oxaliplatin dependent on performance status, clinical lymph node involvement, and potential risk of a non-sphincter-conserving surgical procedure. Radiation therapy was delivered with a 3-D-conformational multiple field technique at a daily dose of 1.8 Gy/fraction. All patients received a central venous access (port-a-cath) for delivering chemotherapy. Chemotherapy consisted of a 2-h oxaliplatin infusion (50 mg/m^2) on the first day of each week of radiotherapy, and five daily continuous infusions of 5-FU (200 mg/m^2).

Toxicity was evaluated using National Cancer Institute's Common Terminology Criteria for Adverse Events version 3.0[10]. Oxaliplatin and 5-FU dose reductions were not planned. For occurrence of hematological toxicity grade 3 or neurological toxicity grade 2, the oxaliplatin administration was interrupted; both chemotherapeutic agents were stopped if grade 3 toxicity was reached. If severe toxicity persisted, did not return to grade 1, or was classified as grade 4, chemotherapy was cancelled but radiotherapy was completed.

Surgery: Five weeks from the end of neoadjuvant treatment, each patient underwent digital rectal examination, total body TC, colonscopy and trans-rectal ecography to evaluate clinical response. Surgery was planned seven to nine weeks after the end of radiochemotherapy treatment. The surgeon chose the type of surgery to perform.

Adjuvant chemotherapy: Adjuvant chemotherapy treatment was left to the oncologist's discretion and was recommended in patients with lymph node metastases.

Pathologic examination of the operative specimen

Pathological staging was designated as pTNM and depended on the data acquired clinically in addition to surgical and pathologic findings. Radial margins were considered positive if there was evidence of microscopic invasion. Downstaging was defined as a reduction of at least one level in T or N staging between the baseline ultrasound exam and histopathological staging. Downsizing was defined as a reduction of lesion diameter between pre-treatment ultrasound evaluation and histopathological results. pCR was defined as the absence of any residual

Table 1 Characteristics of patients *n* (%)

Characteristics	Patients
Performance status	
0	52 (65.00)
1	27 (33.75)
2	1 (1.25)
Localization	
⩽ 5 cm from anal verge	45 (56.25)
> 5 to ⩽ 8 cm from anal verge	19 (23.75)
> 8 cm from anal verge	16 (20.00)
Stage	
ⅡA	19 (23.75)
ⅢA	1 (1.25)
ⅢB	30 (37.50)
ⅢC	30 (37.50)

tumor cells detected in the operative specimen.

Patient recruitment

This study was an extension of a previous study of 51 patients[8]. Between January 2007 and December 2011, 29 new patients were enrolled for a total of 80 participants: 25 females (31.25%) and 55 males (68.75%). The patients' ages ranged between 36-76 years (average = 63.55 years). Patients presented clinically with rectal bleeding (39/80, 48.75%) that may have been accompanied by a change in bowel habits, such as unexplained constipation and diarrhea (10/80, 12.50%). At a pre-treatment evaluation, 75.00% of patients showed pathological tumor-positive lymph nodes; 37.5% of patients were clinically staged as ⅢB and 37.5% as ⅢC. The distance of the inferior margin of the tumor lesion was located in the lower rectum at ⩽ 5 cm from the anal verge in 56.25% of patients. The characteristics of patients are listed in Table 1.

Follow-up

After surgery, all patients were monitored at three-month intervals for the first year and at six-month intervals for the subsequent years. All patients were stratified into five cohorts according to the year of the last treatment. We defined "absolute permanence" as the greatest number of months of permanence in each cohort. Absolute permanence, overall survival (OS) and disease-free survival (DFS) were measured in months from the end of the neoadjuvant treatment.

Statistical analysis

Statistical analysis was performed using the following factors: 1 - sex, 2 - age, 3 - PS, 4 - TNM clinical staging, 5 - cranio-caudal extension of tumor lesion, 6 - tumor location, 7 - total radiotherapy dose, 8 - cycles of associated chemotherapy, 9 - interval between neoadjuvant treatment and surgery, 10 - type of surgery, 11 - toxicity, 12 - OS, 13 - DFS, 14 - pathologic downstaging, and 15 - pCR. Factors from 1 to 11 were considered "causal" or "predictive"; factors from 12 to 15 characterized the "considerable" results of therapy. Standard descriptive statistics were used to evaluate the distribution of each factor. OS and DFS were evaluated in each cohort. To determine the association between downstaging or pCR and predictive factors, the univariate analysis was performed using the non-parametric Bernard test. Statistical tests were one-sided. Changes of OS and DFS according to predictive factors were assessed using a logistic model in multivariate analysis. Statistical analysis was performed using MATLAB software, version 7.5.0.342 (R2007b), and SAS software, version 9.1.

RESULTS

Compliance

Seventy-five patients completed the programmed radiochemotherapy treatment. All patients received the radiotherapy prescribed total dose of 50.4 Gy in 75 patients (93.75%) and 54 Gy in five patients (6.25%). Five patients suspended chemotherapy indefinitely because of chemo-related toxicity after the second cycle (one patient), third cycle (two patients), or fourth cycle (two patients). Twelve patients stopped the planned neoadjuvant treatment because of acute toxicity: five patients interrupted radiation therapy only and seven patients interrupted both treatments. In these patients, radiotherapy was stopped for an average period of 10.58 d (range 2-22 d).

Surgery

Seventy-seven out of 80 patients underwent surgery. A "wait and see" approach was recommended to only one patient; he was unfit for surgery because of type Ⅱ diabetes mellitus and pericarditis co-morbidities. After neoadjuvant treatment, he underwent pelvic RM that indicated a complete clinical regression of the tumor. One patient did not undergo surgery because of liver metastases. One patient had a myocardial infarction two weeks after the end of radiochemotherapy and died.

For those patients who were eligible, surgery was planned an average of 9.30 wk (range 5-24 wk) after the end of neoadjuvant treatment. Low anterior resection was performed in 52 patients, Miles' surgery in 11 patients, and transanal endoscopic microsurgery in nine patients. Three patients had a different surgical approach. Two patients died of intra-operative complications. None had positive radial margins. At the beginning, 45 patients required an abdominoperineal resection due to a distal tumor margin distance of ⩽ 5 cm from the anal verge. Only seven patients underwent Miles' surgery, and sphincter preservation was guaranteed in 84.50% of this subgroup of patients. Post-operative complications were recorded in nine patients; the most common type was perianastomotic fistula (six patients) and other post-operative complications included fever (one patient), intestinal obstruction (one patient), and adhesion (one patient).

Pathologic complete response and downstaging

Downsizing and downstaging was evaluated by comparing clinical staging to pathological staging. Fifty patients showed tumor downsizing of ⩾ 50% and associated

Table 2 Evaluation of overall survival and disease-free survival

Cohort	α	Ω	AP, mo	OS, mo	DFS, mo	OS/AP %	DFS/OS %
2007	16	3	54.00	50.90	46.90	94.28	92.14
2008	22	3	42.90	39.30	34.90	91.40	89.01
2009	18	2	30.80	29.40	22.60	95.53	76.97
2010	13	0	17.40	17.40	15.30	100.00	87.93
2011	11	0	6.30	6.30	5.60	100.00	88.79
Total	80	8	29.80	27.90	23.90	93.45	85.88

α: Survived; Ω: Died; AP: Absolute permanence; OS: Overall survival; DFS: Disease-free survival.

downstaging in 88.00% of tumors. Out of 75 patients surviving surgery, 67 patients (89.33%) had some form of downstaging from preoperative treatment. After surgery, 25 patients (33.33%) harbored tumors that were classified as Stage Ⅰ. Of these, 18 patients (72.00%) had clinical positive nodes at diagnosis. Pathologic complete response, defined as the absence of tumor cells in the operative specimen, was observed in 18 patients (22.50%); only six of them had clinical negative lymph nodes at diagnosis. Six patients had a nearly pathologic complete response (stage ypT1ypN0). During surgery, intra-abdominal metastases were found in one patient only (1.25%).

Adjuvant chemotherapy

Fourteen patients received postoperative chemotherapy. Twelve patients had lymph node-positive tumors previously identified by trans-anal ultrasound (eight patients with cN2 and four patients with cN1). One patient had positive lymph nodes identified by histopathological examination and distant metastases were detected intraoperatively in one patient. Of 16 patients with ypT3ypN0 disease, only four were assigned to adjuvant chemotherapy.

Overall survival and disease-free survival

Until January 2012, surviving patients were monitored with a follow-up program; the average follow-up time was 27.28 mo and the median follow-up time was 28.50 mo (range 2-58 mo). Twenty-four patients were followed for 25-36 mo, 18 patients followed for 37-48 mo, and 11 patients were followed for at least 49 mo.

In the full analysis of study cohort, eight of the 80 patients died. Seven deaths were related to rectal cancer and one death was caused by unrelated causes. Of these, one patient died before surgery, two died during surgery, and four deaths occurred during the follow-up program. Of the latter, two patients had local recurrence 6 and 9 mo, respectively, after the end of neoadjuvant treatment and two patients had distant metastases 13 and 15 mo, respectively, after the end of preoperative radiochemotherapy. Three out of the four patients had stage ⅢB disease at diagnosis.

The absolute permanence (AP), OS, and DFS times were evaluated for each cohort (Table 2 and Figure 2), as well as the permanence/OS and permanence/DFS ratios and the total number of patients surviving (α) or deceased (Ω) at the time of analysis (Table 2). It is important to note that OS times were nearly equivalent to absolute permanence times; the permanence/OS ratios were greater than 90% in all years evaluated. Remarkably, comparisons between OS and DFS demonstrated that the DFS/OS ratio was always higher than 88%, except in the 2009 cohort.

Of 19 patients with a complete response (18 pathological and one clinical), 18 patients are still disease-free survivors. One patient had local recurrence 19 mo after the end of neoadjuvant treatment. In the full analysis of the study participants, locoregional recurrence was observed in seven patients (8.75%) an average of 21.29 mo (range 6-39 mo) after the end of preoperative radiochemotherapy, six patients showed downstaging, and one patient had no benefit from neoadjuvant treatment. Local recurrence was located in the perianastomotic region (six cases) or pre-sacral region (one case). Distant metastases were recorded in 17 cases (21.25%) where eight patients presented pulmonary metastases after an average of 22.75 mo (range 7-38 mo) after the end of neoadjuvant treatment and six patients had liver metastases after an average of 6.83 mo (range 1-16 mo) after the end of radiochemotherapy. Individual cases of brain and ovarian metastases were recorded 6 and 12 mo, respectively, after the end of preoperative treatment. One patient presented distant metastases in multiple locations. Of these 17 patients, only five showed negative lymph nodes at diagnosis.

Toxicity

Acute toxicity: Table 3 summarizes the incidence of acute toxicity. Proctitis, grade G2, was the most common symptom (63.75%) and the earliest manifestation of acute toxicity. On average, proctitis symptoms appeared during the ninth day of radiation therapy (range 2-25 d). Grade G1-2 diarrhea was noted in 31.00% of patients. Grades 3-4 toxicity was seen only in nine patients, with symptoms of diarrhea (three patients), proctitis (four patients), and abdominal pain (two patients). Genitourinary toxicity was observed in 32.50% of patients. Symptoms included an increase in frequency and dysuria, usually during the end of third week of radiation therapy (range 3-26 d). Of the 23 patients with dysuria, 47.83% (11 patients) were classified as G2. Radiation dermatitis was reported in 30 patients; ten patients' symptoms were graded as G1, 17 patients as G2, and three patients as G3. There was a significant correlation between radiation dermatitis and tumors located within 5 cm of the anal verge (*P* value, 0.0076). No hematological toxicity was observed. Hypersensitivity reactions were recorded in six patients. One patient stopped chemotherapy after the second cycle. One patient completed all five prescribed cycles of intensified chemotherapy, but during the last two cycles was administered 5-FU only. Four patients completed the prescribed treatment after taking a one-week break.

Sixteen patients presented neurologic toxicity due to oxaliplatin. Neuropathy was classified as G3 in only one patient, who had sensory loss and paresthesia during the

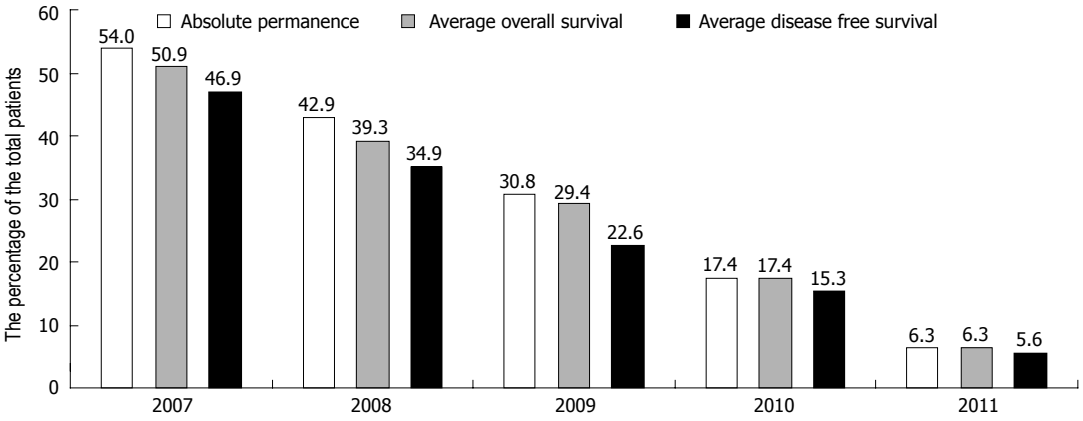

Figure 2 Absolute permanence (white column), overall survival (grey column), and disease-free survival (black column) comparisons. Patient data was stratified by year and is represented as a percentage of the total cohort of patients.

Table 3 Incidence of acute toxicity *n* (%)

	G1	G2	G3
Blood-bone marrow			
Neutrofilis-granulocytes	1 (1.25)		
Allergy-immunology			
Allergic reaction-hypersensitivity	6 (7.5)		
Cardiac arrhythmia			
Palpitation	1 (1.25)		
Constitutional symptoms			
Fatigue	10 (12.5)	5 (6.25)	
Fever	7 (8.75)	1 (1.25)	
Dermatology-skin			
Pruritus-itching	2 (2.5)		
Rash-desquamation	2 (2.5)	6 (7.5)	
Radiation-dermatitis	10 (12.5)	17 (21.25)	3 (3.75)
Gastrointestinal			
Constipation	2 (2.5)	15 (18.75)	
Diarrhea	14 (17.5)	11 (13.75)	3 (3.75)
Nausea	12 (15)		
Proctitis	6 (7.5)	51 (63.75)	4 (5)
Vomiting	5 (6.25)	1 (1.25)	
Neurology			
Neuropathy: sensory	14 (17.5)	1 (1.25)	1 (1.25)
Pain			
Pelvic pain	1 (1.25)	3 (3.75)	
Abdominal pain or cramping	6 (7.5)	2 (2.5)	2 (2.5)
Renal-genitourinary			
Dysuria-painful urination	12 (15)	11 (13.75)	
Urinary frequency	3 (3.75)		
	G3	G4	G5
Cardiovascular			
Vascular thromboembolism	7 (8.75)	2 (2.5)	2 (2.5)

end of the fifth cycle of chemotherapeutic treatment. In total, 11 patients (13.75%) had adverse cardiovascular events. Two patients experienced acute myocardial infarction (IMA), two patients experienced pulmonary embolism, and seven patients experienced deep vein thrombosis requiring anticoagulant therapy. Embolic events G3-4 arose after the end of neoadjuvant-intensified radiochemotherapy by an average of 4.42 mo (range 1-8 mo). One patient died 2 wk after the end of treatment; it was considered an IMA-related death. One patient died during surgery of cardiac infarction.

Late toxicity: Late toxicity was defined as long-term toxic effects occurring at least 6 mo after the end of radiochemotherapy treatment. Considering all enrolled patients, gastrointestinal toxicity was the most evident late side effect and was recorded in 37 patients (46.25%). Fecal incontinence was reported in 32.43% of patients and proctitis in 32.43% of patients. Four patients had diarrhea and five patients had an increase in stool frequency. Seven patients reported sexual dysfunction. Urinary incontinence was observed in three patients with colostomy. In 11 out of 60 patients with acute proctitis, the symptoms continually persisted as G1-2 grade. No correlation was observed between acute toxicity and late toxicity. Neuropathy with loss of deep tendon reflexes and paresthesia that did not interfere with activities of daily living was documented in five patients and acute neuropathic toxicity was reported in 16 patients.

Correlation analysis

We evaluated 11 "predictive" factors: (1): sex; (2): age; (3): PS; (4): TNM clinical staging; (5): cranio-caudal extension of the tumor lesion; (6): tumor location; (7): total radiotherapy dose; (8): cycles of associated chemotherapy; (9): interval between neoadjuvant treatment and surgery; (10): type of surgery; and (11): toxicity. Univariate analysis did not show any significant correlation between "predictive" factors and downstaging or pCR. There was no causal significant correlation between "predictive" factors and OS or DFS in the multivariate analysis.

Two additional statistical analyses were of interest and enhanced the evaluation of the data. First, the linear relationship between the infusion of six cycles of chemotherapy and tumor sizes smaller than 5 cm was statistically significant (*P* value, 0.0334). Second, a causal correlation was found between surgery performed seven weeks before the end of neoadjuvant treatment and OS or DFS, although the data was not stratified by the surgery characteristics.

DISCUSSION

Multimodality treatment in rectal cancer

In the past two decades, several clinical trials have been performed to determine the role of a multimodal approach in treating rectal cancer. These clinical trials faced three problems: (1) determination of temporal sequencing of treatment modalities; (2) integration of radiotherapy and chemotherapy; and (3) radiation dose fractionation. Testing and analysis of new approaches should be aimed at defining therapeutic strategies to improve local tumor control and overall survival benefit[11].

Year after year, local recurrence has represented the prevailing method of treatment failure. Now, with improvement in surgical and radiotherapeutic techniques, local tumor control rates have improved. Neoadjuvant radiotherapy and concurrent 5-FU based chemotherapy, as compared with the same protocol delivered after surgery, has improved local control[4]. The addition of 5-FU to radiation therapy also has been shown to significantly reduce the incidence of local recurrences[4,11,12]. Likewise, survival has improved with standardization of total mesorectal excision surgery[13-16]. The decrease in local recurrence rates emphasizes the need to investigate the risk of distant metastases and requires new treatment strategies to improve distant tumor control.

Although most patients achieve tumor downstaging after preoperative radiochemotherapy, the debate over monochemotherapy *vs* polichemotherapy still remains. Specifically, whether 5-FU infusion, the standard of care in rectal cancer, is better than multichemotherapeutic agents for reducing the risk of distant metastases. The prevalence of distant metastases (24%-30%) stresses the importance of a more effective systemic treatment[11].

Evaluation

In this study, the effects of a multimodal therapy approach for rectal cancer were evaluated. Disease-free survival was considered the most efficient indicator of the absence of disease and is the most robust indicator of effective treatment. In our previously reported study[8], we evaluated the toxicity and efficacy of preoperative-intensified radiochemotherapy, and the subsequent pathologic complete response, downstaging, and sphincter preservation rates. In the current study, the patient cohort from the previous study was expanded with 29 new cases and the overall survival and disease-free survival rates were evaluated and stratified per year.

The aim of this study was to verify efficacy of neodjuvant-intensified treatment. Oxaliplatin (50 mg/m^2 per week) was added to the standard 5-FU chemotherapy, normally given in continuous infusion of 200 mg/m^2 per die during each day of radiation therapy. Weekly administration of oxaliplatin, with the cumulative dose of 300 mg/m^2, was chosen so that its toxic effects were reduced and to optimize its radiosensitizing properties. This intensified radiochemoterapy regime was used to test the hypothesis that it could produce greater OS and DFS within tolerable toxicity. An empirical analysis of the study results confirms our hypothesis; the treatment was well tolerated, with high compliance and a relatively good level of toxicity. All patients had received the total prescribed radiotherapy dosage and at least five cycles of oxaliplatin were administered to 91.25% of patients.

The compliance rate (93.75%) was slightly higher than rates registered in studies in which intensified radiochemoterapy regimes were adopted (range 64%-85%)[17-21]. Most of the acute toxic side effects observed in this study were classified as grade 1-2. Grade 3-4 acute toxicity was reported in 30% of patients and was only slightly higher than data from the STAR-01 trial (24%) and the ACCORD study (25%)[17,18]. Of note, cardiovascular toxicity represented 45.83% of grade 3-4 acute toxic effects.

Thromboembolic risk

Thromboembolic risk could be ascribed to the type of chemotherapy administrated or to surgery. In the literature, there is not enough available data describing this risk, although chemotherapy is recognized as an independent risk factor for a thromboembolic event[22]. Ng *et al*[23] examined the frequency and pattern of cardiotoxicity in 153 patients treated with capecitabine used in addition with oxaliplatin for advanced colorectal cancer and found that 6.7% of patients developed thromboembolic events. Randomized studies, aimed to assess the efficacy of neoadjuvant radiochemotherapy treatment in locally advanced rectal cancer, with or without oxalipaltin, did not analyze cardiovascular toxicity. Chua *et al*[24,25] reported that during induction chemotherapy using capecitabine and oxaliplatin, 10 patients (8.5%) had cardiac or thromboembolic events and four patients died in a phase Ⅱ trial. The incidence rate of that study was slightly lower than the rate reported here (13.75%). The high rate of thromboembolic risk may be associated with properties of oxaliplatin that boost the 5-FU thrombogenetic effect[26]. Certainly, the rise in cardiovascular, chemo-related toxicity must be monitored carefully and kept under control. Because of the risk, neoadjuvant-intensified radiochemotherapy must be interrupted if cardiac symptoms appear.

Toxicity

Excluding cardiovascular toxicity, grade 3 or 4 acute toxic effects occurred only in 13 patients (16.25%) with diarrhea occurring just in three patients (3.75%). In both STAR-01 and ACCORD trials, addition of oxaliplatin to 5-FU based radiochemotherapy increased toxicity rates. In those studies, grade 3-4 diarrhea was recorded in 15% and 13% of patients given oxaliplatin *vs* 4% and 3% of those in the control group, respectively[17,18]. The lowest rates of acute gastrointestinal toxicity in this study were observed in the oxaliplatin regimen, given as 2-h infusion at a dose of 50 mg/m^2 per week. Grade 3-4 long-term toxic effects were not recorded.

Neoadjuvant-intensified radiochemotherapy efficacy

It was difficult to determine if the cause of fecal incontinence and sexual function was due to radiation therapy, surgery, or both treatments. Sixty-four patients, or 80% of the enrolled patients, underwent conservative surgery. Of these patients, 10.45% reported sexual dysfunction and 17.91% fecal incontinence.

The sphincter preservation rate, evaluated in the total patient cohort and in a subgroup of 45 patients with a tumor localization ≤ l5 cm from the anal verge, was 80% and 84.50%, respectively. These results are slightly better than the ACCORD trial, in which conservative surgery was performed in 77.20% of patients, and similar to the STAR-01 study, in which 82% of patients had sphincter preservation. In the literature, the incidence of pCR and negative radial margins, in patients who received preoperative radiochemotherapy with 5-FU and oxaliplatin, ranged from 10% to 24% and from 80.95% to 100%, respectively[18-20,24,25,27,28]. Randomized trials confirmed an improvement in the local tumor control rate. Neoadjuvant treatment has been associated with a reduction of local recurrence of 6%-15%, although the incidence of distant metastases was still 30%[29-32]. A retrospective study showed that pathologic downstaging and pCR were essential for an accurate prediction of disease-free survival and overall survival[33].

In 22.5% of patients, no residual cancer cells were identified and the tumor was staged as pT0N0. Pathological complete response rates reached 23.75% and were evaluated by post-treatment RM. One patient is still a disease-free survivor 21 mo after the end of radiochemotherapy. The majority of patients, 83.75%, had some form of tumor downstaging after preoperative treatment; involvement of the radial margin was never present.

The 5-year overall survival rate (90.91%) in this study was better than results observed in randomized trials of locally advanced rectal cancer, which only observed survival rates of 65%-68%[12,13,26]. The results of this study indicate that intensification of radiochemotherapy using oxaliplatin is effective in preoperative treatment and showed that a strong response to neoadjuvant treatment increased the overall patient survival.

Considering a median follow-up of 28.5 mo (range 2-58 mo) in the total cohort, the cumulative incidence of local recurrences was 8.75% and the incidence of OS and DFS was 90% and 70%, respectively. The most common type of first recurrence was pulmonary metastases, which arose in eight patients. This observation confirmed the need to include CT thorax controls during the follow-up program. Although some questions remain, such as the need for adjuvant chemotherapy and whether oxaliplatin boosts the 5-FU trombogenetic effect, the radiochemotherapy regime used (OXP 50 mg/m^2 and 5-FU 200 mg/m^2) seems to be appropriate to produce good clinical results and to maintain toxicity at tolerable levels.

In conclusion, the results of this study indicate that neoadjuvant-intensified radiochemotherapy in patients with rectal cancer carcinoma improved pathological complete response, negative radial margins, and sphincter preservation. The chemotherapy regime of OXP 50 mg/m^2 and 5-FU 200 mg/m^2 was well tolerated, with few severe toxic effects. Despite the small number of patients enrolled, this study suggests that the addition of oxaliplatin has a beneficial effect on overall survival, likely due to an increase in local control, although there was not a clear increase in distant metastases control. On the basis of these results, the importance and validity of intensified radiochemotherapy to reduce local recurrence should be emphasized. Currently, we are waiting for longer follow-up data from the randomized phase Ⅲ trials. Future studies will address the treatment and management of recurrent distant metastases in rectal cancer.

COMMENTS

Background

Management of rectal cancer needs a multimodality treatment approach. Significant progress has been made in the conventional modalities of surgery, radiotherapy and chemotherapy used to treat this type of cancer. The decrease in local recurrence rates emphasizes the need to investigate the risk of distant metastases and indicates that new treatment strategies are necessary to improve distant control.

Research frontiers

Although most patients achieve a tumor downstaging after preoperative radiochemotherapy, the question of monochemotherapy versus polichemotherapy still remains. Infusion of 5-fluorouracil (5-FU), the standard of care in rectal cancer, is better than multi-chemotherapeutic agents for reducing the risk of distant metastases. The incidence of distant metastases (24%-30%) stresses the importance of a more effective systemic treatment.

Applications

The objective of this study was to determine whether neoadjuvant-intensified radiochemotherapy improved the overall and disease-free survival rates in patients with locally advanced rectal cancer. Typically, improved survival rates have only been achieved with 5-FU treatment.

Terminology

Oxaliplatin is a chemotherapeutic agent. Studies *in vitro* and *in vivo* confirm its radiosensitizing properties.

Peer review

This is a retrospective review in a significant number of patients with favourable results and worth publication.

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BRIEF ARTICLE

Glucomannan for abdominal pain-related functional gastrointestinal disorders in children: A randomized trial

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Abstract

AIM: To assess the efficacy of glucomannan (GNN) as the sole treatment for abdominal pain-related functional gastrointestinal disorders (FGIDs).

METHODS: We conducted a double-blind, placebo-controlled, randomized trial. Patients were recruited among children referred to the Department of Paediatrics, Medical University of Warsaw. Included in the study were children aged 7-17 years with abdominal pain-related FGIDs classified according to the Rome Ⅲ diagnostic criteria. The children were randomly assigned to receive GNN, a polysaccharide of 1,4-D-glucose and D-mannose, a soluble fiber from the Japanese Konjac plant, at a dosage of 2.52 g/d (1 sachet of 1.26 g 2 times a day), or a comparable placebo (maltodextrin) at the same dosage. The content of each sachet was dissolved in approximately 125 mL of fluid and was consumed twice daily for 4 wk.

RESULTS: Of the 89 eligible children, 84 (94%) completed the study. "No pain" and "treatment success" (defined as no pain or a decrease ≥ 2/6 points on the FACES Pain Scale Revised) were similar in the GNN (*n* = 41) and placebo (*n* = 43) groups [no pain (12/41 *vs* 6/43, respectively; RR = 2.1, 95%CI: 0.87-5.07) as well as treatment success (23/41 *vs* 20/43; RR = 1.2, 95%CI: 0.79-1.83)]. No significant differences between the groups were observed in the secondary outcomes, such as abdominal cramps, abdominal bloating/gassiness, episodes of nausea or vomiting, or a changed in stool consistency. GNN demonstrated no significant influence on the number of children requiring rescue therapy, school absenteeism, or daily activities.

CONCLUSION: In our setting, GNN, as dosed in this study, was no more effective than the placebo in achieving therapeutic success in the management of FGIDs in children.

Key words: Functional abdominal pain; Abdominal pain-related functional gastrointestinal disorders; Children

Core tip: This study focused on abdominal pain-related functional gastrointestinal disorders (FGIDs) which are a common problem in children. The aim of the study was to assess the effectiveness of glucomannan (GNN), a soluble fiber of the Japanese Konjac plant, in alleviating the frequency and the severity of pain in children with FGIDs. We have demonstrated through our prospective, double-blind, placebo-controlled, randomized study that GNN in this setting for 4 wk was not effective in treatment of the FGIDs. The obtained results led us to the conclusion that further studies are needed to explore the role of GNN in the pathophysiology of functional disorders.

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INTRODUCTION

Functional abdominal pain (FAP) is one of the most common reasons for a referral to a physician[1]. According to various estimates of the prevalence of FAP, approximately 13% to 38% of children and adolescents report functional abdominal problems[2].

The diagnosis of FAP is symptom-based. The Rome Ⅲ criteria for abdominal pain-related functional gastrointestinal disorders (FGIDs) have been validated and assist clinicians make diagnoses[2]. The vast majority of children with FGIDs are subsequently diagnosed with functional dyspepsia (FD), irritable bowel syndrome (IBS), and childhood abdominal pain (AP)[3,4]. The symptoms associated with these diagnoses have a great impact on the patients' quality of life, daily activities, and school absenteeism, which in turn can result in long-term psychological implications[2]. Therefore, patients as well as caregivers and physicians are interested in safe and reliable treatments to relieve troublesome symptoms. Unfortunately, the medical cause for these symptoms remains unclear, thus limiting currently available therapies. FGIDs continue to represent a significant challenge in approaches to patient management.

For most children with FGIDs, no organic causes for their pain are found on physical examination or during investigations[5]. One of the recent hypotheses regarding the pathophysiology of FGIDs proposes that the biological and environmental factors that alter the enteric flora and visceral perception cause the development of functional disturbances[2,6]. The associations between the enteric microbiota, immune activation, and the role of lumen-mucosa interaction have been explored recently[2,7,8]. However, a precise explanation of the etiology of FGIDs remains to be expounded.

Regardless of the lack of a biophysiological model for FGIDs, a variety of therapeutic options have been tried. Unfortunately, studies have proven that most options do not exert any significant influence on the natural history of FGID development[2]. Two recent Cochrane systematic reviews have revealed weak evidence for the benefits of medication or dietary manipulation in children with functional abdominal pain[9,10]. Only 2 small, randomized clinical trials (Christensen 1982, Feldman 1985) have been conducted in children with FGIDs, and they compared the effects of added dietary fiber with placebo[9,11,12]. The results and conclusions of these trials were not consistent. Nevertheless, many clinicians routinely recommend the use of bulking agents or dietary fiber to stimulate regular bowel movements and to improve the symptoms associated with FGIDs[2].

The goal of our study was to assess the efficacy of glucomannan (GNN) as the sole treatment for FGIDs in children. GNN, a polysaccharide of 1,4-D-glucose and D-mannose, is a soluble fiber from the Japanese Konjac plant. GNN exhibits properties that are typical of dietary fiber. Thus, it may provide a positive effect on many aspects of gut physiology and the appropriate functioning of enteric microbiota in patients with FGIDs[8,13].

MATERIALS AND METHODS

Trial design

We conducted a double-blind, placebo-controlled, randomized trial (RCT) from January 2009 to October 2011. The recommendations of the CONSORT 2010 Statement for reporting parallel group randomized trials were followed[14].

Participants

Patients were recruited among children referred to the Department of Paediatrics, the Medical University of Warsaw, from January 2009 to October 2011. The RCT included children aged 7-17 years with abdominal pain-related FGIDs classified according to the Rome Ⅲ diagnostic criteria (Table 1)[5]. Patients with organic gastrointestinal diseases (as established by the medical history, complete blood count, urinalysis, stool examination for occult blood, ova and parasites, blood chemistries, abdominal ultrasound, breath hydrogen testing, and endoscopy, if needed), other chronic disease, or growth failure were excluded from the study. Additionally, during the time of the study, subjects were instructed not to take any medications that could influence the enteric flora, including antibiotics and commercially available probiotic or prebiotic preparations.

Interventions

At the randomization visit, the inclusion criteria were checked. Potentially eligible patients were evaluated using a full review of their clinical histories and the results of a physical examination. The included subjects were randomized into a group receiving either GNN (Dicoman Junior, Vitis Pharma, Poland) at a dosage of 2.52 g/d, *i.e.*, 1 sachet of 1.26 g 2 times a day, or a group receiving a comparable placebo (maltodextrin) at the same dosage. The content of each sachet was dissolved in approximately 125 mL of fluid and was consumed twice daily for 4 wk. The manufacturer had no role in the conception, design, or conduct of the study or in the data analysis.

Subjects were instructed to ingest the preparation twice a day, in the morning and in the evening, for 4 wk and to record any symptoms in a questionnaire at the end of the study. At randomization, parents received 28 sachets for the first 2 wk of the study. After this period, the parents were contacted to examine the children's compliance with the treatment, provide them with the next 28 sachets, and schedule the exact timing of a final visit. The assessment of all outcome measures was based on the patients' questionnaires collected at the final visit. Additionally, the subjects and/or caregivers were asked to report the following information in their diaries: compli-

Table 1 Study population baseline characteristics *n* (%)

	Placebo	Glucomannan
Patients recruited	45	44
Male/female	21/22	21/20
Age mean, yr	11.3 ± 2.5	11.6 ± 3.0
Self-reported frequency of pain[1]		
Pain 1-3 times per month		
Pain 1-2 times per week	12 (27.9)	9 (21.9)
Pain > 2 times per week	13 (30.2)	9 (21.9)
Pain every day	14 (32.6)	14 (34.1)
	4 (9.3)	9 (21.9)
Severity of pain[1]		
"face 0"	0 (0)	0 (0)
"face 1"	5 (11.6)	2 (4.8)
"face 2"	18 (40.8)	13 (31.7)
"face 3"	15 (34.8)	16 (39.0)
"face 4"	5 (11.6)	8 (19.5)
"face 5"	0 (0)	2 (4.8)
Self-reported absenteeism from school	21	21
Self-reported alterations in daily activities	29	27
Number of subjects who required rescue therapy	20	25

[1]Wilcoxon's test.

ance with consumption of the product and information on any other treatment given to the child during the study. The diary was constructed in a simple, understandable mode. The FACES Pain Scale Revised was used to assess the severity of pain[15]. The scale consists of 6 faces that reflect different levels of pain, ranging from a relaxed face on the left (no pain, indicating a score of 1 points - "face 0") to a face showing intense pain on the right (highest pain possible, indicating a score of 6 points - "face 5").

Outcomes

The primary outcome measures included the proportion of patients with self-reported no pain and that of patients with treatment success, which was defined as no pain or a decrease of $\geqslant$ 2/6 points on the FACES Pain Scale Revised[15]. The subjective assessment of pain frequency, abdominal cramps, abdominal bloating/gassiness, the number of episodes of nausea or vomiting; changes in stool consistency (loose or constipated stools) during the study were the secondary outcome measures. Furthermore, the frequencies of school absenteeism and changes in daily activities were assessed, as was the percentage of children requiring rescue therapy. Finally, all adverse effects were recorded, and their possible relation to the study product consumption was evaluated. Compliance was assessed by direct questioning of the subjects or their caregivers during clinic visits.

Randomization

Block randomization, with a block size of 6, was performed using a computer-generated random number list prepared by an investigator with no clinical involvement in the trial. The sequence was concealed until all data were analyzed.

Blinding

Both the participants and the researchers conducting the study were blinded. The study intervention product was prepared centrally by the hospital pharmacy at the Medical University of Warsaw with the assistance of independent personnel who were not involved in the conduct of the trial. The active product and the placebo were packaged in identical sachets and labeled with one of two codes, each allocated to the experimental product or placebo. This procedure was performed by an independent pharmacist, who was the only person aware of the codes' meanings. The randomization numbers had been previously generated, and every patient eligible for inclusion received a consecutive number from the list. The appearance and texture of the dry placebo product were identical to those of the active product. When mixed with water, GNN turned into a substance of jelly-like consistency; however, this only occurred if the solution was not consumed within a few minutes, which was the recommended time limit for consumption.

Statistical analysis

The sample size was based on the treatment success outcome (*i.e.*, the proportion of participants with no pain or a decrease of $\geqslant$ 2/6 points on the FACES Pain Scale Revised). It was estimated that an initial sample size of 80 patients would be sufficient to reveal a difference in the treatment effect of 30% (70% of the participants receiving GNN compared with 40% of the participants receiving placebo) considering that alpha = 0.05 and a power (beta) of 80%. The number of 80 for the children accounted for approximately 10% withdrawals or losses.

The computer software "R" [version 2.13.1 (2011-07-08)] was used for the analysis. Analyses of continuous data were performed with a parametric analysis (Student's *t*-test) in the case of a normal distribution of variables. The Mann-Whitney test was implemented for non-normally distributed variables. The χ^2 or Fisher's exact test were used, as appropriate, for the analysis of dichotomous outcomes. The RR or the mean difference (MD) with a 95%CI was calculated using the computer software StatsDirect [version 2.7.8b (2011-11-09)]. The differences between the study groups were considered to be statistically significant when the *P* value was < 0.05 or when the 95%CI: for the RR did not exceed 1.0 or, for the MD, did not exceed 0. The results were analyzed using an Available Case Analysis (ACA).

Ethical considerations

Parents and children were fully informed about the schedule and the aims of the study. Informed consent was obtained from at least one caregiver of each child included in the study and from all children older than 12 years of age.

The Ethics Committee of The Warsaw Medical University approved the study. The trial was registered at ClinicalTrials.gov (http://clinicaltrial.gov), number NCT 01495806.

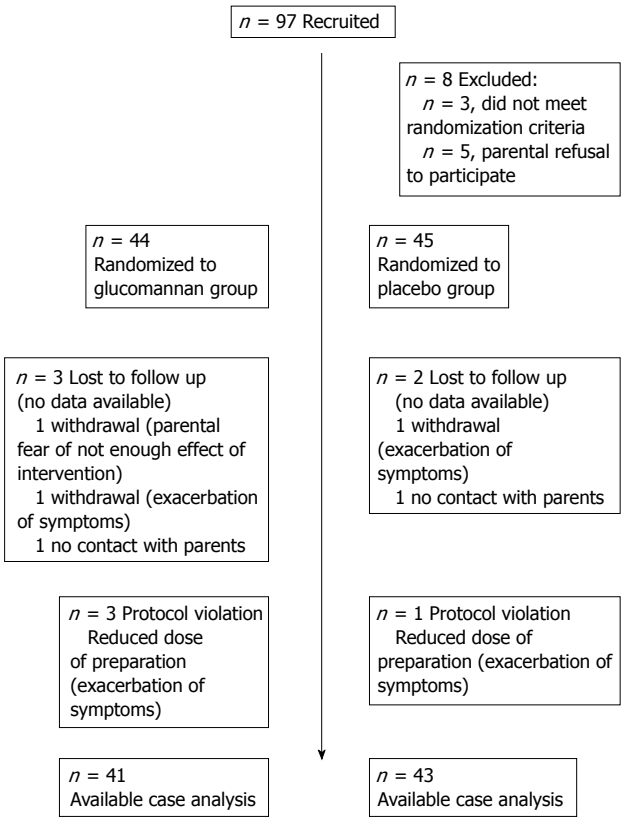

Figure 1 Flowchart of patients participating in the study. Declaration of funding interests: This study was funded in full by the Medical University of Warsaw.

RESULTS

Patients' characteristics and compliance

Figure 1 shows a flowchart of the subjects' progression through the study. Of the 97 eligible children, 89 underwent randomization. Among them, 44 children were assigned to the experimental GNN group, and 45 children were assigned to the placebo group. Of the 89 randomized children, 84 (94%) completed the study. There was no significant difference in the drop-out rate between the 2 groups. The baseline demographic and clinical characteristics were similar between the experimental and placebo groups (Table 1).

Primary outcomes

Overall, 18 of the 84 (21%) subjects reported the primary outcome measure of 'no pain'. The subjects in the GNN group were more likely to experience "no pain" (29%) than the subjects in the placebo group (14%); however, the difference was not statistically significant (RR = 2.1, 95%CI: 0.87-5.07).

"Treatment success" (defined as no pain or a decrease $\geqslant$ 2/6 points on the FACES Pain Scale Revised) was similar between the study groups. Of the 41 children in the GNN group, 23 (56%) experienced treatment success, compared with 20 of the 43 (47%) in the placebo group (RR = 1.21, 95%CI: 0.79-1.83).

Originally, we had also planned to conduct a subgroup analysis (*e.g.*, FD, FAP, IBS). However, due to the small number of patients included in the subgroups and the overlap of symptoms between the patients with various types of functional disorders, we decided against conducting this analysis.

Secondary outcomes

No significant differences between the GNN and placebo groups were observed in the secondary outcome measures, such as the subjective assessment of gastrointestinal symptoms: (1) Abdominal cramps (32% *vs* 49%, respectively; $P = 0.12$); (2) Abdominal bloating/gassiness (44% *vs* 51%, respectively; $P = 0.39$); (3) Number of episodes of nausea or vomiting (7% *vs* 2%, respectively; $P = 0.34$/24% *vs* 33%, respectively; $P = 0.31$); or (4) Change in stool consistency (loose stools: 29% *vs* 39%, respectively; $P = 0.33$; constipated stools: 27% *vs* 21%, respectively; $P = 0.53$).

Rescue therapy

The percentage of patients requiring rescue therapy was similar in both groups (GNN 19% *vs* placebo 14%, respectively; $P = 0.53$).

Subjects' activities

The GNN supplementation showed no significant influence on the frequency of school absenteeism (10% *vs* 14%, respectively; $P = 0.56$) or on changes in daily activities (27% *vs* 19%, respectively; $P = 0.37$) during the study.

Adverse effects

The GNN was well tolerated, and no adverse effects were recorded in any of the patients. However, 4 patients in the GNN group complained of an exacerbation of symptoms (1 discontinued the therapy; 3 required a dose reduction) compared with 2 patients in the placebo group (1 discontinued therapy; 1 required a dose reduction). Nonetheless, it was difficult to establish causality in these cases because the course of FGIDs was defined by exacerbations and periods of recovery.

DISCUSSION

This prospective, double-blind, placebo-controlled, randomized study showed that GNN, a soluble fiber of the Japanese Konjac plant, as used in this study and setting for 4 wk, was not effective in reducing the frequency or severity of pain in children with abdominal pain-related FGIDs. Although "no pain" was more likely to occur in the GNN group, the difference was of borderline statistical significance.

The study groups did not differ with regard to any of the secondary outcomes, such as the proportion of patients with gastrointestinal symptoms during the study period, the need for rescue therapy, and the occurrence of adverse effects.

Fiber as a treatment option for FGIDs

The optimal management strategy for abdominal pain-related FGDs in children is a matter of ongoing debate. Because of their obscure pathophysiology, the management of FGIDs remains challenging. This difficulty has prompted interest in new and safe treatment options, among them dietary interventions.

Given their safety profiles, prebiotics (especially soluble fiber) appear to be attractive therapeutic options for FGIDs[16]. Dietary fiber is not digested by human enzymes but is instead fermented by the flora of the large intestine[17,18]. There are several reasons why these agents might, in theory, prove to be beneficial in the management of functional disorders. First, some studies have demonstrated the positive effects of prebiotics on changes in the intestinal microbiota through the selective stimulation of the growth of potentially protective bacteria (bifidobacteria and, in part, lactobacilli) and the simultaneous inhibition of potentially pathogenic microorganisms[8,19-23]. Second, fiber increases biomass, feces weight and defecation frequency, which can alter the volume and composition of the stool and gas[8,13,18,24]. These changes in intestinal contents can affect the gastrointestinal symptoms associated with functional disturbances[2,8,18]. Additionally, a reduction in the pH and the release of short-chain organic acids stabilize the intestinal environment[8,16,19,25]. Finally, prebiotic-induced changes could indirectly modulate various parameters of the immune system, such as the NK-cell activity, the secretion of Il-10 and interferon, and the lymphocyte proliferation that may establish intestinal regularity[8,18,26].

Dietary fiber consumption has remained low in many populations, especially in children. The American Academy of Pediatrics recommends a daily dietary fiber intake for children of 0.5 g/kg body weight, up to 35 g/d[27]. Williams *et al*[28] proposed a minimum daily fiber intake equivalent to the age in years plus 5 g/d for children older than 2 years of age.

Few studies have evaluated the response of functional abdominal disorders to prebiotics. Furthermore, most data on the possible use of prebiotics in the management of functional disorders and the rationale for their use are derived from the studies of adults with IBS[16,19,26]. Until now, there have been only 3 published papers (Christensen 1982, Christensen 1986, Feldman 1985), which described 2 trials and assessed the effect of fiber supplementation on FGIDs in children[9]. These 2 trials, which met the inclusion criteria for the Cochrane systematic review, concerned the dietary interventions for recurrent abdominal pain (RAP) and irritable bowel syndrome (IBS) in children[9]. Both studies involved a total of 92 children with recurrent abdominal pain, and they evaluated the effect of fiber supplementation on the improvement in gastrointestinal complaints[11,12]. According to this meta-analysis, the pooled odds ratio for improvement in the frequency of abdominal pain was 1.26 (95%CI: 0.25-6.29)[9]. Feldman *et al*[12] (52 children recruited) described changes in the intensity of pain; however, the differences between the fiber and placebo groups were not statistically significant. Christensen *et al*[11] (40 children recruited) reported the mean number of episodes of pain during the study period, but the differences in the results between groups were also not statistically significant. The lack of recent findings concerning the potential application of fiber in the management of children with FGIDs instigated our decision to examine the effectiveness of GNN, which is composed of a dietary fiber, *i.e.*, water-soluble polysaccharide.

Dose and duration of treatment

The optimal dose and treatment duration of GNN therapy have not yet been clearly established. Recently, we used GNN to treat functional constipation in children, where it proved to be relatively safe[13]. In the present study, regardless of the dietary recommendations for daily fiber intake, we used only one standard dose of fiber for all participants to provide a relatively easy administration regimen for the clinicians and parents. We applied a daily intake of 2.52 g/d, which exceeded the minimum dose of fiber suggested in the literature for therapeutic purposes[13].

Considering the chronic nature of functional disorders, the chosen duration of treatment (4 wk) appears to be optimal for evaluating GNN's potential therapeutic influence on gastrointestinal complaints. The administration of GNN, a soluble fiber preparation, was the sole intervention implemented, allowing one to draw conclusions regarding its effectiveness without any confounding influence from other treatments.

Participants

Evaluating children as study participants is a complicated issue because they complain of nonspecific chronic abdominal pain and constitute a heterogeneous group of patients. To minimize the heterogeneity of our study's population, the diagnosis was based on the well-recognized Rome Ⅲ criteria[5].

The trial was conducted in a pediatric department oriented towards the diagnosis and treatment of children with functional abdominal pain. Nevertheless, the possibility that only less affected patients were included in the study could not be entirely eliminated. The pattern of the response to treatment may differ from that in patients with more severe courses of functional disturbances.

Strengths and limitations of the study

We used the appropriate methods to generate the allocation sequence and allocation concealment. We then strived to maintain the blinding of the selection, treatment, monitoring, data management, and data analyses throughout the study. In addition, the follow-up was appropriate. Data were obtained from more than the 94% of the participants. All of these features minimize the risk of systematic bias.

The lack of an apparent effect of GNN compared with the placebo may be explained by an inadequate dose

and an overly brief treatment period. Although the optimal dose and duration of treatment for GNN have not yet been established, a tentative conclusion can be drawn: subjects with abdominal pain-related FGIDs require a modification of the GNN dose and an extension of the period of supplementation to improve their gastrointestinal symptoms. Additionally, nutritional habits can modify the natural course of abdominal complaints. We realized that precise data on the daily fiber intake would be useful. However, collecting that type of information would have required the use of a food diary. We abstained from this process for 2 major reasons. First, our study population of children aged 7-17 years included children who attended school. Collecting reliable information from children in this age group represents a particularly challenging task. Second, the use of a food diary, especially in this group of patients, provides only an approximate evaluation of dietary intake. Moreover, the validity of paper diary records is sometimes questionable. Well-known problems with paper diaries include poor adherence and retrospective recording[29]. As there were no differences between the groups in terms of the baseline characteristics, we may expect that the randomization was implemented properly and that the daily fiber intake was similar in both groups.

It is known that success in treating patients with abdominal pain-related FGIDs depends on the relationship established between a patient and a physician. An "active listening approach" and an enthusiastic, positive, and encouraging attitude towards treatment help improve subjects' responses to both the therapeutic attempts and the placebo[30]. The placebo effect in adults ranges from 10%-70% for FD and from 0%-84% for IBS[6]. We used a placebo control group, which is considered an essential requirement for interventional studies[31]. According to our previous experiences based on earlier studies conducted in children with functional abdominal pain and functional constipation, we were able to predict the high proportion of children who were responsive to the placebo[3,13]. Considering our positive patient-physician relationship, we expected a drop-out rate of approximately 10% (6% at the end of the trial), in contrast to the 20% drop-out rate estimated for most trials. Thus, paradoxically, these positive relationships can be a potential weakness of our investigation.

Finally, another potential limitation of this trial is that although the overall number of patients was adequate, the study did not allow the analysis of the data for a specific diagnosis of abdominal pain-related FGIDs, *e.g.*, FD, IBS or FAP.

Consequently, an obvious next step in future research should be to identify the characteristics of children with specific abdominal pain-related FGIDs who respond to GNN treatment. Identifying this group of patients will allow for the selection of the optimal GNN dose to improve long-term gastrointestinal symptoms. Further studies are also needed to explore the role of GNN in the pathophysiology of functional disorders.

In our setting, GNN as dosed in this study was no more effective than the placebo in achieving therapeutic success in the management of abdominal pain-related FGIDs in children.

COMMENTS

Background

Abdominal pain-related functional gastrointestinal disorders (FGIDs) are widespread complaints in the pediatric population, affecting from 13% to 38% of children and adolescents. The symptoms associated with FGIDs have a great impact on patients' quality of life and their daily activities, which in turn can result in long-term psychological implications. Therefore a variety of therapeutic options have been considered to date.

Research frontiers

Many clinicians routinely recommend the use of bulking agents or dietary fiber to stimulate regular bowel movements and to improve symptoms associated with FGIDs. However, only two small, randomized clinical trials (Christensen 1982, Feldman 1985) conducted in children with FGIDs, which compared the effects of added dietary fiber with placebo, were identified. The results and conclusions of these trials were not consistent.

Innovations and breakthroughs

Development of the optimal management strategy for abdominal pain-related FGIDs in children is difficult. Soluble fiber seems to be an attractive therapeutic option for FGIDs. Only a small number of studies that have evaluated the response of functional abdominal disorders to fiber are available. Additionally, the optimal dose and treatment duration have not been clearly established yet. In the present study, the standard daily dose (2.52 g/d) of glucomannan was used for all participants in order to provide a relatively easy administration regimen for clinicians and parents. The dose was equal to the minimum dose of fiber suggested in the literature for therapeutic purposes. Because of the chronic nature of functional disorders, the 4 wk duration of treatment was chosen to evaluate glucomannan's potential therapeutic influence on gastrointestinal complaints. The most important contribution of this study and its strength is its methodology. The adequate methods for generation of the allocation sequence, allocation concealment and blinding of selection, treatment, monitoring, data management, and data analyses were used throughout the study. Data were obtained from more than the 94% of the participants. All of these features minimize the risk of systematic bias.

Applications

The study results suggest that, glucomannan was no more effective than placebo in achieving therapeutic success in the management of abdominal pain-related FGIDs in children.

Terminology

Glucomannan, a polysaccharide of 1,4-D-glucose and D-mannose, is a soluble fiber from the Japanese Konjac plant. Glucomannan exhibits properties that are typical of dietary fiber. There are several reasons why these agents might, in theory, prove to be beneficial in the management of functional disorders. Some studies have demonstrated positive effects of fiber on changes in the intestinal microbiota by selective stimulation of the growth of potentially protective bacteria with simultaneous inhibition of potentially pathogenic microorganisms. Fiber increases biomass, feces weight and defecation frequency, which can in turn alter the volume and composition of stool and gas. These changes in intestinal contents can affect the gastrointestinal symptoms associated with functional disturbances.

Peer review

This study employs a simple, methodologically sound model. The main conclusion of the study is the lack of influence of glucomannan supplementation on the level and duration of pain in children with FGIDs.

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BRIEF ARTICLE

Efficacy and safety of 0.4 percent sodium hyaluronate for endoscopic submucosal dissection of gastric neoplasms

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Abstract

AIM: To evaluate the efficacy and safety of sodium hyaluronate solution (SH) in endoscopic submucosal dissection (ESD) of gastric neoplasms.

METHODS: A prospective multicenter randomized, double blind, controlled trial was designed and utilized in this study. A total of 76 patients with 5-20 mm sized gastric neoplasms were enrolled at three academic hospitals in South Korea from June 2011 to October 2011. Patients were randomly assigned to the 0.4% sodium hyaluronate or control groups. All lesions underwent endoscopic ESD. ESD was performed with 0.4%SH and normal saline (NS) solution for submucosal injection. Efficacy was assessed using *en bloc* resection and the number of additional injections. Secondary evaluation variables were the volume of injection material, steepness of mucosal elevation, bleeding rate, procedural time and operator satisfaction. Finally, the safety was assessed by analyzing adverse events during the study.

RESULTS: The usefulness rate in the 0.4%SH group and the controlled group had statistically significant difference under intention to treat (ITT) analysis (90.91% *vs* 61.11% $P = 0.0041$). Under per protocol (PP), the usefulness rate is statistically significant different (93.10% *vs* 61.76%, $P = 0.0036$). The difference in volume of the solution injected between 0.4%SH group and the controlled group and NS group was also statistically significant under intention to treat and per protocol analysis (ITT: 0.03 ± 0.02 mL *vs* 0.06 ± 0.03 mL, $P = 0.0003$, PP: 0.03 ± 0.02 mL *vs* 0.06 ± 0.03 mL, $P = 0.0004$). Satisfaction above the grade good was significantly higher in the SH group under intention to treat and per protocol analysis (ITT: 90.91% *vs* 61.11%, $P = 0.0041$, PP = 93.11% *vs* 61.77%, $P = 0.0022$). Adverse events above grade 3 were not noticed in either group. All adverse events were treated and were judged as not associated with the submucosal injection solutions.

CONCLUSION: 0.4%SH solution is a safe and effective agent that doesn't cause any significant adverse events and is useful for submucosal injection during ESD.

Key words: Sodium hyaluronate; Endoscopic submucosal dissection; Gastric neoplasm; Endoscopic mucosal resection

Core tip: Saline-assisted endoscopic mucosal resection is an established method for excision of nonpolypoid early neoplastic lesions of the gastrointestinal tract. However, it is sometimes difficult to maintain a desired level of tissue elevation after injection of saline, especially when using a one-channeled endoscope. Adequate elevation of the mucosa and sufficient elevation time is achieved more effectively when a material more viscous than normal saline (NS) is used. The 0.4% sodium hyaluronate solution (SH) used in this study provides a more effective and prolonged cushion effect for large lesions without serious adverse events compared to NS. Therefore, endoscopic submucosal dissection (ESD) with SH is more useful than ESD with NS.

Kim YD, Lee J, Cho JY, Kim SW, Kim SH, Cho YK, Jang JS, Han JS, Cho JY. Efficacy and safety of 0.4 percent sodium hyaluronate for endoscopic submucosal dissection of gastric neoplasms. *World J Gastroenterol* 2013; 19(20): 3069-3076 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3069.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3069

INTRODUCTION

Endoscopic mucosal resection (EMR) using submucosal saline injection is an established method for excision of nonpolypoid early neoplastic lesions of the gastrointestinal tract[1,2]. Numerous resection methods have been developed using the submucosal injection technique. In 1998, Hosokawa developed an IT knife (insulated tipped electrosurgical knife) useful for endoscopic submucosal dissection (ESD)[3], which has made *en bloc* resection of not only elevated lesions but depressive lesions without ulcers and flat lesions possible, compared to EMR. The procedure has minimal limitations regarding location and size of the lesions. The technical advancement of ESD combined with development of many endoscopic accessories has made ESD a standard treatment of early gastric cancer in selected cases.

The submucosa is a thin connective tissue layer with a lax structure compared to the mucosa or the muscularis propria. The injection of solutions to this layer forms a bulla and lifts the lesion above. Submucosal injection during ESD facilitates the removal of the lesions, provides a safety cushion during resection, and prevents perforations during the procedure. The most commonly used material is normal saline (NS). However, it is sometimes difficult to maintain a desired level of tissue elevation after an injection of saline, especially when using a one-channeled endoscope[4].

An ideal submucosal injection solution would have a prolonged cushion effect, and be easily available and inexpensive, nontoxic, and easy to inject[5]. Sodium hyaluronate solution (SH) is a macromolecular polysaccharide composed of *D*-glucuronate and *N*-acetyl-*D*-glucosamine. The high viscosity, elasticity, and lack of antigenicity or toxicity[6-8] have led to its extensive use in ophthalmologic surgical procedures and intra-articular injections. Since Yamamoto *et al*[9] reported the use of SH in porcine stomach yielding a more distinct and prolonged submucosal elevation compared to NS, numerous studies have used SH for difficult cases.

The authors aimed to compare the usefulness rate and safety of 0.4%SH in ESD as compared to NS in a multicenter prospective randomized double-blind control study.

MATERIALS AND METHODS

Patient selection

From June 2011 to October 2011, 76 patients with less than 20 mm sized early gastric cancer or gastric adenoma were enrolled in 3 independent academic hospitals in South Korea. Experienced endoscopists with more than 300 cases of ESD experience performed the ESD. Criteria for inclusion in the study were: (1) patients between the ages of 20 and 80; (2) patients with 5- to 20-mm early gastric cancer or gastric adenoma; and (3) patients who gave written informed consent. The limitation of the lesion size to less than 20 mm was based on the absolute indication criteria of ESD. Exclusion criteria of the study were: (1) residual or recurrent lesion; (2) lesions accompanied by ulcers; (3) undifferentiated cancer; (4) advanced malignant neoplasm; (5) patient with pacemakers; (6) history of hypersensitivity to hyaluronic acid; (7) serum creatinine $\geqslant$ 1.5 mg/dL or creatine clearance $\leqslant$ 50 mL/min; (8) patients with severe liver disease; (9) severe cardiovascular disease; (10) patient taking immunosuppressants including prednisolone or anti-cancer agents; (11) alcohol- or drug-addicts; (12) pregnant or lactating patients; and (13) patients judged by a physician as inappropriate for inclusion.

Study design

The clinical trial was approved by the Korean Food and Drug Administration (KFDA) and the study protocol was approved by the Institutional Review Board of each center. Written informed consent was obtained from all the patients.

The study was double-blinded by having an independent investigational device manager that supplied the solutions used for the study. The endoscopist was blind to the material used. During the procedure, an experienced assistant did the injection of fluids to the submucosa. Although the endoscopist could visualize the elevation effect, the difference in pressure during injection of the material was known only to the injecting assistant. The injecting assistant did not take any part in evaluating the satisfaction grade of the materials. All lesions were resected through ESD. The margin of the lesion was marked through electrocautery and submucosal injection was done at the normal mucosa adjacent to the lesion. ESD was initiated after an adequate amount of solution was injected to lift the lesion. The maximum amount of

0.4%SH solution (Endo-Mucoup, BMI Korea, Co., Ltd) for the procedure was limited to 40 mL, which is 1/10 the nontoxic level administered into the peritoneum. The amount of SH solution supplied for the procedure was limited to 40 mL and any additional amount of injection needed used NS. Necessary medication or procedure for the treatment of coexisting disorders of the patients was allowed. Except for the use of epinephrine and indigo carmine, any other material for submucosal injection solution was prohibited.

The study was designed as a multicenter, randomized, double blind, and placebo-controlled trial. The primary outcome of the study was *en bloc* complete resection and additional injections of the solution used. *En bloc* complete resection was defined as *en bloc* resection with negative resection margins confirmed histopathologically. Additional resection was defined as the number of additional injects required to maintain the mucosal lifting for the procedure. The usefulness rate was defined as the percentage of *en bloc* complete resection with 1 or less additional injection during the procedure (Table 1). The 40 mL of SH was decided for the efficacy evaluation as it is 1/10 the nontoxic level administered into the peritoneum.

Secondary outcomes of the study included: (1) volume of the solution injected; (2) the steepness of the lift; (3) presence or absence of bleeding; (4) procedure time; and (5) satisfaction of the solution. The volume of the solution used for evaluation was the total amount of the solution divided by the area of the lesion (long diameter × short diameter). The steepness of the lift was graded as steep, mild, or non lifted. The percentage of each grade was used for evaluation. Bleeding was defined as the need for electrocauterization before the incision and after injecting the submucosal solution. The procedure time was defined as the period from the marking of the margins to the completion of the excision. Satisfaction rate of the solution was comprehensively assessed by evaluating the *en bloc* complete resection rate and the number of additional injections (Table 1).

The safety of the solution was assessed through 5 grades of symptoms and signs unwarranted during the study. Grade 1 adverse event was defined as symptoms or signs that do not require treatment and do not inhibit daily activities. Grade 2 adverse events were defined as symptoms or signs that required treatment but did not inhibit daily activities. Grade 3 adverse events were when the patient experienced substantial discomfort leading to limitation of daily activities and requiring admission. Life-threatening adverse events were defined as grade 4. Death was defined as grade 5. For each adverse event, the clinician assessed the association of the response to the submucosal injection solution as definitely related, probably related, possibly related, probably not related, definitely not related, or unknown.

Sample size

This multi center randomized, double-blind, placebo-controlled study was designed with a level of significance $\alpha = 0.05$ and detection power $1 - \beta = 0.8$ to test the superiority of the 0.4%SH solution compared to NS for the lift and maintenance of the submucosa during ESD. The calculated sample size was 76 patients. Thirty-four patients were analyzed in each group assuming a 10% rate of non-comparability in the two groups.

Statistical analysis

Statistical analysis was done using SAS software 9.1 version (SAS Institute, Cary, NC). Intention to treat analysis and per protocol analysis was used in the study. The clinical usefulness was assessed using both methods, with intention to treat as the primary method. Per protocol analysis was used for demographic data and adverse effects. Continuous variables for the two groups were compared using *t*-test and χ^2 test or the Fisher's exact test, which was used for categorical variables. The primary outcome, the usefulness rate, was analyzed using the χ^2 test and 95%CIs are given. The secondary outcomes, volume of the solution used and procedure time were analyzed using *t*-test. Presence or absence of bleeding and satisfaction of the solution was assessed using χ^2 test. Safety evaluation using the total number of the adverse effects and the rate of patients with more than 1 adverse effect was done using the χ^2 test. Null hypotheses of no difference were rejected if *P*-values were less than 0.05.

RESULTS

Seventy-six patients who underwent screening examination and who gave informed written consent were included in this study. Using randomization, 37 patients were assigned to the 0.4%SH group and 39 patients were assigned to the control group that used NS for submucosal injection. All these patients were included in the intention to treat analysis. Eight patients from the 0.4%SH group and 5 patients from the control group were either lost to follow up or had a clinical trial protocol violation; the remaining 63 patients were included in the per protocol analysis (Figure 1). There were no statistically significant differences in the patients' age, sex, percentage of gastric cancer, or location of the lesion between the two groups (Table 2).

Clinical usefulness

Usefulness, the primary outcome of the study, was assessed using both intention to treat analysis and per protocol analysis, with intention to treat as the primary method. Under intention to treat analysis, the usefulness rate of the 0.4%SH group (90.91%, 30/33) was found to be significantly greater than the control group (61.11%, 22/36) ($P = 0.0041$). Using the per protocol analysis, the usefulness rate of the 0.4%SH group and the control were 93.10% (27/29) and 61.76% (21/34), respectively. The difference of the usefulness rate using per protocol analysis was also statistically significant ($P = 0.0036$). The usefulness according to the site of the gastric neoplasm,

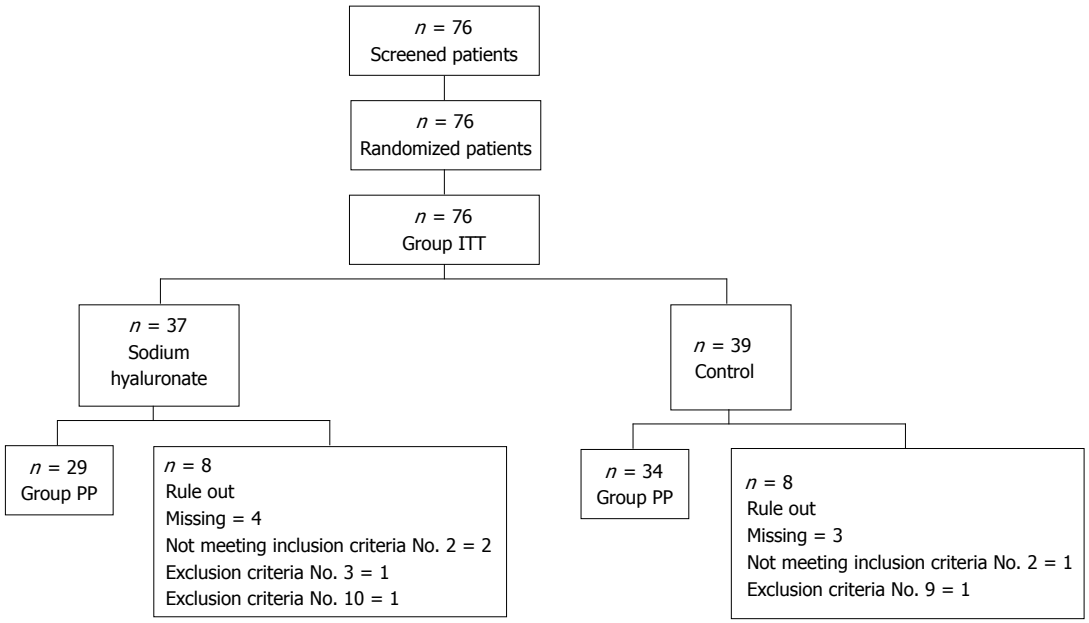

Figure 1 Trial profile. Inclusion criteria No. 2, 5 mm ⩽ adenoma or adenocarcinoma ⩽ 20 mm; exclusion criteria No. 3, undifferentiated adenocarcinoma; exclusion criteria No. 9, severe functional abnormalities of cardiovascular system; exclusion criteria No. 10, concomitant medication with systemic prednisolone, anticancer agents or immunosuppressive agents. ITT: Intention to treat; PP: Per protocol.

Table 1 Measurement of usefulness and satisfaction

Complete *en bloc* resection	Additional injection	Usefulness	Satisfaction
Complete	0	Usefulness	Excellent
Complete	1	Usefulness	Good
Complete	⩾ 2	Useless	Moderate
Incomplete or not evaluated	-	Useless	Poor

using the intention to treat (ITT) analysis revealed that 0.4%SH was statistically significantly useful when used during procedures for gastric neoplasms at the body and angle. However, there was no statistically significant difference in procedures done at the antrum. Using a per-protocol analysis, 0.4%SH showed statistically significant usefulness in all the sites of the stomach.

Secondary outcomes of the study were analyzed including volume of the solution injected, the steepness of the lift, presence or absence of bleeding, procedure time, and satisfaction of the solution (Table 3).

The volume of the solution used for evaluation was the total amount of the solution divided by the area of the lesion (long diameter × short diameter). The volume of the solution injected for the 0.4%SH group (0.03 ± 0.02 mL) was significantly less compared to the control group (0.06 ± 0.03 mL) ($P = 0.0003$). The difference in volume of the solution injected between the 0.4%SH group and NS group using per protocol analysis was also statistically significant ($P = 0.0004$).

The procedure time was defined as the period from the marking of the margins to the completion of the excision. The procedure time analyzed by intention to treat showed 23.42 ± 16.76 min in the 0.4%SH group and 21.64 ± 16.52 min in the control group. Using the per protocol analysis the procedure times were 23.79 ± 17.51 min in the 0.4%SH group and 19.71 ± 12.65 min in the control group. Although the procedure time for the 0.4%SH group was shorter compared to the control group, the difference was not statistically significant.

The steepness of the lift was graded as steep, mild, or non lifted in the two study groups and showed no statistically significant differences.

The absence of bleeding during injection of the submucosal solution was higher in the 0.4%SH group (96.97%, 32/33) compared to the control group (88.89%, 32/36) using the intention to treat analysis. Using the per protocol analysis, the absence of bleeding was 100% (29/29) in the 0.4%SH group and 88.24% (30/34) in the control group. However, the difference was not statistically significant in both analyses.

Satisfaction with the solution was comprehensively assessed by evaluating the *en bloc* complete resection rate and the number of additional injections. ESD with *en bloc* complete resection and no additional submucosal injection was defined as excellent. *En bloc* complete resection with 1 additional submucosal injection was defined as good. Satisfaction above the grade good was significantly higher in the 0.4%SH group (90.91%, 30/33) compared to the control group (61.11%, 22/36) using intention to treat analysis ($P = 0.0175$). Using per protocol analysis showed statistically significant difference in the satisfaction above the grade good in 93.11% (27/29) and 61.77% (21/34) in the 0.4%SH group and control group, respectively ($P = 0.0022$).

The ESD that had additional submucosal injections was analyzed by the site of the lesion using the usefulness rate, volume of solution injection and procedure time (Table 4).

Table 2 Demographic characteristics of the patients at base line

	Sodium hyaluronate	Control	*P*-value
Age	62.59 ± 9.23	62.44 ± 9.93	0.943
Sex (male/female)	25/12	26/13	0.933
Diagnosis, *n* (%)			
Adenocarcinoma	5 (13.51)	7 (17.95)	0.869
Adenoma	31 (83.78)	31 (79.49)	
Atypia	1 (2.7)	1 (2.56)	
Size (mm)	14.2 ± 5.47	13.5 ± 4.35	0.293
Location, *n* (%)			
Antrum	23 (62.16)	29 (74.35)	0.465
Angle	3 (8.10)	3 (7.69)	
Body	11 (29.72)	7 (17.94)	

Table 3 Summary of results *n* (%)

	Sodium hyaluronate	Control	*P*-value
ITT			
Usefulness rate	30/33 (90.91)	22/36 (61.11)	0.004
Volume of injection	0.03 ± 0.02	0.06 ± 0.03	0.000
Procedural time	23.42 ± 16.76	21.64 ± 16.52	0.658
Steepness			0.228
Steep	26 (78.79)	27 (75.00)	
Mild	6 (18.18)	4 (11.11)	
Non lifted	1 (3.03)	5 (13.89)	
Absence of bleeding	32/33 (96.97)	32/36 (88.89)	0.359
Satisfaction	30/33 (90.91)	22/36 (61.11)	0.002
PP			
Usefulness rate	27/29 (93.10)	21/34 (61.76)	0.004
Volume of injection	0.03 ± 0.02	0.06 ± 0.03	0.000
Procedural time	23.79 ± 17.51	19.71 ± 12.65	0.288
Steepness			0.474
Steep	24 (82.76)	26 (76.47)	
Mild	4 (13.79)	4 (11.76)	
Non lifted	1 (3.45)	4 (11.76)	
Absence of bleeding	29/29 (100.00)	30/34 (88.24)	0.118
n (%)	27/29 (93.11)	21/34 (61.77)	0.002

ITT: Intention to treat; PP: Per protocol.

The sites of the lesion were divided into the antrum group and body/angle group. Under intention to treat analysis on antrum, there is no significant difference of the usefulness rate in the 0.4%SH group (90.00%, 18/20) compared to the control group (66.67%, 18/27) (P = 0.086). But using per protocol analysis, the antrum had a significantly higher usefulness rate in the 0.4%SH group (93.75%, 15/16) compared to the control group (65.38%, 3/8) (P = 0.036). The usefulness rate analyzed in the lesions located at the body/angle using intention to treat analysis was 92.30% (12/13) in the 0.4%SH group compared to the 66.67% (6/9) in the control group. The difference was statistically significant (P = 0.007). Per protocol analysis also revealed a statistically significant higher usefulness rate of 92.30% (12/13) in the 0.4%SH group compared to 37.50% (3/8) in the control group (P = 0.007).

The amount of volume injected at the antrum using intention to treat analysis was 0.03 ± 0.02 mL in the 0.4%SH group and 0.06 ± 0.04 mL in the control group. The smaller amount used in the 0.4%SH group was statistically significant (P = 0.003). Using per protocol analysis, the difference in the amount of volume injected 0.4%SH group (0.03 ± 0.02 mL) and control group (0.06 ± 0.04 mL) was statistically significant (P = 0.011).

The amount of volume injected at the body/angle using intention to treat analysis was 0.03 ± 0.02 mL in the 0.4%SH group and 0.05 ± 0.02 mL in the control group. The smaller amount used in the 0.4%SH group was statistically significant (P = 0.047). Using per protocol analysis, the difference in the amount of volume injected 0.4%SH group (0.03 ± 0.02 mL) and control group (0.05 ± 0.03 mL) was statistically significant (P = 0.039).

The procedure time using both intention to treat analysis and per protocol analysis based on the site of the lesion showed no statistically significant differences.

Table 4 Summary of results on location

	Sodium hyaluronate	Control	*P*-value
ITT			
Usefulness rate			
Antrum	18/20 (90.00%)	18/27 (66.67%)	0.086
Body/angle	12/13 (92.30%)	6/9 (66.67%)	0.007
Volume of injection			
Antrum	0.03 ± 0.02	0.06 ± 0.04	0.003
Body/angle	0.03 ± 0.02	0.05 ± 0.02	0.047
Procedural time			
Antrum	19.40 ± 7.46	18.41 ± 11.67	0.741
Body/angle	28.08 ± 25.47	31.33 ± 24.68	0.768
PP			
Usefulness rate (%)			
Antrum	15/16 (93.75%)	17/26 (65.38%)	0.036
Body/angle	12/13 (92.30%)	3/8 (37.50%)	0.007
Volume of injection			
Antrum	0.03 ± 0.02	0.06 ± 0.04	0.011
Body/angle	0.03 ± 0.02	0.05 ± 0.03	0.039
Procedural time			
Antrum	19.06 ± 6.78	18.23 ± 11.88	0.800
Body/angle	28.08 ± 25.47	24.50 ± 14.70	0.723

ITT: Intention to treat; PP: Per protocol.

Adverse events

Adverse events were seen in 8 out of the 76 patients, with 13 incidents in the intention to treat analysis. Grade 1 adverse events were noticed in 12.50% (5/37) of the SH group and 2.44% (1/39) of the control group. Grade 2 adverse events were seen in 7.50% (3/37) and 9.76% (4/39) of the 0.4%SH group and control group, respectively. Adverse events above grade 3 were not noticed in both groups. Gastrointestinal symptoms were the most common adverse events with 10 out of the 13 incidents. Five cases of nausea, 3 cases of vomiting, and each 1 case of dyspepsia and hematemesis were noticed. All adverse events were treated and were judged as not associated with the submucosal injection solutions.

DISCUSSION

ESD is now a standard treatment for early gastric neo-

plasms in the gastrointestinal tract. The EMR used currently are strip-biopsy method or lift and cut EMR[10], endoscopic resection after local injection of a solution of hypertonic saline and epinephrine[11], endoscopic double-snare polypectomy[12], EMR using an over-tube[13], strip-biopsy using two small diameter endoscopes[14], EMR with a cap-fitted panendoscope[15], an EMR using a ligation device[16,17]. The submucosal injection of solutions during the resection of gastrointestinal neoplasm is an essential part of the resection procedure[18-21]. Tanabe *et al*[10] introduced strip biopsy with submucosal injection using NS as a safety cushion. Since Ikeda *et al*[22] injected a mixture of hypertonic saline and epinephrine to lift the lesion in 1986, lifting the lesion with a safety cushion underneath the lesion has become an essential procedure in EMR. ESD can resect a larger lesion without regard to the location of the lesion compared to EMR. To achieve an *en bloc* resection of a large lesion, adequate lift of the lesion is needed for a prolonged period. Numerous solutions are under research and have been used for mucosal resection. These agents include NS, mixture of NS and epinephrine, hypertonic saline (3.8%), hypertonic glucose solution (20%, 50%), 10% glycerin + 5% fructose + 0.9% NS, SH, hydroxypropyl methycellulose, and a fibrinogen mixture. NS is easily available, cheap, and causes minimal tissue injury due to its isotonic property. However, NS is easily absorbed to the tissue and multiple injections are needed. The mixture of NS and epinephrine (1:10000) is most widely used. The steepness and maintenance of the cushion is not significantly different from NS, but the mixture of epinephrine causes vasoconstriction of the vessels leading to hemostasis. The addition of indigo carmine to the mixture assists in identifying the submucosa and muscularis propria during resection of the deep margins. However, during ESD, the cushion is easily dissipated and multiple injections are needed. This leads to a prolonged procedure time and the risk of perforation is high when resecting the lesion without adequate cushion. Therefore, an ideal agent would have a higher viscosity to maintain the cushion longer and require fewer additional injections during the procedure. Although hypertonic saline and hypertonic glucose solution had a steeper elevation compared to NS, this was not statistically significant. The hypertonic solution has a propensity to cause tissue injury and may delay healing of the artificial ulcer after the procedure.

SH has a prolonged cushion-effect and causes minimal tissue injury. However, the solution is expensive and has special requirements for storage. Hydroxypropyl methycellulose has a similar viscosity and tensile strength compared to SH and has an adequate cushion-effect, is inexpensive, and is easy to store. However, the synthetic material can cause cross-antigen reaction in the body. Fibrinogen mixture is a 340 kDa high molecular glycoprotein separated from blood. The viscosity is high and has a microvascular hemostatic effect. However, the high viscosity requires a large diameter injection tip. Lee *et al*[23] report that the fibrinogen mixture is superior to NS in the procedure time, total volume of solution injection, and additional injection rates in ESD.

SH is considered the best solution for submucosal dissection. However, its use is limited due to the high cost of the solution. The molecular weight and dilution rate of SH is continuously researched to decrease the cost and find the dilution rate for effective lifting. Fujishiro *et al*[24,25] report that the optimal viscosity and tensile strength was 1% 1900-kDa solution. However, 1% 800-kDa, 0.5% 1900-kDa, 0.5% z800-kDa, and 0.25% 1900-kDa solutions all showed similar results. Concentration of the solution below 1% still maintained adequate viscoelasticity while not increasing osmolarity or causing tissue injury. Despite the fact that it is an ideal substance for submucosal injection, the high cost and special requirements for storage led to a mixture of it with other hypertonic solutions.

This study evaluated the clinical usefulness and safety of 0.4%SH compared to NS. All procedures for the resection of the lesions were ESD. The size of the lesion was limited to 2cm due to the absolute indication of ESD for early gastric cancers without lymph node metastasis[14]. This led to the limitation of gastric adenoma size to less than 2cm. The primary outcome, usefulness rate, analyzed using intention to treat and per protocol analysis revealed higher and statistically significant rates in the 0.4%SH group. Secondary outcome of the study including volume of the solution injected, the steepness of the lift, presence or absence of bleeding, procedure time, and satisfaction with the solution were analyzed. The volume of the solution injected was statistically significantly decreased and satisfaction of the solution was significantly superior in the 0.4%SH group. The limitation of the size in the inclusion criteria may have limited the analysis of the resection rate in the two groups. The usefulness rate may have showed a more significant difference in the two groups as the size of the lesion is increased. The procedure time of the two groups in this study showed no statistically significant difference. This may have been due to the fact that the size of the lesion was limited to less than 2 cm. The experienced clinicians participating in this study may have decreased the difference in time required for the 2 groups. However, if larger lesions with scars were included in the study, even experienced clinicians may show a difference in the procedure time for the 2 groups due to the multiple injections that may be required to maintain the cushion-effect during the procedure. Therefore, a prospective study including gastric adenoma that has no limitation of size in *en bloc* resection using ESD may show a difference in the usefulness rate, volume of solution injected and satisfaction with the solution. The procedure time may show a significant difference because 0.4%SH had a prolonged cushion-effect and requires less additional injections compared to NS. There were grade 1 and grade 2 adverse events observed in this study. However, there was no statistically significant difference in the two groups and clinicians judged that the adverse events were not related to the solution

injected. Therefore, the use of 0.4%SH for submucosal injections during ESD is considered safe.

This study has a size limitation of 2 cm. Gotoda *et al*[26] reported that due to the lack of lymph node metastasis, mucosal gastric cancer less than 3 cm accompanied by an ulcer and submucosal gastric cancer invading the SM1 layer less than 3 cm are also indications of endoscopic therapy. This has formed the basis of the expanded criteria for endoscopic therapy in early gastric cancer. However, the current study was approved by the KFDA for absolute indications of ESD for early gastric cancer. The size of the gastric adenomas was also limited for evaluation of the two materials without bias.

The recent development of endoscopic accessories with more skilled clinicians has led to *en bloc* resection of lesions without regard to the location and size. This has led to a more extended indication of ESD and a solution with a long lasting cushion-effect is needed. Using NS for lesions that are easily approachable and small is cost-effective. However, for lesions that are large and difficult to approach, the procedure time could be prolonged with more frequent complications. In these cases, using 0.4%SH for the submucosal injection would be more cost-effective.

In conclusion, the ideal agent would have a prolonged cushion effect, be easily available, nontoxic, easy to inject, and inexpensive. SH has a long-lasting cushion effect compared to the widely used NS and leads to a safe and effective procedure. SH has no significant adverse events compared to NS and is safe. With the recent advances in the indication of ESD in early gastric cancer, development of endoscopic accessories, and more trained clinicians, the resection of large lesions without concern regarding the location of the lesion is possible. Therefore, with appropriate measures taken regarding price and storage issues, 0.4%SH may further enhance the ease and safety of ESD.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) has made *en bloc* resection of early gastric neoplasms possible. A sufficient amount of submucosal fluid cushion is one of the important requisites for ESD. The ideal injection agent should provide a long-lasting submucosal cushion.

Research frontiers

The submucosal injection during ESD is a requisite for safety during and after ESD. An ideal submucosal injection solution would have a prolonged cushion effect, be easily available, nontoxic, easy to inject, and inexpensive. Numerous solutions are being studied to determine if they are safe and effective in the procedure.

Innovations and breakthroughs

There are no studies evaluating the efficacy and safety of sodium hyaluronate solution (SH) solution in ESD for gastric neoplasm in a Korean population.

Applications

0.4%SH solution gives gastric mucosa long lasting lifting compared to normal saline (NS), thereby ESD using it is more effective compared to the control group. The solution is safe when comparing adverse events to the NS group. Therefore, 0.4%SH is a useful agent for submucosal injection during ESD.

Peer review

This is a randomized controlled trial on efficacy and safety of 0.4% sodium hyaluronate for gastric ESD. It is worthy pubishing.

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BRIEF ARTICLE

Robotic cholecystectomy with new port sites

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Abstract

AIM: To introduce robotic cholecystectomy (RC) using new port sites on the low abdominal area.

METHODS: From June 2010 to June 2011, a total of 178 RCs were performed at Ajou University Medical Center. We prospectively collected the set-up time (working time and docking time) and console time in all robotic procedures.

RESULTS: Eighty-three patients were male and 95 female; the age ranged from 18 to 72 years of age (mean 54.6 ± 15.0 years). All robotic procedures were successfully completed. The mean operation time was 52.4 ± 17.1 min. The set-up time and console time were 11.9 ± 5.4 min (5-43 min) and 15.1 ± 8.0 min (4-50 min), respectively. The conversion rate to laparoscopic or open procedures was zero. The complication rate was 0.6% (n = 1, bleeding). There was no bile duct injury or mortality. The mean hospital stay was 1.4 ± 1.1 d. There was a significant correlation between the console time and white blood cell count (r = 0.033, P = 0.015). In addition, the higher the white blood cell count (more than 10000), the longer the console time.

CONCLUSION: Robotic cholecystectomy using new port sites on the low abdominal area can be safely and efficiently performed, with sufficient patient satisfaction.

Key words: Robotic cholecystectomy; Port sites; Operation time; Abdominal area; Gallbladder disease

Core tip: The robotic procedure is safe; however, it is not acceptable as a standard operation for gallbladder disease because of its lack of benefits for patients as a result of the high cost and prolonged operating time. In the previous studies, port sites of robotic cholecystectomy were located on the supraumbilical area, similar to laparoscopic surgery. In this study, we changed the port placements from the upper abdominal area to the lower abdominal area.

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INTRODUCTION

Laparoscopic cholecystectomy is a standard technique for treatment of gallbladder diseases[1]. However, there are some disadvantages to using laparoscopic techniques[2], and laparoscopic surgery can have a steep learning curve[3]. To overcome these limitations of laparoscopic techniques, the robotic-assisted procedure de-

veloped, the 3-dimensional view, magnification, tremor suppression, and flexibility of the instruments[4,5] have allowed precise operating techniques in general surgery[6-8]. Since the first robotic-assisted cholecystectomy was performed in 1997, many reports have been published, including comparative[8-10] and non-comparative studies[6,11-15]. All authors agreed on the safety and feasibility of the robotic procedure. However, most of them concluded that this procedure is not acceptable as a standard operation because of the lack of benefits for patients due to the high cost and prolonged operating time. In this regard, the benefits of the robotic procedure in gallbladder diseases have not yet been established.

Based on the above reports, port sites of robotic cholecystectomy were located on the supraumbilical area, similar to laparoscopic surgery. As a result, we performed robotic cholecystectomy, changing the port placements from the upper abdominal area to the lower abdominal area. In this study, we examined robotic cholecystectomy using port sites located on the low abdominal area and evaluated its surgical outcomes.

MATERIALS AND METHODS

From June 2010 to June 2011, a total of 178 robotic cholecystectomies were performed at Ajou University Medical Center. We prospectively collected the set-up time (working time and docking time) and console time in all robotic procedures. The initial indications of surgery included gallbladder polyp or symptomatic gallstones. Exclusion criteria were the presence of acute cholecystitis and previous history of extensive upper abdominal surgery. Informed consent was obtained for the robotic cholecystectomy. We retrospectively reviewed the medical records of all patients and analyzed data, including demographic information, clinical presentation, results of laboratory studies, operative records, postoperative complications, and postoperative hospital stay.

In this study, the operating time was defined as the time from skin incision to wound closure. The working time extended from the first skin incision until the decision to bring the da vinci into place was made. The docking time spanned the setup of the robot onto the surgical field. The set-up time was defined as the time from skin incision until the start the dissection. The console time was defined as the time from the start of dissection until the moment the gallbladder was completely freed from the liver.

The robotic-assisted operations were performed with the 4-arm da Vinci robot system (Intuitive Surgical, CA, United States). The operating team consisted of one operating surgeon and one assisting resident. The assisting resident replaced instruments and paced clips during cholecystectomy. Robotic cholecystectomy was performed using a three or four port technique. A total of three trocars were utilized as shown in Figure 1. First, a 12-mm trocar was inserted through a vertical incision below the umbilicus using an open method. CO_2 gas was introduced through this trocar to obtain an intraperitoneal pressure of 12 mm Hg. All other ports were placed under direct visualization. The 8-mm ports were placed 7 to 10 cm distant from the endoscope. An additional fourth trocar (3 or 5 mm) was placed in the right anterior axillary line in the upper quadrant in cases of severe inflammation of gallbladder and thickening of the gallbladder wall and used for retraction and clip/loop placement.

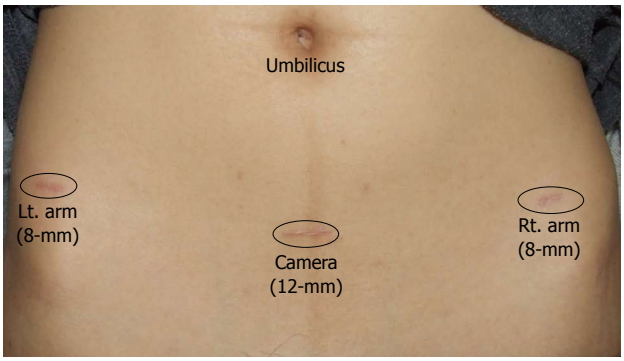

Figure 1 Port sites of robotic cholecystectomy. A 12-mm trocar was inserted through a vertical incision below the umbilicus using an open method. The 8-mm ports were placed 7 to 10 cm distant from the endoscope.

The patient was then placed in reverse Trendelenburg position with the right side up. The da vinci surgical robot was then brought into position and docked. The maryland forceps was inserted into the right robotic positioner, and a cadiere grasper was placed into the left positioner. The dissection was performed according to the critical view method as described by Strasberg *et al*[16]. After clear identification of the cystic duct and cystic artery, only the cystic duct was ligated manually with clips. In contrast, the cystic artery was coagulated just around the gallbladder and not ligated. The gallbladder was dissected from the fossa and placed in an endoscopic retrieval bag. Once fully dissected, the gallbladder was removed through the umbilical port in an endopouch. The robot was then withdrawn, and the 12-mm port site was closed with absorbable sutures.

Patients were discharged 1 d after surgery if sufficiently recovered and if pain and nausea had receded. All patients were seen for examination and reassessment at the outpatient clinics 1 wk after surgery. Laboratory tests were performed only if indicated.

Statistical analysis

Statistical analysis was performed with independent *t*-test, and Spearman's correlation. *P*-value < 0.05 was considered statistically significant.

RESULTS

Clinical findings

Eighty-three patients were male and 95 female; the age

Table 1 Clinical characteristics

	RC (n = 178)
Age (yr)	40.1 ± 9.8
Gender (male/female)	83/95
Laboratory findings	
White blood cell count (/m^3)	7483.4 ± 2670.8
AST (IU/L)	62.9 ± 124.0
ALT (IU/L)	68.9 ± 134.4
Total bilirubin (mg/dL)	1.0 ± 0.9
Combined diseases	
Diabetes mellitus	5
Hypertension	29
Ischemic heart disease	0
COPD	0

RC: Robotic cholecystectomy; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; COPD: Chronic obstructive pulmonary disease.

Table 2 Operation time of robotic cholecystectomy

	RC (n = 178)
Operation time (min)	52.4 ± 17.1
Set-up time (min)	11.9 ± 5.4
Working time (min)	7.6 ± 4.2
Docking time (min)	4.3 ± 2.5
Console time (min)	15.1 ± 8.0

RC: Robotic cholecystectomy.

ranged from 18 to 72 years of age (mean 54.6 ± 15.0 years). Table 1 shows the clinical characteristics of patients who underwent robotic cholecystectomy. The associated diseases were hypertension (n = 29), diabetes mellitus (n = 5), hypothyroidism (n = 2), and hepatitis (n = 2). The previous operations were appendectomy (n = 10), C-section (n = 10), and hysterectomy (n = 1). Endoscopic retrograde cholangiopancreatogram was performed in 18 patients. After surgery, two patients in robotic cholecystectomy (RC) group were diagnosed with gallbladder (GB) cancer. A 53-year-old man who was diagnosed with a gallbladder mass underwent cholecystectomy with lymph node dissection. Unfortunately, tumor penetrated the serosa layer of the gallbladder (T3N0). We recommended re-operation; however, he refused the surgery in our hospital and did not follow up. The other patient was a 60-year-old man who had undergone surgery for gallbladder polyp and was diagnosed with T1a gallbladder cancer. There was no recurrence for 8 mo after surgery.

Surgical outcomes

All robotic procedures were successfully completed. The mean operation time was 52.4 ± 17.1 min. The set-up time and console time were 11.9 ± 5.4 min (5-43 min) and 15.1 ± 8.0 min (4-50 min), respectively (Table 2). The conversion rate to laparoscopic or open procedures was zero. The complication rates was 0.6% (n = 1, bleeding) (Table 3). The patient who had complications was a

Table 3 Surgical outcomes of robotic cholecystectomy

	RC (n = 178)
Complications	
Bleeding	1
Bile duct injury	0
Open conversion	0
Total hospital stay (d)	2.9 ± 1.8
Postoperative hospital stay (d)	1.4 ± 1.1

RC: Robotic cholecystectomy.

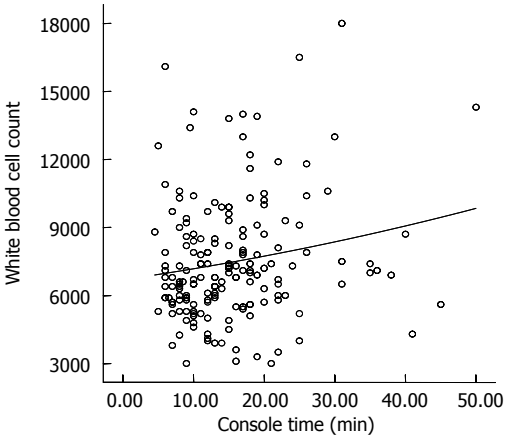

Figure 2 Correlation between console time and white blood cell count. There was a significant correlation between console time and white blood cell count (r = 0.182, P = 0.015).

34-year-old female who underwent re-operation on postoperative day 1; previous incisions on the low abdominal area were employed during the surgery. We identified the focus of bleeding on the gallbladder bed and coagulated the bleeder. She was finally discharged from the hospital without any symptoms. There was no bleeding associated with the cystic artery. There was no bile duct injury and mortality. The mean postoperative hospital stay was 1.43 ± 1.16 d.

We analyzed the relationship between console time and other factors. The results showed that the only significant correlation was between console time and white blood cell count (r = 0.182, P = 0.015) (Figure 2). In addition, the higher the white blood cell count (more than 10000), the longer the console time (Table 4).

DISCUSSION

Since 2005, when the robotic system was first introduced in South Korea, other investigators have also reported experience with surgical robotics in a variety of surgical procedures, including cholecystectomy, gastrectomy, and thyroidectomy[17-19]. However, hepato-biliary surgeons in South Korea stopped performing robotic-assisted cholecystectomy because of the lack of advantages for patients compared to its high cost[8,9,20]. In our hospital, we experienced the first fully robotic procedure in a pa-

Table 4 Relationship between console time and inflammation of gallbladder

	WBC (< 10000) (*n* = 151)	WBC (≥ 10000) (*n* = 27)	*P* value
Console time (min)	14.6 ± 7.5	18.3 ± 9.9	0.025

WBC: White blood cell.

tient who was diagnosed with choledochal cyst; the patient underwent resection of the cyst and intracorporeal hepaticojejunostomy. After that, we decided to change the trocar placements when using the robotic system in gallbladder diseases. Before beginning robotic surgery, Professor Kim (Kim WH) also enrolled in the Intuitive Surgical da Vinci training course.

In the present study, we used only three of four arms of the da Vinci system to reduce the instrument-related costs. Most importantly, port sites were also much lower than the umbilical line. The 12-mm camera port site was located almost 10 cm from the umbilicus. The other two 8-mm port sites were located around the right and left anterior superior iliac spine. This line can be called the "Panty line" or "Bikini line". All patients were satisfied with both the degree of postoperative pain and scarring. However, we experienced a problem in that the length of the robotic arms were too short to dissect the gallbladder, especially in big or obese patients. Indeed, Cadière *et al*[21]. Previously suggested that the robotic approach requires new operative strategies and a change in the pattern of trocar placement. Most surgeons will likely agree that, although Nio *et al*[9]. Reported on altered positioning of trocars in robot-assisted laparoscopic cholecystectomy, their locations were not different from the laparoscopic technique. In the early period of the study, we included patients who were diagnosed with gallbladder polyp or minimal symptomatic gallstones. As the number of cases increased, we attempted robotic procedures in cases of inflamed gallbladder, such as acute cholecystitis, empyematous cholecystitis, and gangrenous cholecystitis. According to previous reports, some authors did not perform robotic procedures in acute cholecystitis[11,22,23]. In study by Ruurda *et al*[14], the rates of acute cholecystitis were 17%, and there was one conversion to an open procedure, caused by the surgeons' inability to expose the gallbladder sufficiently because of severe cholecystitis. In the present study, there was no conversion to laparoscopic or open procedures after including cases of acute cholecystitis.

Thus far, most authors have reported that the operation time of robotic procedures was much longer than cases that used laparoscopic technique. The operation time varied, with a range of 55-152 min[13,14,21,23-28]. The reason for the various results in those studies was that the definition of the operation time was heterogeneous; the operation time was defined as the time from skin incision to skin closure, or it included anesthesia or time in the robotic room. In the present study, the operation time was 81.3 ± 19.0, similar to other results[14,21,24-28]. Marescaux *et al*[24] reported that the median time for dissection was 25 min (range 14-109 min) and the overall operative time was 108 min, similar to times of conventional laparoscopy. In this study, the console time was 15.1 ± 8.0 min, similar to another report[24]. The console time in cases of inflamed gallbladder was longer than that of cases of non-inflamed gallbladder.

The conversion rates of robotic procedures in other studies have been almost zero[9,13,21,22,25,27]; our data showed similar results. However, Miller *et al*[28] reported that conversion to conventional laparoscopic techniques was necessary due to malfunction of graspers in three consecutive procedures. The other reasons for conversion were in cases of acute cholecystitis[24], presence of severe adhesions, and poor visualization[26]. In this study, we found that the fourth 5-mm instrument was effective in grasping and retracting the edematous gallbladder. Until now, we have experienced no conversion after the indications were expanded to include severe acute cholecystitis. There are two published reports of 8-mm port-site hernias, so it should be recommended that any port greater than 5 mm in diameter should be routinely sutured closed[22,29]. We did not experience an 8-mm port-site hernia, likely due to the short follow-up period after surgery.

To our knowledge, this study is the first large series of robotic cholecystectomy in South Korea. Some advantages of robotic cholecystectomy from this study are as follows. First, patient satisfaction regarding a lower lying wound was very high because of the absence of a scar on the upper abdominal area. Second, the subjective perception of the surgeons is that the robotic system makes dissection easier at Callot's triangle. Furthermore, the use of the robotics allowed the surgeon to remain in an ergonomic position throughout the procedure. This could reduce the fatigue experienced during prolonged or difficult operations, especially in cases of severe acute cholecystitis. Third, the robotic procedure is safely performed in patients who underwent upper abdominal surgery, because an adhesiolysis can be easily performed throughout the lower lying port sites.

In summary, robotic cholecystectomy using the new port sites on the low abdominal area can be safely and efficiently performed, with sufficient patient satisfaction. However, we recommend that the more difficult cases (due to acute inflammation) were likely excluded from participation in an early period.

COMMENTS

Background

To overcome the limitations of laparoscopic techniques, the robotic-assisted procedure developed, the 3-dimensional view, magnification, tremor suppression, and flexibility of the instruments have allowed precise operating techniques in general surgery. The robotic procedure is safe; however, it is not acceptable as a standard operation for gallbladder disease because of the lack

of advantages for patients compared to its high cost.

Research frontiers

Since the first robotic-assisted cholecystectomy was performed in 1997, the benefits of the robotic procedure in gallbladder diseases have not yet been established. In the previous studies, port sites of robotic cholecystectomy were located on the supraumbilical area, similar to laparoscopic surgery. As a result, we performed robotic cholecystectomy, changing the port placements.

Innovations and breakthroughs

Recent reports have highlighted the importance of changing the port placements from the upper abdominal area to the lower abdominal area. This is the first study to report that robotic cholecystectomy with lower lying ports can be safely and efficiently performed.

Applications

This study may represent a new strategy for surgical intervention in the treatment of patients with gallbladder diseases.

Terminology

Da vinci robot system (Intuitive Surgical, CA, United States) is composed of the surgeon's viewing and control console and a movable cart with four articulated robot arms.

Peer review

This study is a feasibility study that demonstrates that robotically assisted cholecystectomy may be performed. The technical details of port placement by the authors will make it helpful to the general surgeon who wishes to use the robot to assist with cholecystectomy.

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BRIEF ARTICLE

High-intensity focused ultrasound ablation: An effective bridging therapy for hepatocellular carcinoma patients

Tan To Cheung, Sheung Tat Fan, See Ching Chan, Kenneth SH Chok, Ferdinand SK Chu, Caroline R Jenkins, Regina CL Lo, James YY Fung, Albert CY Chan, William W Sharr, Simon HY Tsang, Wing Chiu Dai, Ronnie TP Poon, Chung Mau Lo

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Abstract

AIM: To analyze whether high-intensity focused ultrasound (HIFU) ablation is an effective bridging therapy for patients with hepatocellular carcinoma (HCC).

METHODS: From January 2007 to December 2010, 49 consecutive HCC patients were listed for liver transplantation (UCSF criteria). The median waiting time for transplantation was 9.5 mo. Twenty-nine patients received transarterial chemoembolization (TACE) as a bringing therapy and 16 patients received no treatment before transplantation. Five patients received HIFU ablation as a bridging therapy. Another five patients with the same tumor staging (within the UCSF criteria) who received HIFU ablation but not on the transplant list were included for comparison. Patients were comparable in terms of Child-Pugh and model for end-stage liver disease scores, tumor size and number, and cause of cirrhosis.

RESULTS: The HIFU group and TACE group showed no difference in terms of tumor size and tumor number. One patient in the HIFU group and no patient in the TACE group had gross ascites. The median hospital stay was 1 d (range, 1-21 d) in the TACE group and two days (range, 1-9 d) in the HIFU group ($P < 0.000$). No HIFU-related complication occurred. In the HIFU group, nine patients (90%) had complete response and one patient (10%) had partial response to the treatment. In the TACE group, only one patient (3%) had response to the treatment while 14 patients (48%) had stable disease and 14 patients (48%) had progressive disease ($P = 0.00$). Seven patients in the TACE group and no patient in the HIFU group dropped out from the transplant waiting list ($P = 0.559$).

CONCLUSION: HIFU ablation is safe and effective in the treatment of HCC for patients with advanced cirrhosis. It may reduce the drop-out rate of liver transplant candidate.

Key words: Ablation; Bridging therapy; Cirrhosis; Hepatocellular carcinoma; High-intensity focused ultrasound; Liver transplant; New technology

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INTRODUCTION

Deceased donor liver transplantation provides one of the best treatments to patients with hepatocellular carcinoma (HCC) and cirrhosis. The numbers of donations and cases performed are on a rising trend. However, the scarcity of liver grafts in many parts of the world, especially Asia, leads to a significant dropout rate of patients from liver transplant waiting lists, particularly patients with HCC and a low model for end-stage liver disease (MELD) score[1]. In order to reduce the dropout rate, different bridging therapies have been proposed. Among them, transarterial chemoembolization (TACE) and radiofrequency ablation (RFA) are most popular. Despite of treatment applied before liver transplantation, the dropout rate for TACE ranged from 15% to 35% in different studies[1,2]. RFA seems to have produced better results but the dropout rate also ranged from 5.8% to 14% in various studies[3,4].

High-intensity focused ultrasound (HIFU) ablation is one of the latest treatments. It provides a totally non-invasive therapy to HCC and is viable even in patients with severe cirrhosis. In one of our previous studies, it achieved a complete ablation rate of 82.4% for HCCs smaller than 3 cm in with just one treatment session. It is well tolerated even in patients with advanced cirrhosis and age[5]. The current study is the first study that investigates whether HIFU therapy can be safely performed in HCC patients with cirrhosis and whether it can reduce the dropout rate of liver transplant candidates.

MATERIALS AND METHODS

From January 2007 to December 2010, 49 consecutive HCC patients were listed for deceased donor liver transplantation (UCSF criteria). The diagnosis of HCC was confirmed by histology, elevated level of serum alpha-fetoprotein (> 400 ng/mL), or typical radiological appearance of lesion shown by contrast computed tomography or contrast magnetic resonance imaging. The median waiting time for transplantation was 9.5 mo. Patients who were listed for transplantation received TACE as a bridging therapy before transplantation. HIFU ablation has been used as a standard local ablative therapy since 2006 for HCC patients who have poor liver function and cannot tolerate hepatectomy[5]. This is a retrospective study performed with prospectively collected data. Informed consent to treatment and to the use of data for research was obtained beforehand.

Five patients received HIFU ablation and 29 patients received TACE as a bringing therapy. Fifteen patients received no treatment before transplantation. Another five patients with the same tumor staging (within the UCSF criteria) who were not on the transplant waiting list but received HIFU ablation were included for comparison. All patients were comparable in terms of Child-Pugh and MELD scores, tumor size and number, and cause of cirrhosis.

Contraindications to TACE included main portal vein thrombosis, arteriovenous shunting, Child-Pugh C cirrhosis, and extrahepatic metastasis. Cisplatin was used as the chemotherapeutic agent and was delivered with Lipiodol, followed by Gelfoam particle embolization. Selective cannulation and embolization of the feeding arteries of the tumors were performed whenever possible. During the procedure, 10 mL of Lipiodol was mixed with 10 mg of cisplatin into a 20 mL emulsion. Depending on the tumor size and number, 4-60 mL of the Lipiodol emulsion was injected into the catheter placed in the artery supplying the tumor, or into the hepatic artery proper beyond the gastroduodenal artery for bilobar disease. Light embolization of the feeding artery was then performed with pellets sized 1 mm × 2 mm mixed with 40 mg of gentamycin. Gelfoam injection was stopped when the blood flow in the artery supplying the tumor slowed down but before occlusion occurred. TACE was repeated every 2 to 3 mo. Patients were monitored every month for hepatic and renal functions and alpha-fetoprotein level. TACE was terminated if there was evidence of further derangement of liver function (bilirubin > 50 μmol/L, ascites not controlled by diuretics, or hepatic encephalopathy), progression of disease, extrahepatic metastasis, or any other major complication.

HIFU ablation was offered to patients with poor liver function or decompensated cirrhosis as documented by (1) presence of gross ascites; (2) disease at Child-Pugh B or above; and (3) tumor located at site considered difficult for percutaneous RFA. The treatment probe can target lesions as deep as 10 cm beneath the skin; any lesion within this range can be ablated. Contraindications to HIFU ablation included serum bilirubin level above 100 μmol/L and subcutaneous tissue thicker than 3.5 cm as adipose tissue would absorb a substantial amount of energy from the energy pathway.

All HIFU treatments were carried out by experienced hepatobiliary surgeons and radiologists. The JC HIFU system (Chongqing Haifu Technology, Chongqing, China) was used. The system comprises a real-time diagnostic imaging unit, a therapeutic unit, a degassed water circulation unit, and a computer system. The real-time diagnostic imaging unit provides direct visualization of the tumor. The therapeutic unit consists of an ultrasound energy transducer which focuses the ultrasound energy at a 12-cm focal point. The degassed water circulation unit provides a medium for ultrasound transmission outside the body. The computer system controls these three units.

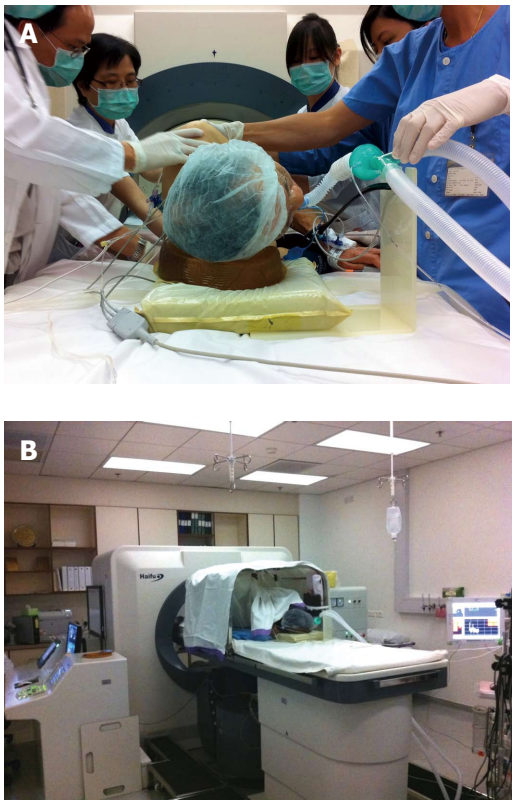

Figure 1 Locations of patient and control panel to the treatment console. A: The patient was placed in a right lateral position on the treatment console; B: The control panel is located next to the treatment console.

Before operation, the skin of the patient was cleaned by 70% alcohol followed by degassed water to remove all the grease from the skin. A dose of antibiotics (1.2 g of amoxicillin/clavulanic acid) was given on induction. A dose of proton pump inhibitor was also given on induction. Every patient was subjected to general anesthesia to aid comfort as the whole procedure could last for 3 h and the patient had to lie still and endure long periods of breath-holding. In addition, general anesthesia allowed manipulation of tumor location by the Valsalva maneuver during the procedure. If the tumor was at the dome of the liver, artificial right pleural effusion was induced before treatment. If the tumor was located at the right lobe of the liver, the patient was put in a right lateral position (Figure 1A). For better ultrasound conduction, the patient was put in a water bath. The surgeon and the radiologist controlled the operation from the control panel next to the treatment unit (Figure 1B). The treatment was performed under real-time ultrasound image guidance. The lesion was localized by a 3.6-MHz diagnostic ultrasound probe (Philips) incorporated at the center of the transducer. Parallel slices of the target tumor with 5-mm separations were planned and then ablated slice by slice with focused ultrasound energy produced by the transducer operating at 0.8 MHz. Grey-scale changes of the ablated sites were observed during the ablation procedure, indicating the temperature change inside the target lesion.

After the procedure, the patient was usually sent to the general ward and was closely monitored for vital signs, particularly body temperature. Transient hypothermia could occur as the patient had been immersed in a water bath. For patients who had received artificial pleural effusion, radiograph of the chest was taken to check for pneumothorax.

Tumor response was categorized according to the RECIST criteria: (1) complete response was denoted by disappearance of all target lesions; (2) partial response was denoted by at least a 30% decrease in the sum of the largest diameters of the target lesions; (3) progressive disease was denoted by at least a 20% increase in the sum of the largest diameters of the target lesions or appearance of one or more new lesions; and (4) stable disease was denoted by the absence of sufficient shrinkage of tumor qualified as partial response and the absence of sufficient increase of tumor qualified as progressive disease[6]. Contrast computed tomography or contrast magnetic resonance imaging was performed one month after the HIFU treatment and then every three month to evaluate tumor response before transplantation.

Statistical analysis

The baseline characteristics of patients were expressed as medians with range. The Mann-Whitney *U* test was used to compare continuous variables and a χ^2 test was used to compare discrete variables. Statistical significance was denoted by $P < 0.05$. All statistical calculations were made with the SPSS/PC + computer software (SPSS, Chicago, IL, United States).

RESULTS

The TACE group and the HIFU group had no difference in age, hepatitis B virus infection, or hepatitis C virus infection. The two groups of patients had similar liver function in terms of serum levels of albumin, aspartate aminotransferase and alanine aminotransferase, prothrombin time, indocyanine green retention rate, and international normalized ratio. One patient in the HIFU group had gross ascites before treatment. No patient in the TACE group had gross ascites. The two groups showed no difference in terms of tumor size and tumor number (Table 1). Table 2 shows the Child-Pugh and MELD scores of the patients. The median number of sessions of TACE was 3 (range, 1-7).

The median hospital stay was 1 d (range, 1-21 d) in the TACE group and two days (range, 1-9 d) in the HIFU group ($P < 0.000$). Seven patients in the TACE group dropped out from liver transplant waiting list. One of them developed extrahepatic metastasis and six of them had local progression of disease rendering them unqualified for transplantation. No patient in the HIFU group dropped out during the study period ($P = 0.559$).

According to the RECIST criteria, nine patients (90%) had complete response and one patient (10%) had partial response in the HIFU group. In the TACE group, only

Table 1 Patient demographics

	HIFU (n = 10)	TACE (n = 29)	P value
Age (yr)	59.5 (49-76)	57 (43-65)	0.107
Sex (male/female)	7/3	24/5	0.399
Child-Pugh A disease	3 (30)	17 (58.6)	0.267
Child-Pugh B disease	6 (60)	12 (41.4)	
Child-Pugh C disease	1 (10)	0	
Carrier of hepatitis B virus	5 (50)	28 (96.5)	0.002
Carrier of hepatitis C virus	4 (40)	1 (3.4)	0.011
Serum bilirubin (μmol/L)	14.5 (6-36)	25 (4-49)	0.074
Serum albumin (g/dL)	32 (27-38)	34 (20-43)	0.606
Platelet count (10^9/L)	67 (28-166)	59 (23-144)	0.688
Aspartate transaminase (U/L)	52 (29-141)	47 (15-104)	0.440
Alanine transaminase (U/L)	44 (26-109)	36 (9-132)	0.376
Alpha-fetoprotein (ng/mL)	8 (2-160)	24 (1-1151)	0.101
International normalized ratio	1.25 (0.9-1.5)	1.3 (1.0-1.5)	0.960
Largest tumor size (cm)	2.6 (1.2-4.0)	2.0 (0.8-4.3)	0.252
Tumor number	1 (1-2)	1 (1-3)	0.172

Data are expressed as absolute n (%) or median (range). HIFU: High-intensity focused ultrasound; TACE: Transarterial chemoembolization.

Table 2 Patients' model for end-stage liver disease and Child-Pugh scores

	HIFU (n = 10)	TACE (n = 29)
MELD score (P = 0.687)		
14	0	2
13	1	4
12	1	4
11	4	7
10	1	3
9	1	3
8	0	1
7	1	4
6	1	1
Child-Pugh score (P = 0.096)		
5	0	10
6	3	7
7	5	4
8	1	5
9	0	2
10	1	1

MELD: Model for end-stage liver disease; HIFU: High-intensity focused ultrasound; TACE: Transarterial chemoembolization.

one patient (3%) had response to the treatment while 14 patients (48%) had stable disease and 14 patients (48%) had progressive disease (P = 0.00).

Three out of the five patients in the HIFU group subsequently received transplantation. The median waiting time was nine months (range, 3-36 mo). Histopathological examination showed coagulation necrosis with no active tumor cells in two of the excised livers. One patient had 90% necrosis of the HCC. None of the patients who had received HIFU ablation as a bridging therapy developed complication due to intolerance of the procedure. The other two patients who were still waiting for transplantation had stable disease during the study period.

DISCUSSION

The incidence of HCC is increasing throughout the world. The annual incidence of HCC in hepatitis B carriers is around 0.5%. The incidence in patients with liver cirrhosis is even higher at around 2.5% annually[7,8]. Hepatitis-B-related cirrhosis is common in Asia, where HCC is endemic. Other risk factors for the development of HCC include hepatitis C infection, alcoholic cirrhosis, genetic hemochromatosis, and primary biliary cirrhosis. These patients should be offered regular surveillance in order to identify small tumors that may be potentially treatable. However, most patients with small HCCs have no symptoms. Resection is the main hope of cure for HCC but is only possible in 25% of the patients because the disease is usually so advanced at presentation and is frequently associated with cirrhosis[9,10]. For patients with unresectable HCC, liver transplantation appears to be the only viable option. The chance of receiving a liver graft varies worldwide. Liver donation rate is highest in Spain where there are 33.7 donations per one million of the population. In contrast, the donation rates in Asia range from only 0.05 to 4.3 donations per one million of the population. The general lack of suitable deceased donors makes successful liver transplantation for HCC difficult[11,12]. In order to maximize the benefit of utilizing this scarce resource, different liver graft allocation systems are adopted worldwide. The principle of allocation is to prioritize the sickest and yet maintain the highest survival rate possible. As a corollary, patients with very high MELD scores have priority. In most countries, patients with unresectable HCC and yet lower MELD scores have a low priority.

The results of liver transplantations in the early period of development were not satisfactory, with a 5-year survival rate below 40%. This urged recognition of poor prognostic factors in liver transplantation in patients with HCC[13]. Mazzaferro *et al*[14,15] showed that a subgroup of patients with radiological evidence of a single tumor smaller than 5 cm in diameter or two to three tumors each smaller than 3 cm in diameter had better survival outcome. The Milan criteria were established in 1996 and have led to the improvement of the 5-year survival rate to 83%. At many transplant centers, patients with tumor status beyond the Milan criteria are not accepted for transplantation and those on transplant waiting lists are delisted if their tumors enlarge to beyond the criteria, ensuring that liver grafts are allocated to patients predicted to have longer survival[16].

In order to make sure patients receive appropriate treatment before transplantation so as to remain listed, different bridging therapies have been tried. This is particularly important for patients whose treatment options are limited by poor liver reserve and portal hypertension. TACE and RFA are the most popular bridging therapies.

TACE is a standard bridging therapy at some centers, achieving a rate of down-staging of tumors of around 40%. However, about 20% of patients develop tumor progression after TACE, rendering them delisted[1,2,17,18]. RFA is an effective thermal ablative treatment modality and is widely practiced to treat small HCCs. RFA is

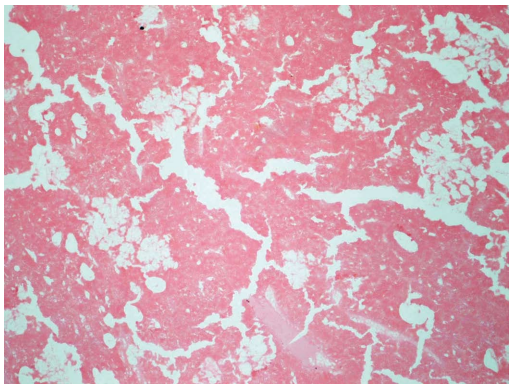

Figure 2 The tumor nodule shows coagulative necrosis. The necrotic tumor tissue shows a barely preserved architectural pattern and loss of cellular details. Hematoxylin and eosin stain, × 200.

also used as a bridging therapy. Successful tumor downstaging is observed in 70%-85% of patients. However, the dropout rate after RFA is around 14%[3,19-21]. TACE and RFA seem to be effective bridging therapies, but only for selected patients. They are not safe for patients with liver decompensation such as gross ascites and thrombocytopenia.

The concept of using ultrasound energy as a penetrating force to destroy something remote originated in last century and was summarized by Kremkau[22]. In the 1950's, researchers bought the phenomenon of piezoelectricity to the clinical setting, treating Parkinson's disease and other neurological conditions with focused ultrasound energy[23-26].

Nowadays clinical HIFU ablation for liver tumors utilizes a unique frequency of ultrasound wave, 0.8-3.5 MHz, which can be focused at a distance from the therapeutic transducer. The accumulated energy at the focused region induces necrosis of the target lesion by elevating the tissue temperature to above 60 ℃[27,28]. Temperature outside the focus point remains static as particle oscillation remains minimal. This is an advantage of HIFU over RFA in which inadvertent collateral damage is unavoidable. Patients with derangement of liver function and thrombocytopenia usually show intolerance of RFA[29]. A bridging therapy must not cause further liver decompensation. The slow process of heating by HIFU energy propagation followed by resting leads to little tissue destruction beyond the focused point.

The presence of gross ascites facilitates HIFU treatment. As ultrasound energy travels much better in water than in air, ascites encourages energy propagation to the target lesion. In addition, the presence of ascites acts as a cushion of coolant inside the peritoneal cavity and prevents the muscle wall and skin absorbing too much energy from the beam pathway where subcutaneous tissue burn could happen.

HIFU ablation is a totally extracorporeal non-invasive treatment modality using focused ultrasound energy that is capable of causing coagulative necrosis of the target lesion via intact skin without the need of surgical incision.

The unique needleless design of the HIFU system makes HIFU ablation superior to RFA, as percutaneous needle penetration may induce hemorrhage from a hypervascular tumor in a patient with coagulopathy and a low platelet count. Furthermore, without needle puncture, there is no risk of direct tumor seeding to the surrounding major vessels[30]. For tumors located at the dome of the liver, open RFA would be required if HIFU ablation is not used.

Figure 2 demonstrates the effect of HIFU ablation on the HCC in one of the excised liver in the series in the present study. Coagulative necrosis of the tumor was observed microscopically on almost the whole cut surface. Although histological examination showed the presence of viable tumor cells at a few focuses, there was no gross tumor progression. HIFU ablation was a successful bridging therapy in this case in which TACE and RFA were not considered acceptable treatment due to the poor liver reserve.

Repeated sessions of HIFU or adjuvant TACE should be performed to enhance the effect of tumor necrosis when liver function allows and the waiting time is prolonged.

Wu *et al*[31] reported the results of HIFU treatment for 68 patients with liver tumors. Thirty patients subsequently received liver resection. In histological examination, all the lesions showed complete coagulation necrosis.

We recently published the results of HIFU treatment for 49 patients with HCC. The complete ablation rate was comparable to that of RFA, ranging from 79.5% to 82.4%. The complication rate was around 8.2%. The complications were mainly mild skin edema and injury due to energy accumulation at the ultrasound beam pathway. The treatment was well tolerated in most of the patients. The median hospital stay was 4 d[5].

In conclusion, to the best of our knowledge, we are the first liver transplant center investigating HIFU ablation as a bridging therapy before liver transplantation. In this study, HIFU ablation was shown to be an effective thermal ablation method. The treatment stopped gross tumor progression in a patient with severe cirrhosis when TACE and RFA were contraindicated. HIFU treatment can potentially reduce the dropout rate of patients from the transplant waiting list. In a broader sense, HIFU may prolong the survival of selected patients with decompensated cirrhosis for which liver transplantation is not an option.

COMMENTS

Background

The lack of liver grafts in many places, particularly Asia, is the major obstacle to liver transplantation for patients with hepatocellular carcinoma (HCC). An effective bridging therapy which is well tolerated by patients with decompensated liver cirrhosis is important and much needed.

Research frontiers

High-intensity focused ultrasound (HIFU) ablation is a relatively new technique which provides a non-invasive treatment for HCC. However, its efficacy and safety in candidates of liver transplantation have not been known. In this study, the authors demonstrated that it is an effective treatment modality for liver

transplant candidates who have decompensated cirrhosis.

Innovations and breakthroughs

This is the first original study on the effect of HIFU ablation as a bridging therapy for liver transplantation. Unlike radiofrequency ablation, HIFU ablation does not require any needle puncture and so eliminates the risk of disease dissemination. It can even be performed in patients with gross ascites without the risk of precipitating liver decompensation. In the study, the ablation caused no adhesion. The absence of adhesion renders the subsequent liver transplantation easier.

Applications

With its advantages, safety and efficacy as demonstrated in this study, HIFU ablation should be considered as a treatment option for HCC. This study already proved that it is a safe and effective bridging therapy before liver transplantation for HCC.

Terminology

HIFU ablation utilizes a unique frequency of ultrasound wave of 0.8 to 3.5 MHz, causing a cavitation effect in the target lesion.

Peer review

The authors studied the effect of HIFU ablation on HCC performed before liver transplantation. The study demonstrated that HIFU ablation is an effective ablation modality which is totally non-invasive. It can effectively reduce the drop-out rate of liver transplant candidates by giving effective control on their tumors. The histological examination of the excised livers provided evidence that necrosis is effective in an *in vivo* model, which makes this article unique of its kind.

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BRIEF ARTICLE

Development and application of a real-time polymerase chain reaction method for *Campylobacter jejuni* detection

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Abstract

AIM: To develop a real-time polymerase chain reaction (PCR) method to detect and quantify *Campylobacter jejuni* (*C. jejuni*) from stool specimens.

METHODS: Primers and a probe for real-time PCR were designed based on the specific DNA sequence of the *hipO* gene in *C. jejuni*. The specificity of the primers and probe were tested against a set of *Campylobacter spp.* and other enteric pathogens. The optimal PCR conditions were determined by testing a series of conditions with standard a *C. jejuni* template. The detection limits were obtained using purified DNA from bacterial culture and extracted DNA from the stool specimen. Two hundred and forty-two specimens were analyzed for the presence of *C. jejuni* by direct bacterial culture and real-time PCR.

RESULTS: The optimal PCR system was determined using reference DNA templates, 1 × uracil-DNA glycosylase, 3.5 mmol/L $MgCl_2$, 1.25 U platinum *Taq* polymerase, 0.4 mmol/L PCR nucleotide mix, 0.48 μmol/L of each primer, 0.2 μmol/L of probe and 2 μL of DNA template in a final volume of 25 μL. The PCR reaction was carried as follows: 95 ℃ for 4 min, followed by 45 cycles of 10 s at 95 ℃ and 30 s at 59 ℃. The detection limit was 4.3 CFU/mL using purified DNA from bacterial culture and 10^3 CFU/g using DNA from stool specimens. Twenty (8.3%, 20/242) *C. jejuni* strains were isolated from bacterial culture, while 41 (16.9%, 41/242) samples were found to be positive by real-time PCR. DNA sequencing of the PCR product indicated the presence of *C. jejuni* in the specimen. One mixed infection of *C. jejuni* and *Salmonella* was detected in one specimen and the PCR test for this specimen was positive.

CONCLUSION: The sensitivity of detection of *C. jejuni* from stool specimens was much higher using this PCR assay than using the direct culture method.

Key words: *Campylobacter jejuni*; Real time polymerase chain reaction; Application

Core tip: In the present study, we developed an effective real-time polymerase chain reaction method based on the specific DNA sequence of the *hipO* gene in *Campylobacter jejuni* (*C. jejuni*). The detection limit of this assay is 4.3 CFU/mL. A study of 242 clinical stool specimens from diarrheal patients indicated that this method is more sensitive than direct bacterial culture for the identification of *C. jejuni* from stool specimens.

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INTRODUCTION

Campylobacter spp., *Salmonella spp.*, *Yersinia spp.*, *Shigella spp.*, and *Escherichia coli* (*E. coli*) O157 are the leading causes of human bacterial gastroenteritis worldwide[1]. *Campylobacter jejuni* (*C. jejuni*) is the main species of Campylobacter that affects humans[2,3]. *Campylobacter* infections have been investigated and followed with considerable interest since the early 1980s in China, and there have been numerous reports on *C. jejuni* infection in patients with diarrhea. However, reports and the frequency of isolation of *C. jejuni* from diarrheal patients have decreased since the late 1990s. Improvement in sanitary conditions may explain this decline in numbers; however, the difficulty in detecting this fastidious pathogen might be another contributor to the decreased number of reports and frequency of isolation. Our recent pilot study revealed that the ratio of isolation of this pathogen from stool specimens of diarrheal patients was considerably different among surveillance spots in different laboratories (2%-15%, unpublished). Sensitive and accurate detection of this pathogen is important, both for the treatment of patients and for prompt epidemiological investigation.

With the development of genomic DNA sequencing, online databases, and bioinformatic analyses, nucleic acid-based methods, particularly polymerase chain reaction (PCR) methods, have become promising tools for the rapid, reliable, and sensitive detection and diagnosis of pathogenic infection[4-8]. In this study, we developed a real-time PCR assay to detect *C. jejuni*. We then combined this PCR assay with DNA sequence analysis and compared this method with the direct culture method to detect *C. jejuni* in stool specimens obtained from patients with diarrhea. The results obtained in this study provide a proof of concept for PCR-based detection of *C. jejuni* infection in patients with diarrhea, may lead to the development of a pre-screening approach for isolating *C. jejuni*.

MATERIALS AND METHODS

Sample collection and culture-based detection

Two hundred and forty-two stool specimens were collected from diarrheal patients (with ages ranging from 6 to 72 years) in the outpatient facilities of 11 hospitals. The specimens were transferred to a laboratory within 4 h for bacterial culture. The remainder of the samples was frozen at -80 ℃ for DNA extraction. A sterile cotton swab was twisted in each stool sample and streaked directly on selective Skirrow agar plates consisting of Columbia agar base (CM331, Oxoid, Basingstoke, United Kingdom), selective supplement (SR0069, Oxoid), and 5% defibrinated sheep blood. The plates were incubated in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2) at 42 ℃ for 48 h. Suspected colonies were picked and identified by gram staining, oxidase and catalase tests, and hippurate hydrolysis analysis, according to our previous report[9]. All specimens were also examined for the presence of *Shigella spp.*, *Salmonella spp.*, *Yersinia spp.* and *E. coli* O157 by culture on xylose lysine deoxycholate agar, deoxycholate hydrogen sulfide lactose agar, cefsulodin-irgasan-novobiocin agar, and sorbitol MacConkey agar, respectively. Suspected colonies were identified biochemically using API 20E strips, and the cultures were confirmed up to the species level by further serotype or PCR analysis, as previously described[10,11].

DNA preparation from bacterial culture

Reference genomic DNA was extracted from the *Campylobacter* isolates cultured in this study by using a QIAamp DNA mini kit (Qiagen, Düsseldorf, Germany). DNA templates from other reference pathogens were gifts from Drs. Huaiqi Jing and Biao Kan. The reference DNA templates are shown in Table 1.

Primers and probe design

Specific DNA fragments of the *hipO* gene were compared using BLASTn and Vector NTI suite 6.0 software. The primers and probe sets were designed and synthesized by Shanghai Huirui Biotechnology Co., Ltd. The sequences of the primers and probe used in this study were: hipO-F, 5'-CGGATAGTTATAGTATTGAAGTTATTGG-3', hipO-R, 5'-GAAGCAGCATAAATAGGATCTTTTG-3', and hipO-P, 5'-FAM-TTCTGGAGCACTTCCATGACCACC-BHQ1-3'.

Optimization for real-time PCR

The optimal PCR conditions were determined by testing a series of conditions with standard *C. jejuni* template. The standard PCR curve was constructed using genomic DNA from *C. jejuni* strain NCTC11168. The detection limits of this assay from pure culture and stool specimens were determined using 10-fold dilutions of quantified reference *C. jejuni* genomic DNA templates (1×10^0 to 1×10^6 CFU/mL) and the same amount of bacteria inoculated into stool specimens that were previously confirmed to contain no *Campylobacter*. The specificity of this assay was verified using genomic DNA from other enteric pathogens. The *C. jejuni*-inoculated samples were also cultured to compare the detection limits.

Evaluation of repeatability

To evaluate the reproducibility of the assay, five serial dilutions with three replicates per concentration were performed for two separate *C. jejuni* genomic DNA templates (NCTC11168 and ATCC33560). The serial dilutions were also performed on three different *C. jejuni* strains (81116, ATCC49349 and ICDCCJ07001) simultaneously. The curves were constructed on the basis of the log pg/mL of the genomic DNA and threshold cycle (Ct). Reproducibility was assessed using the SD of the Ct value and the SD of the slope of each dilution curve, respectively.

PCR detection of C. jejuni from stool specimens and DNA sequencing of PCR products

After direct culture, the stool specimens were stored at -80 ℃ until DNA extraction using a QIAamp DNA stool mini kit (Qiagen), which was performed in accordance with the manufacturer's instructions. Conventional PCR with the universal primers targeting conserved bacterial 16s rRNA gene sequences was carried out to evaluate the validity of the template used in this study. Real-time PCR was performed in a 25-μL volume with 2 μL of purified DNA obtained directly from stool specimens. All the samples with Ct < 35 were considered positive. Each of the positive PCR products, except for the ones from the culture-positive specimens, was confirmed with conventional PCR using the same primers, and the PCR products were purified and sequenced. Online sequence BLAST was performed using the NCBI BLASTN suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

RESULTS

Bacterial culture

Twenty *C. jejuni* (8.3%, 20/242) isolates, 34 (14%, 34/242) *Salmonella* isolates, and one (0.4%, 1/242) *Shigella* isolate were obtained by direct plating of the 242 stool specimens. *Salmonella* was the most frequently isolated pathogenic genus from the stool specimens and *C. jejuni* was the second most frequently isolated. No *Yersinia* or *E. coli* O157 isolates were found. In another laboratory, serotyping methods were used to further identify *Salmonella* and *Shigella* colonies.

Optimized PCR assay

The optimal PCR system was determined using reference DNA templates, 1 × uracil-DNA glycosylase, 3.5 mmol/L $MgCl_2$, 1.25 U platinum *Taq* polymerase, 0.4 mmol/L PCR nucleotide mix, 0.48 μmol/L of each primer, 0.2 μmol/L of probe, and 2 μL of DNA template in a final volume of 25 μL run with the following parameters: 95 ℃ for 4 min, followed by 45 cycles of 10 s at 95 ℃ and 30 s at 59 ℃. Fluorescence signals were measured at the end of the annealing step of every cycle. PCR products were verified using DNA electrophoresis through 2% agarose gels. The size of the PCR product was consistent with the expected size (85 bp) in each case. The specificity of the primers and probe were tested against *Campylobacter spp.* and other enteric pathogens. Only *C. jejuni* showed positive reactions. All the other templates gave negative results, including other *Campylobacter spp.*

Detection limit and stability of the PCR assay

Serial dilutions from 10^0 to 10^6 CFU/ml of the target template were subjected to the real-time PCR assay (Figure 1A). The standard curve based on the dilutions of genomic DNA showed a linear relationship between Log CFU/mL and threshold cycles (Ct; Figure 1B). Copy numbers could be obtained by the following equation using the standard curve: Y(Ct) = -3.993X (log CFU/mL) + 37.51 (R2 = 0.998). When Ct = 35, the detection limit of the PCR assay for pure culture was approximately 4.3 CFU/mL. However, when Ct = 35, the detection limit for *C. jejuni* in the stool specimens was 10^3 CFU/g, and we could not obtain a linear relationship between log CFU/g and Ct using serial bacterial inoculation in different stool specimens. The curves constructed from the serial dilution replicates of the individual *C. jejuni* templates and those generated from the three different *C. jejuni* templates are shown in Figure 2. The variation of the Ct value and the slopes of the curves indicated that this assay could be stably reproduced.

Table 1 Reference DNA templates used in this study

Bacteria	Strain name
Campylobacter jejuni	NCTC11168
Campylobacter jejuni	81116
Campylobacter jejuni	ATCC33560
Campylobacter jejuni sub *doylei*	ATCC49349
Campylobacter jejuni	ICDCCJ07001
Campylobacter coli	ATCC33559
Campylobacter coli	WHO-10.2
Campylobacter fetus	ATCC27374
Campylobacter lari	ATCC35221
Escherichia coli O157:H7	Isolate-1
Enterotoxic Escherichia coli	ETEC10407
Enteroaggregative Escherichia coli	EAECO42
Enteroinvasive Escherichia Coli	EIEC44825
Enteropathogenic Escherichia coli	Isolate-2
Salmonella Serovar typhi	CT18
Typhoid-paratyphoid A	Isolate-3
Salmonella typhimurium	Isolate-4
Cholera O1	Isolate-5
Shigella	Isolate-6
Salmonella enteritidis	Isolate-7
Yersinia enterocolitica O3	52203
Vibrio parahemolyticus	Isolate-8
Enterococcus	Isolate-9
Listeria monocytogenes	Isolate-10

PCR detection from stool specimens

The detection limit from both bacterial culture and real-time PCR (with Ct < 35) for the inoculated stool samples was 10^3 CFU/g; therefore, a Ct value of ⩽ 35 was considered positive for the real-time PCR assay in this study. Forty-one (16.9%, 41/242) samples were found to be positive by the real-time PCR method. All culture-positive specimens showed positive PCR results (Ct < 34). DNA sequencing of the positive PCR products for the culture-negative samples indicated the presence of *C. jejuni*. The samples containing *Salmonella* and *Shigella* isolates tested negative by real-time PCR (Ct > 40), except for one sample that contained both *C. jejuni* and *Salmonella* (Ct = 27).

DISCUSSION

C. jejuni is one of the major causes of food borne disease

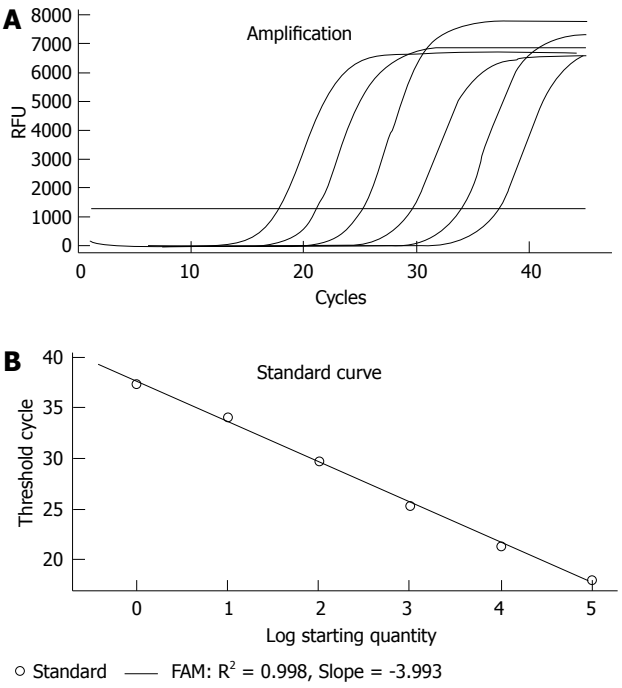

Figure 1 Real time polymerase chain reaction standard curve and the linear relationship between *Campylobacter jejuni* quantity and polymerase chain reaction cycle threshold. A: The standard curve was constructed using genomic DNA from *Campylobacter jejuni* isolates. Serial dilutions ranging from 100 to 106 CFU/mL of target template were subjected to real time polymerase chain reaction assay; B: The linear relationship between Log CFU/mL and threshold cycles. RFU: Relative fluorescence units.

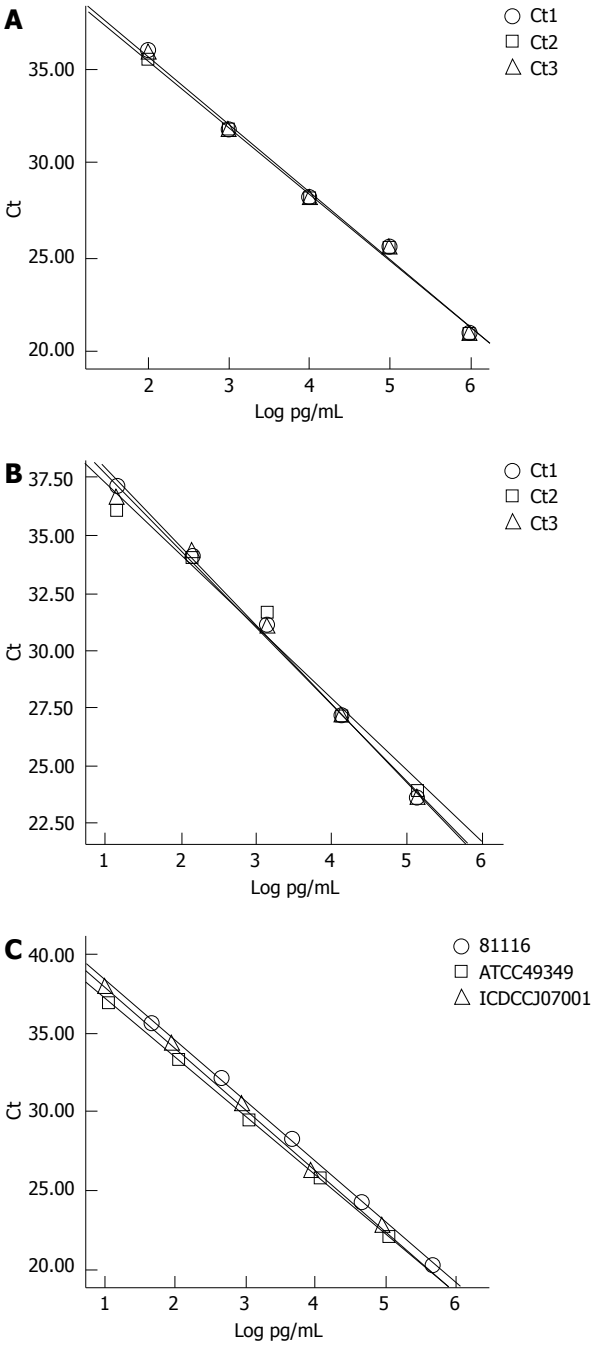

Figure 2 Stability tests. A: Curves constructed based dilutions of the genomic DNA of *Campylobacter jejuni* (*C. jejuni*) NCTC11168; B: Curves constructed based on the dilution of the genomic DNA of *C. jejuni* ATCC33560; C: Curves generated from the serial dilution of the genomic DNA of *C. jejuni* 81116, ATCC49349 and ICDCCJ07001. Ct: Threshold cycle.

worldwide[12]. It is an important pathogen of acute bacterial diarrhea in humans and has been associated with the development of Guillain-Barré syndrome, a post-infectious polyneuropathy[13,14]. Routine detection of *Campylobacter* species in the clinical setting is currently based on culture-based detection and subsequent phenotypic identification. Its detection is difficult because of its special growth requirements, low infectious doses, and potential for entering a viable, but not cultivable, state[15,16]. The traditional "gold standard" diagnostic methods for *Campylobacter* infection include bacterial culture and culture-based biochemical tests. These methods are both time-consuming and laborious. In addition to the fastidious culture requirements, the discrimination and differentiation of *Campylobacter spp.* are complicated and error prone. For example, distinguishing *C. jejuni* from *C. coli* is usually done on the basis of the biochemistry test for hippurate hydrolysis. Only *C. jejuni* gives a positive reaction, but previous studies have found that 10% of *C. jejuni* isolates failed to hydrolyze hippurate under laboratory conditions[17,18]. The accurate identification of *Campylobacter* up to species level is an essential prerequisite for many epidemiological studies.

In the present study, we developed a real-time PCR assay based on the specific DNA fragment of the *hipO* gene from *C. jejuni*, which could identify *C. jejuni* directly and differentiate it from other pathogens, especially from other *Campylobacter spp.*, in stool specimens from diarrheal patients. The specificity of the PCR system was verified with 27 reference enteric pathogens (Table 1). The detection limit of this PCR assay is less effective for stool samples than for pure culture. Loss of template during DNA extraction or inhibitors present in the stool specimens could reduce the efficiency[19]. Two hundred and forty-two stool specimens from patients with diarrhea were tested by the direct culture and by the PCR assay. All the culture-positive samples gave positive PCR results and samples containing other pathogens tested negative by PCR (Ct > 40). The DNA sequence of the

positive PCR products indicated the presence of *C. jejuni* in the specimens. The sensitivity of this PCR assay (100%, 41/41) was much higher than that of direct bacterial culture (49%, 20/41). The result from the mixed infection sample indicated that this PCR assay is considerably accurate. The results presented in this study contrast with those reported by certain previous studies, in which PCR results were found to have the same or less sensitivity compared with the direct culture method for the detection of *C. jejuni* from stool specimens[20,21]. However, Maher *et al*[22] used a DNA probe-based PCR assay and were able to identify 94% (17/18) and 32% (35/109) of *Campylobacter* infections from culture-positive and culture-negative stool specimens, respectively. Furthermore, Bessède *et al*[23] proved the weakness of the culture methods compared with PCR and immunoenzymatic methods of detection of *Campylobacter*. Notably, many aspects of the culture-based method, such as the choice of the selective media used for culture, need to be improved[24]. Although the sensitivity of the PCR system was high in this study, false-negative PCR results may still occur because of the presence of inherent inhibitors or because of very low numbers of organisms.

Interestingly, when *C. jejuni* were inoculated into stool specimens, the detection limit by the PCR assay was 10^3 CFU/g. The PCR detection limit from stool samples was similar to that reported in a previous study, but the detection limit of the direct culture methods mostly depended on the culture methods used, particularly the choice of plating medium, which may influence the efficiency of *C. jejuni* isolation during direct plating of fecal samples[25,26]. Instant plating of the inoculated samples in this study might increase the laboratory isolation capacity. Meanwhile, the non-uniformity of the clinical stool specimens in terms of physical matter, target organisms, the associated fecal flora and transportation status would result in variability of the culture results. In this study, although we used selective medium under specific microaerobic atmosphere conditions, some samples were contaminated with *Proteus* bacilli, and the suspected colonies could not be picked because of the rapid spread of the contaminants. *Proteus* bacilli may be one of the agents that reduced culture sensitivity, and the use of cotton swabs rather than inoculating loops for streaking on plates might have increased this contamination. In this study, we used QIAamp DNA stool mini kits to extract the DNA; the QIAamp kit has the highest sensitivity and requires the least manipulation of template DNA prior to PCR. More research is required to develop an in-house procedure for DNA extraction from different specimens and for optimizing the extraction process to increase the desired yield.

In conclusion, the real-time PCR assay developed in this study is sufficiently sensitive and accurate for *C. jejuni* detection from stool specimens. Although the PCR results do not permit its use as the standard method of diagnosing *C. jejuni* infection in the clinical setting, this newly developed method will benefit large-scale epidemiology investigations and pre-screening for bacterial isolation.

COMMENTS

Background

Campylobacter jejuni (*C. jejuni*) is a major food-borne pathogen. In addition to enteritis, *C. jejuni* infection can also cause Guillain-Barré syndrome, an autoimmune neuropathy in humans. The "gold standard" diagnostic methods for *C. jejuni* infection include bacterial culture and culture-based biochemical tests. Primary *in vitro* culture of *C. jejuni* is difficult because of its special growth requirements, low infectious doses, and potential for entering a viable, but not cultivable, state. In addition to its fastidious culture conditions, current methods for discrimination and differentiation of *Campylobacter spp.* are complicated and fallible. Specific DNA detection, particularly real-time polymerase chain reaction (PCR), is a promising tool for the rapid, reliable, and more sensitive detection and diagnosis of *C. jejuni* infection. In this study, the authors developed and validated a real-time PCR assay method for the detection of *C. jejuni* from stool specimens.

Research frontiers

C. jejuni is a fastidious organism and requires microaerophilic conditions for *in vitro* culture. Nucleic acid-based methods for the detection of *C. jejuni* infection are research hotspots, particularly PCR methods, which are promising tools for the rapid, reliable, and more sensitive detection of *C. jejuni* for laboratory-based diagnosis.

Innovations and breakthroughs

In the present study, the authors developed a sensitive and specific real-time PCR assay using a DNA fragment of the *hipO* gene in *C. jejuni*, which can directly differentiate *C. jejuni* from other pathogens and other *Campylobacter spp.* from stool specimens obtained from diarrheal patients.

Applications

The real-time PCR assay developed in this study is sensitive and accurate for the detection of *C. jejuni* from stool specimens. This method will benefit both large-scale epidemiology investigations and the clinical diagnosis of *C. jejuni* infection in humans.

Peer review

This is an excellent and well-written manuscript. The authors are to be congratulated on developing and validating their own in-house PCR-based technique to detect *C. jejuni* in stool specimens. The *hipO* gene is specific to *C. jejuni*; therefore, by definition, this is a highly specific test.

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BRIEF ARTICLE

Prediction of risk factors for lymph node metastasis in early gastric cancer

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Abstract

AIM: To explore risk factors for lymph node metastases in early gastric cancer (EGC) and to confirm the appropriate range of lymph node dissection.

METHODS: A total of 202 patients with EGC who underwent curative gastrectomy with lymphadenectomy in the Department of Surgery, Xinhua Hospital and Ruijin Hospital of Shanghai Jiaotong University Medical School between November 2003 and July 2009, were retrospectively reviewed. Both the surgical procedure and the extent of lymph node dissection were based on the recommendations of the Japanese gastric cancer treatment guidelines. The macroscopic type was classified as elevated (type Ⅰ or Ⅱa), flat (Ⅱb), or depressed (Ⅱc or Ⅲ). Histopathologically, papillary and tubular adenocarcinomas were grouped together as differentiated adenocarcinomas, and poorly differentiated and signet-ring cell adenocarcinomas were regarded as undifferentiated adenocarcinomas. Univariate and multivariate analyses of lymph node metastases and patient and tumor characteristics were undertaken.

RESULTS: The lymph node metastases rate in patients with EGC was 14.4%. Among these, the rate for mucosal cancer was 5.4%, and 8.9% for submucosal cancer. Univariate analysis showed an obvious correlation between lymph node metastases and tumor location, depth of invasion, morphological classification and venous invasion (χ^2 = 122.901, P = 0.001; χ^2 = 7.14, P = 0.008; χ^2 = 79.523, P = 0.001; χ^2 = 8.687, P = 0.003, respectively). In patients with submucosal cancers, the lymph node metastases rate in patients with venous invasion (60%, 3/5) was higher than in those without invasion (20%, 15/75) (χ^2 = 4.301, P = 0.038). Multivariate logistic regression analysis revealed that the depth of invasion was the only independent risk factor for lymph node metastases in EGC [P = 0.018, Exp (B) = 2.744]. Among the patients with lymph node metastases, 29 cases (14.4%) were at N1, seven cases were at N2 (3.5%), and two cases were at N3 (1.0%). Univariate analysis of variance revealed a close relationship between the depth of invasion and lymph node metastases at pN_1 (P = 0.008).

CONCLUSION: The depth of invasion was the only independent risk factor for lymph node metastases. Risk factors for metastases should be considered when choosing surgery for EGC.

Key words: Gastric neoplasm; Lymph node metastasis; Risk factors; Gastrectomy; Lymphadenectomy

Core tip: Early gastric cancer (EGC) is defined as a lesion confined to the mucosa or the submucosa, irrespective of the presence of regional lymph node metastases. In this study, we retrospectively evaluated the distribution of metastatic nodes in a two-center cohort of 202 patients with EGC. To assess nodal status in EGC, we applied an index calculated by the multiplication of the incidence of metastases in the respective node stations. Univariate and multivariate analyses were applied to confirm the clinicopathological factors associated with lymph node metastases, and to provide a basis for choosing the optimal surgical treatment and for determining the appropriate range of lymph node dissection.

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INTRODUCTION

Early gastric cancer (EGC) is defined as a lesion confined to the mucosa or the submucosa, irrespective of the presence of regional lymph node metastases[1]. Five-year survival rates in EGC tend to be greater than 90%, with lymph node status being the most important prognostic factor[2,3]. In patients with EGC and lymph node metastases, a 5-year survival rate of 87.3% has been reported, compared to 94.2% in those without nodal involvement[4]. Considering the low rate of lymph node metastasis in EGC[5,6], the Japanese guidelines recommend endoscopic mucosal resection, reduction surgery D1 plus No. 7 and 8a and D1 plus No. 7, and 8a and 9 lymph node resection, for the treatment of patients with phase T1 disease. However, this approach is controversial outside Japan. At present, the general consensus is that endoscopic mucosal resection (EMR) or distal gastrectomy plus limited lymph node resection can be undertaken in most patients with mucosal cancer, and a distal gastrectomy plus D2 lymph node resection should be performed in most patients with submucosal cancer[2,3].

Recently, in order to reduce operative and post-operative complications, and to improve quality of life, less invasive surgical alternatives, such as EMR, endoscopic submucosal dissection, laparoscopy-assisted gastrectomy and limited surgery, are used for the treatment of EGC. Although there has been substantial research on the prediction of risk factors for lymph node metastases in EGC, no definitive criteria are available. In addition, controversy surrounds the indications for local treatment in EGC, and limited surgery and the appropriate extent of lymphadenectomy.

In this study, we retrospectively evaluated the distribution of metastatic nodes in a two-center cohort of 202 patients with EGC. To assess nodal status in EGC, we applied an index calculated by the multiplication of the incidence of metastases in the respective node stations. Univariate and multivariate analyses were performed to confirm the clinicopathological factors associated with lymph node metastases, and to provide a basis for choosing the optimal surgical treatment and for determining the appropriate range of lymph node dissection.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of Shanghai Jiaotong University. All patients provided written informed consent.

Patients

A total of 202 patients with EGC, as defined by the Japanese Classification of Gastric Carcinoma[7], who underwent curative gastrectomy with lymphadenectomy in the Department of Surgery, Xinhua Hospital and Ruijin Hospital of Shanghai Jiaotong University Medical School between November 2003 and July 2009, were retrospectively reviewed. Of these, there were 132 men and 70 women, ranging in age from 25 to 87 years (mean 58.1 ± 12.9 years). Mucosal tumors were found in 122 patients (60.4%) and submucosal tumors in 80 (39.6%). Lymph node involvement was detected in 29 patients (Table 1).

Surgery

All operations were performed with curative intent. Curative surgery was defined as the removal of all gross tumors and the demonstration of tumor-negative surgical margins by microscopic examination of the entire circumference. Surgical procedures comprised 171 distal gastrectomies, 24 proximal gastrectomies and seven total gastrectomies. Proximal gastrectomy involved resection of the proximal half of the stomach via an abdominal approach, with an intraabdominal esophagogastric anastomosis. Following a total gastrectomy with D2 lymph node dissection, an esophagojejunostomy was used routinely for Roux-en-Y reconstruction. Proximal and distal resection margins were evaluated intraoperatively to confirm freedom from disease. Both the surgical procedure and the extent of lymph node dissection were based on the recommendations of the Japanese gastric cancer treatment guidelines[7]. A total of 2926 lymph nodes (LNs), with a median of 14.5 LNs per patient, were removed. No patient received neoadjuvant therapy before surgery.

Pathological examination

In both hospitals, the surgical team immediately examined the lymph nodes macroscopically, which were then divided and classified into lymph node stations, as defined by the Japanese Classification of Gastric Carcinoma. No

Table 1 Demographics of 202 patients with early gastric cancer

	Patients (n = 202)
Age	
< 60 yr	109
⩾ 60 yr	93
Sex	
Male	132
Female	70
Tumor site	
Upper	25
Middle	98
Lower	79
Size of tumor	
⩽ 2 cm	97
> 2 cm	105
No. of resected nodes	
< 15	114
⩾ 15	88
Tumor depth	
Mucosal	122
Submucosal	80
Macroscopic type	
Elevated	25
Flat	21
Depressed	156
Histologic type	
Differentiated	121
Undifferentiated	81
Lyphovessel ambolus	
+	2
-	200
Vessel ambolus	
+	5
-	197
Nerve invasion	
+	0
-	202
CEA	
+	1
-	201

CEA: Carcinoembryonic antigen.

size limitation was imposed for lymph node harvesting. Specimens were fixed in formalin, stained with hematoxylin and eosin, and sent for histopathological evaluation, following which the number of histologically confirmed lymph nodes was recorded for each lymph node station. Each lymph node was embedded in paraffin and at least two sections were performed. Immunhistochemistry for micrometastasis was not performed.

Tumor size was recorded as the maximum diameter. The depth of infiltration was measured at the deepest point of penetration of the cancer cells. The macroscopic type was classified as elevated (type Ⅰ or Ⅱa), flat (Ⅱb), or depressed (Ⅱc or Ⅲ), according to the Japanese Classification of Gastric Carcinoma[7]. Of the 202 patients, 26 (12.9%), 21 (10.4%) and 155 (76.7%) were elevated, flat or depressed, respectively. Histopathologically, papillary and tubular adenocarcinomas were grouped together as differentiated adenocarcinomas, and poorly differentiated and signet-ring cell adenocarcinomas were regarded as undifferentiated adenocarcinomas. Overall, tumors were differentiated in 121 patients and undifferentiated in 81.

The relationship between various clinicopathological factors and the presence or absence of lymph node metastases was then examined. Clinicopathological parameters included patient age (< 60 years or ⩾ 60 years), sex, tumor location (U = upper third, M = middle third, or L = lower third of the stomach), tumor size (maximum dimension ⩽ 20 mm or > 20 mm), macroscopic type (elevated, flat, or depressed), depth of invasion (mucosal or submucosal), histological type (differentiated or undifferentiated), carcinoembryonic antigen levels (CEA, < 5 ng/mL or ⩾ 5 ng/mL), lymphatic invasion (present or absent) and venous invasion (present or absent). Evaluation of these factors was undertaken according to the Japanese Classification of Gastric Carcinoma established by the Japanese Research Society for Gastric Cancer.

Statistical analysis

Descriptive data are presented as the mean ± SD. For between group comparisons, continuous variables were analyzed using the Student's t test, and categorical variables with the χ^2 test. Factors found to be significant ($P < 0.05$) in univariate analysis were included in subsequent multivariate logistic regression analysis, in order to identify independent variables associated with lymph node metastases. All statistical analyses were undertaken using the Statistical Package for the Social Sciences (SPSS) for Windows, Version 17.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Univariate analysis of lymph node metastases in EGC and clinicopathological factors

Univariate analysis was performed on the relationship between lymph node metastases and clinicopathological factors. The findings revealed a close relationship between tumor location, depth of invasion, morphological classification, venous invasion and lymph node metastases ($\chi^2 = 122.901$, $P = 0.001$; $\chi^2 = 7.14$, $P = 0.008$; $\chi^2 = 79.523$, $P = 0.001$; $\chi^2 = 8.687$, $P = 0.003$, respectively). There was no correlation between lymph node metastases and sex, age, tumor size, number of retrieved lymph nodes, histological type, lymphatic invasion, nervous invasion, and serum levels of carcinoembryonic antigen (CEA) (Table 2).

In patients with mucosal cancers, no significant differences in the occurrence of lymph node metastases were found in relation to sex, age, tumor location, tumor size, number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, venous invasion, nervous invasion and CEA levels (Table 2).

In patients with submucosal cancers, there was no significant difference in the occurrence of lymph node metastases in relation to sex, age, tumor location, tumor size, number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, nervous invasion and CEA levels. However, the lymph node

Table 2 Univariate analysis of lymph node metastases in early gastric cancer and clinicopathological factors *n* (%)

Clinicopathological factors	LN (+)	LN (-)	*P*[1]
The entire study population			
Age			0.887
< 60 yr	16 (14.7)	93 (85.3)	
⩾ 60 yr	13 (14.0)	80 (86.0)	
Sex			0.213
Male	16 (12.1)	116 (87.9)	
Female	13 (18.6)	57 (81.4)	
Tumor site			0.001
Upper	2 (8.0)	23 (92.0)	
Middle	15 (15.3)	83 (84.7)	
Lower	12 (15.2)	67 (84.8)	
Size of tumor			0.240
⩽ 2 cm	11 (11.3)	86 (88.7)	
> 2 cm	18 (17.1)	87 (82.9)	
No. of resected nodes			0.580
< 15	15 (13.2)	99 (86.9)	
⩾ 15	14 (15.9)	74 (84.1)	
Tumor depth			0.008[a]
Mucosal	11 (9.0)	111 (91.0)	
Submucosal	18 (22.5)	62 (77.5)	
Macroscopic type			0.001[a]
Elevated	4 (16)	21 (84)	
Flat	3 (14.3)	18 (85.7)	
Depressed	22 (14.1)	134 (85.9)	
Histologic type			0.332
Differentiated	15 (12.4)	106 (87.6)	
Undifferentiated	14 (17.3)	67 (82.7)	
Lyphovessel ambolus			0.149
+	1 (50.0)	1 (50.0)	
-	28 (14.0)	172 (86.0)	
Vessel ambolus			0.003[a]
+	3 (60.0)	2 (40.0)	
-	26 (13.2)	171 (86.8)	
Nerve invasion			N
+	0 (0)	0 (0)	
-	29 (14.4)	173 (85.6)	
CEA			0.681
+	0 (0)	1 (100)	
-	29 (14.4)	173 (85.6)	
Mucosa cancer			
Age			0.234
< 60 yr	8 (11.8)	60 (88.2)	
⩾ 60 yr	3 (5.6)	51 (94.4)	
Sex			0.578
Male	6 (7.9)	70 (92.1)	
Female	5 (10.9)	41 (89.1)	
Tumor site			0.976
Upper	1 (10.0)	9 (90.0)	
Middle	6 (9.4)	58 (90.6)	
Lower	4 (8.3)	44 (91.7)	
Size of tumor			0.142
⩽ 2 cm	3 (5.1)	56 (94.9)	
> 2 cm	8 (12.7)	55 (87.3)	
No. of resected nodes			0.580
< 15	7 (10.3)	61 (89.7)	
⩾ 15	4 (7.4)	50 (92.6)	
Macroscopic type			0.539
Elevated	2 (11.1)	16 (88.9)	
Flat	0 (0)	11 (100)	
Depressed	9 (9.7)	84 (90.3)	
Histologic type			0.073
Differentiated	4 (5.3)	71 (94.7)	
Undifferentiated	7 (14.9)	40 (85.1)	
Lyphovessel ambolus			N
+	0 (0)	0 (0)	
-	11 (9.0)	111 (91.0)	
Vessel ambolus			N
+	0 (0)	0 (0)	
-	11 (9.0)	111 (91.0)	
Nerve invasion			N
+	0 (0)	0 (0)	
-	11 (9.0)	110 (91.0)	
CEA			0.752
+	0 (0)	1 (100)	
-	11 (9.1)	110 (90.9)	
Submucosal cancer			
Age			0.512
< 60 yr	8 (19.5)	33 (80.5)	
⩾ 60 yr	10 (25.6)	29 (74.4)	
Sex			0.129
Male	10 (17.9)	46 (82.1)	
Female	8 (33.3)	16 (66.7)	
Tumor site			0.265
Upper	1 (6.7)	14 (93.3)	
Middle	9 (26.5)	25 (73.5)	
Lower	8 (25.8)	23 (74.2)	
Tumor size			0.768
⩽ 2 cm	8 (21.1)	30 (78.9)	
> 2 cm	10 (23.8)	32 (76.2)	
No. of resected nodes			0.203
< 15	8 (17.4)	38 (82.6)	
⩾ 15	10 (29.4)	24 (70.6)	
Macroscopic type			0.742
Elevated	2 (28.6)	5 (71.4)	
Flat	3 (30.0)	7 (70.0)	
Depressed	13 (20.6)	50 (79.4)	
Histologic type			0.725
Differentiated	11 (23.9)	35 (76.1)	
Undifferentiated	7 (20.6)	27 (79.4)	
Lyphovessel ambolus			0.346
+	1 (50.0)	1 (50.0)	
-	17 (21.8)	61 (78.2)	
Vessel ambolus			0.038[a]
+	3 (60.0)	2 (40.0)	
-	15 (20.0)	60 (80.0)	
Nerve invasion			N
+	0 (0)	0 (0)	
-	18 (22.5)	62 (77.5)	
CEA			N
+	0 (0)	0 (0)	
-	18 (22.5)	62 (77.5)	

[1]Statistically significant. Tumor site: middle and lower *vs* upper; Tumor depth: submucosa *vs* mucosa; Macroscopic type: flat and depressed *vs* elevated; Vessel ambolus: + (present) *vs* - (absent), [a]$P < 0.05$. In patients with mucosal cancers, no significant differences in the occurrence of lymph node metastases were found in relation to the clinicopathological factors. CEA: Carcinoembryonic antigen.

metastases rate in patients with venous invasion (60%, 3/5) was higher than in those without invasion (20%, 15/75), and the difference was significant ($\chi^2 = 4.301$, $P = 0.038$) (Table 2). Venous invasion, as a source variable, was therefore used in the logistic regression model. This revealed that it was not an independent risk factor for lymph node metastases in submucosal cancer [B = 1.792, SE = 0.957, Wals = 3.502, $P = 0.061$, Exp (B) = 6.000] (Table 2).

Multivariate analysis of lymph node metastases in EGC

Multivariate analysis revealed that the depth of invasion was an independent risk factor for lymph node metastases [$P = 0.018$, Exp (B) = 2.744]. Venous invasion was

also an important influencing factor [P = 0.116, Exp (B) = 4.147, Table 3]. Tumor location, depth of invasion, morphological classification, and venous invasion had no significant impact on nodal involvement rates.

Relationship between depth of invasion and number of metastatic lymph nodes

There was no significant difference between mucosal and submucosal tumors in terms of number of retrieved lymph nodes, using the independent sample t test (t = 0.350, df = 200, P = 0.727, mean difference = 0.534). The number of metastatic lymph nodes in those with mucosal tumors was slightly higher than in those with submucosal tumors. The results of dissected nodes were as follows: mucosa (n = 122), 14.70 ± 9.894; submucosa (n = 80), 14.16 ± 11.656, P = 0.727; for the metastasis nodes, mucosa (n = 122), 3.91 ± 5.576; submucosa (n = 80), 3.72 ± 3.102, P = 0.908. The difference was not significant.

Number of retrieved lymph nodes and lymph node metastasis ratios for involved lymph nodes at each nodal station in EGC

The metastatic ratio is the ratio of metastatic nodes to the total number of dissected nodes and was recorded for each nodal station for all regional lymph nodes. There were 110 metastatic lymph nodes, an incidence of 3.8%. Among them, more lymph nodes were retrieved in stations No. 3, 4, 6, 7, 1, 9, 8 and 12. The number of retrieved lymph nodes in above stations was between 108 and 861, and the metastatic ratio was between 0% and 5.9%. Fewer lymph nodes were retrieved in stations No. 5, 13, 2, 11, 14, 10 and 15. The retrieved number was between 13 and 78, and the metastatic ratio was between 0% and 34.8%. Only one patient received station No. 16 lymph node dissection, and no metastasis was found. According to the Japanese Classification of Gastric Carcinoma[7], 29 cases (14.4%) were at N1, seven cases were at N2 (3.5%), and two cases were at N3 (1.0%). A direct skip to N2, without moving through N1, occurred in two cases (1.0%). There were no skips to N3 without going through N2. The incidence of lymph node metastases in each station, from high to low, was as follows: station No. 14 (34.8%), No. 2 (11.1%), No. 6 (5.9%), No. 3 (4.8%), No. 11 (3.7%), No. 4 (3.4%), No. 5 (2.6%), No. 8 (2.5%), No. 9 (2.2%), No. 7 (1.5%), and No. 1 (0.6%). There were no station No. 10 and No. 11 lymph node metastases in two patients who had undergone a total gastrectomy combined with a splenectomy (Table 4).

Lymph node metastasis ratios and incidence at each station in upper third, middle third and lower third gastric cancers

The extent of metastases in 25 cases with upper third gastric cancer was as follows: four cases were at N1 (2.0%), an incidence of 3.4%, and a metastatic rate of 16.0%; one case was at N2 (0.5%), an incidence of 4.5%, and a metastatic rate of 4.0%; and no metastases was found at N3. At N1, lymph node metastases occurred in stations No. 2, 3, and 4, but not in station No. 1. The incidence of metastases was 5.3%, 5.5%, and 1.6% in stations No. 2, 3, and 4, respectively, and the metastatic rate was 4.0%, 8.0%, and 4.0%, respectively. At N2, lymph node metastases occurred only in station No. 8, and the incidence of metastases was 16.7%, and the metastatic rate was 4.0% (Table 5).

The extent of metastases in middle third gastric cancers was as follows: 18 cases (8.9%) occurred at N1, an incidence of 3.5% and a rate of 18.4%; five cases occurred at N2 (2.5%), an incidence of 1.4%, and a rate of 5.1%; and two cases occurred at N3 (1.0%), an incidence of 14.3%, and a rate of 2.0%. At N1, station No. 1, 3, 4, 5, and 6 had lymph node metastases, a rate of 1.0%, 9.2%, 5.1%, 1.0% and 2.0%, respectively. At N2, station No. 8, 9 and 11 had lymph node metastases, a rate of 1.0%, 3.1%, and 1.0%, respectively. At N3, only station No. 2 and 14 had lymph node metastases, a rate of 1.0%. No distal lymph node metastasis was identified (Table 5).

The extent of metastases in lower third gastric cancers was: 20 cases (9.9%) had metastases at N1: an incidence of 5.7%, and a rate of 25.3%; two cases (1.0%) had metastases at N2: an incidence of 1.8%, and a rate of 2.5%; there were no cases of metastases at N3 or at

Table 3 Multivariate analysis of lymph node metastases in early gastric cancer for the entire study population

	RR	95%CI	P[1]
Tumor site	1.159	0.84-1.478	0.644
Invasion depth	2.744	2.316-3.172	0.018[a]
Macroscopic type	0.864	0.57-1.158	0.620
Vessel amblous	4.147	3.242-5.052	0.116
Constant term	0.037	-2.568	0.010

[1]Statistically significant, invasion depth: submucosa *vs* mucosa, [a]P < 0.05.

Table 4 Number of retrieved lymph nodes and lymph node metastasis ratios for involved lymph nodes at each nodal station in early gastric cancer

Node group	No. of dissected nodes	No. of metastasis nodes	Incidence of lymphnode metastasis (%)
No. 1	165	1	0.6
No. 2	27	3	11.1
No. 3	861	41	4.8
No. 4	670	23	3.4
No. 5	78	2	2.6
No. 6	358	21	5.9
No. 7	263	4	1.5
No. 8	120	3	2.5
No. 9	134	3	2.2
No. 10	16	0	0.0
No. 11	27	1	3.7
No. 12	108	0	0.0
No. 13	58	0	0.0
No. 14	23	8	34.8
No. 15	13	0	0.0
No. 16	5	0	0.0
Total	2926	110	3.8

Table 5 Lymph node metastasis ratios and incidence at each station in upper third, middle third and lower third gastric cancers

Node group	Upper			Middle			Lower		
	pN category	Incidence	Ratio	pN category	Incidence	Ratio	pN category	Incidence	Ratio
No. 1	pN1	0.0 (0/52)	0.0 (0/25)	pN1	7.4 (1/73)	1.0 (1/98)	pN2	0.0 (0/40)	0.0 (0/79)
No. 2	pN1	5.3 (1/19)	4.0 (1/25)	pN3	28.6 (2/7)	1.0 (1/98)	M	0.0 (0/1)	0.0 (0/79)
No. 3	pN1	5.5 (7/128)	8.0 (2/25)	pN1	3.8 (17/445)	9.2 (9/98)	pN1	5.9 (17/288)	8.9 (7/79)
No. 4	pN1	1.6 (1/62)	4.0 (1/25)	pN1	4.3 (15/347)	5.1 (5/98)	pN1	2.7 (7/261)	6.3 (5/79)
No. 5	pN3	0.0 (0/6)	0.0 (0/25)	pN1	2.6 (1/38)	1.0 (1/98)	pN1	2.9 (1/34)	1.3 (1/79)
No. 6	pN3	0.0 (0/21)	0.0 (0/25)	pN1	2.2 (4/182)	2.0 (2/98)	pN1	11.0 (17/155)	8.9 (7/79)
No. 7	pN2	0.0 (0/21)	0.0 (0/25)	pN2	0.0 (0/141)	0.0 (0/98)	pN2	4.0 (4/101)	1.3 (1/79)
No. 8	pN2	16.7 (2/12)	4.0 (1/25)	pN2	1.6 (1/63)	1.0 (1/98)	pN2	0.0 (4/45)	0.0 (0/79)
No. 9	pN2	0.0 (0/5)	0.0 (0/25)	pN2	3.5 (3/85)	3.1 (3/98)	pN2	0.0 (4/44)	0.0 (0/79)
No. 10	pN2	0.0 (0/2)	0.0 (0/25)	pN3	0.0 (0/3)	0.0 (0/98)	M	0.0 (0/11)	0.0 (0/79)
No. 11	pN2	0.0 (0/4)	0.0 (0/25)	pN2	7.1 (1/14)	1.0 (1/98)	pN2	0.0 (0/9)	0.0 (0/79)
No. 12	pN3	0.0 (0/6)	0.0 (0/25)	pN2	0.0 (0/61)	0.0 (0/98)	pN2	0.0 (0/41)	0.0 (0/79)
No. 13	M	0.0 (0/10)	0.0 (0/25)	pN3	0.0 (0/31)	0.0 (0/98)	pN3	0.0 (0/17)	0.0 (0/79)
No. 14	M	0.0 (0/2)	0.0 (0/25)	pN3	41.2 (7/17)	1.0 (1/98)	pN2	25.0 (1/4)	1.3 (1/79)
No. 15	M	0.0 (0/1)	0.0 (0/25)	M	0.0 (0/9)	0.0 (0/98)	M	0.0 (0/3)	0.0 (0/79)
No. 16	M	0.0 (0/0)	0.0 (0/25)	pN3	0.0 (0/5)	0.0 (0/98)	pN3	0.0 (0/0)	0.0 (0/79)

Table 6 Correlation between lymph node metastases at pN1 and pN2 and clinicopathological factors

	pN1			pN2		
	-	+	*P*	-	+	*P*
Tumor size (cm)			0.24			0.295
≤ 2.0	86 (88.7)	11 (11.3)		95 (97.9)	2 (2.1)	
> 2.0	87 (82.9)	18 (17.1)		100 (95.2)	5 (4.8)	
Macroscopic type			0.969			0.259
Elevated	21 (84.0)	4 (16.0)		24 (96.0)	1 (4.0)	
Flat	18 (85.7)	3 (14.3)		19 (90.5)	2 (9.5)	
Depressed	134 (85.9)	22 (14.1)		152 (97.4)	4 (2.6)	
Invasion depth			0.008			0.334
Mucosal	111 (91.0)	11 (9.0)		119 (97.5)	3 (2.5)	
Submucosal	62 (77.5)	18 (22.5)		76 (95.0)	4 (5.0)	
Differentiate			0.332			0.349
Differentiated	106 (87.6)	15 (12.4)		118 (97.5)	3 (2.5)	
	67 (82.7)	14 (17.3)		77 (95.1)	4 (4.9)	
Undifferentiated						
Lymphovascular invasion			0.126			0.804
No	174 (87.0)	26 (13.0)		194 (97.0)	6 (3.0)	
Yes	1 (50.0)	1 (50.0)		2 (100.0)	0 (0.0)	

distal lymph nodes. In N1, the metastatic rate, from high to low, was 8.9%, 8.9%, 6.3% and 1.3% respectively, and incidence was 5.9%, 11.0% 2.7%, and 2.9%, respectively in stations No. 3, 6, 4, and 5. In N2, lymph nodes in stations No. 7 and 14 were most frequently involved, while no metastases occurred in stations No. 1, 8, 9, 11, and 12. At N2, lymph node metastases occurred in station No. 7, in a depressed type and differentiated submucosal tumor with a diameter 3.0 cm. Lymph node metastases occurred in a mucosal tumor in station No. 14 with a diameter 6.0 cm. It was a type 0-Ⅲ differentiated cancer (Table 5).

Correlation between lymph node metastases at pN1 and pN2 and clinicopathological factors

Univariate analysis of variance revealed a close relationship between the depth of invasion and lymph node metastases at pN1 ($P = 0.008$). There was no obvious relationship between the depth of invasion and lymph node metastases at pN2 ($P = 0.334$). There was no significant correlation between tumor size, morphological classification, differentiation, lymphatic invasion and the presence of lymph node metastases at pN1 and pN2 (Table 6).

DISCUSSION

Five-year survival rates in EGC tend to be greater than 90%, with lymph node status the most important prognostic factor. In those with lymph node metastases, a 10-year survival rate of 72% has been reported, compared to 92% for those without nodal involvement[2]. Although research has explored the issue of predicting risk factors for lymph node metastases in EGC, as yet there are no definitive criteria. In addition, controversy surrounds indications for local treatment and a limited surgical approach, and the range of lymph node dissection. Hence, in this study, we aimed to explore the risk factors for the development of lymph node metastases in EGC, to confirm the optimal range of lymph node dissection, and to provide a basis for a rational approach to surgical management.

Reported rates of lymph node metastases in EGC range from 5.7% to 20%[8-16]. However, Hayes *et al*[17] reported a rate over 40% during surgery. Depending on the depth of invasion, EGC can be classified as mucosal or submucosal, with a rate of lymph node involvement of 0%-21%[11,18,19] for mucosal tumors, and 16.5%-30%[2,3,19-22] for submucosal tumors. Based on anatomical and histological characteristics, there is a close relationship between the depth of tumor invasion and lymph node metastases in EGC. Once the tumor has invaded the submucosal layer, the rate of lymph node metastases increases significantly. In our group of 202 patients with EGC, lymph node metastases occurred in 29. Among these, there were 11 cases of mucosal and 18 cases of submucosal cancer. The rate of involved lymph nodes was higher in those with submucosal cancer than

in mucosal cancer (22.5% *vs* 9.0%, $\chi^2 = 7.14$, $P = 0.008$).

In our group, male patients predominated, accounting for 65.3%, while was similar to other reports[18,23-26]. Studies to date suggest that EGC frequently occurs in the lower third of the stomach[18,22,26]. In our study, 48.5% of cases originated in the middle third, which was similar to the report from Fujimoto *et al*[14]. Also in our group, 52.0% of patients had tumors greater than 2 cm. Larger tumors have higher rates of lymph node metastases. Of the 29 cases with lymph node metastases, the tumor size in 18 was greater than 2 cm, accounting for 62.1% of all metastases. Morphological classification was mainly of the depressed type (77.2%). Among these, type 0-Ⅱc and type 0-Ⅲ accounted for approximately 50%. Histologically, most tumors were differentiated, accounting for 59.9%, which was similar to that reported by Abe *et al*[27].

Although the lymph node metastatic rate in EGC is relatively low, it has been shown that the presence of lymph node metastases predicts a poor prognosis[28-32]. Thus, many researchers have attempted to investigate the relationship between nodal involvement and clinicopathological factors. The size of the primary tumor, undifferentiated histopathological characteristics, lymphatic or venous invasion, and a cancerous ulcer are associated with nodal metastases in EGC[8,9,12,14,32-42]. Univariate analysis confirmed a correlation between tumor location, depth of invasion, morphological classification, venous invasion and lymph node metastases in EGC in our study ($\chi^2 = 122.901$, $P = 0.001$; $\chi^2 = 7.14$, $P = 0.008$; $\chi^2 = 79.523$, $P = 0.001$; $\chi^2 = 8.687$, $P = 0.003$, respectively), while nodal metastases were not associated with sex, age, tumor size, number of retrieved lymph nodes, histological type, lymphatic invasion, nervous invasion, and CEA levels. Boku *et al*[12] reported an association between lymph node metastases and the tumors arising from the distal third of the stomach, indicating that cancers arising from this section have a worse prognosis. In our study, tumors originating in the upper third of the stomach had a nodal metastatic rate of 8%, compared to 15.3% for the middle third and 15.2% for the lower third, which was similar to that reported by Boku *et al*[12]. Reports have suggested both a lack of association between the occurrence of lymph node metastases and mucosal tumors of the upper and middle third of the stomach[19], and a marked increase in nodal involvement with distal gastric cancers[43]. Data from our study revealed a lymph node metastases rate in mucosal and submucosal tumors of 9.0% and 22.5%, respectively, which was similar to other reports[19,44]. At present, endoscopic submucosal resection is often used for patients with mucosal cancer, while data from Hölscher contradicts this approach[19]. Our data indicates that, prior to surgery, determination of the depth of invasion is important for predicting lymph node metastases. Preoperative staging technology, particularly endoscopic ultrasonography, can determine the depth of invasion, but there can be errors of judgment[11,16,45,46]. In Japan, the morphological classification of EGC is summarized as elevated, flat and depressed type. The rate of lymph node metastases in the elevated subtype was relatively high, being 16% in our study. The rate of lymph node metastases tends to be markedly higher in patients with venous or lymphatic invasion[46]. However, our results did not concur with this, which might be explained by the fact that the number of specimens varied, and the quantity of venous or lymphatic invasion that we included was low.

It is generally acknowledged that tumor size is closely related to lymph node metastases. Hölscher[19] found no evidence of lymph node metastases in mucosal cancers less than 1 cm, and in submucosal cancers. Moreover, lymph node metastases have not been identified in patients with mucosal cancers less than 2 cm[19]. Tumors greater than 2 cm appear to be independent risk factors for lymph node metastases[19,22,47,48]. In our group, although the rate of lymph node metastases in patients with tumors greater than 2 cm (17.1%) was clearly greater than that observed with smaller tumors (11.3%), the difference was not significant, which is similar to the results from Lee *et al*[18].

Histological type is also closely related to nodal status[9,18,35,41,42]. In our group, the rate of lymph node metastases in non-differentiated tumors was higher than in differentiated cancer, 17.3% and 12.4%, respectively. However, a non-differentiated tumor was not an independent risk factor for lymph node metastases. Abe *et al*[49] suggest that, apart from tumor size, submucosal invasion and lymphatic invasion, a correlation also exists between females and the occurrence of lymph node metastases. In females, the biological behavior of gastric cancer tends to be more invasive. Moreover, it has also been shown that the extent of the invasiveness of gastric cancer cannot be fully explained by tumor size, depth of invasion or lymphatic invasion[49]. A possible explanation for the fact that gastric cancer tends to be more invasive in females could be related to endogenous estrogen levels, which might promote tumor growth.

In recent years, endoscopic surgery has become one of the standard procedures, and is indicated for patients with mucosal tumors, tumors less than 2 cm in size, and those without lymph node metastases. Our results suggest that the rate of lymph node metastases is markedly higher in those with cancers of the middle and lower third of the stomach, and those with submucosal tumors, who are therefore not suitable for endoscopic surgery.

Multivariate analysis found that only depth of invasion was an independent risk factor for lymph node metastases in EGC [$P = 0.018$, Exp (B) = 2.744], which is consistent with previous studies[18,26,31,32,35,37,50-55]. Multivariate analysis by Kim *et al*[3] on 748 cases of EGC, indicated that tumor size, poorly differentiated tumors and submucosal cancers were all independent risk factors for lymph node metastases. Hyung *et al*[56] suggest that poorly differentiated tumors, submucosal cancer, tumor size, and venous or lymphatic invasion are independent risk factors for nodal metastases. Many other studies have reached similar conclusions, namely that tumor

size, depth of invasion, histological type, morphological classification, venous or lymphatic invasion, are all independent risk factors for lymph node metastases in EGC[3,14,22,26,27,47,48,50,56-62].

A consideration of the number of lymph node is related to the extent of surgical intervention, which is also related to the depth of tumor invasion. Thus the depth of tumor invasion is also associated with the number of lymph nodes: as the depth increases, the number of lymph nodes also increases. In our group, all 202 cases of EGC had a radical gastrectomy. Post-operatively, 2892 regional lymph nodes were located. In 122 cases, where tumor invasion was limited to the mucosal layer, 1790 lymph nodes were found, with an average of 14.67 per case. In 80 cases with tumor invading the submucosa, 1102 lymph nodes were detected, with an average of 13.77 per case. The difference between the number of lymph nodes in mucosal and in submucosal cancer was not significantly different ($P = 0.727$). Five hundred and sixty lymph nodes were found in 29 cases with lymph node metastases, an average of 19.31 per case. In our study, there was a relationship between the number of lymph nodes and the presence of lymph node metastases. In 29 patients with metastases, the number of involved nodes with tumor depth limited to the mucosa was similar to that observed for submucosal tumors, with no significant difference between the two groups.

Our results showed that there was no obvious correlation between lymph node metastases in mucosal cancer, and sex, age, tumor location, tumor size, the number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, nervous invasion and CEA levels. Currently, there is much interest in the optimal management approach for patients with submucosal tumors and involved lymph nodes[36-40]. In a univariate analysis, An *et al*[22] demonstrated that tumor size, histological type, the Lauren classification, the depth of tumor invasion, lymphatic invasion and nervous invasion, were all relevant in terms of the risk of lymph node metastases in submucosal cancer. Of these factors, tumor size and lymphatic invasion are independent risk factors for nodal involvement[22,36-39]. In our study, there was no obvious correlation between the presence of lymph node metastases in patients with submucosal cancer, and sex, age, tumor location, tumor size, the number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, nervous invasion, and CEA levels. Nonetheless, the lymph node metastases rate (60%, 3/5) in those with venous invasion was higher than that observed in patients without invasion (20%, 15/75), and the difference was significant. However, an analysis of venous invasion, as a source variable, in the logistic regression model, did not confirm it as an independent risk factor for lymph node metastases. An *et al*[22] considered that, for 19.4% of patients with submucosal cancer and lymph node metastases, laparoscopic subtotal distal gastrectomy plus lymph node dissection improved both the resection rate and quality of life. Controversy continues as to which surgical approach is the optimal for the management of patients with submucosal cancer[40].

Most lymph node metastases in EGC are limited to N1 and/or N2. In line with previous reports[34,63], and our own experience, approximately 80%-90% of nodal metastases in EGC are limited to the N1. The proportion of involved N2 and N3 lymph nodes is lower at 10%-19% and 0%-1%, respectively[22,34,63,64]. This appears to support R2 resection[65]. In our group, of 29 patients with lymph node metastases, all were N1, seven were N2 (24.1%), and two were N3 (6.9%). These results are all higher than those reported by An *et al*[22]. Among them, the two N3 patients had the following clinicopathological features: both were mucosal cancers; the tumor size was relatively large, at 6 cm and 2.5 cm, respectively; and both tumors were depressed subtypes. Both patients underwent a radical gastrectomy plus a D2 lymph node dissection. Even in EGC, extensive lymph node metastases can occur. Surgeons should avoid conventional limited surgery, and assess the range of possible surgical resections[22]. Kunisaki *et al*[55] believe that, for most cases of mucosal cancer, the routine D2 lymph node dissection might also be too invasive. Therefore, the method and range of lymph node dissection should be based on clinicopathological features before and after surgery. However, Yoshikawa *et al*[44] suggest that, during surgical treatment of mucosal cancers, apart from perigastric lymph node dissection, it is necessary to perform lymph node dissection at the coeliac trunk and beside the common hepatic artery. Our results support this recommendation. In our group, there was one case of mucosal cancer with a station No. 8 lymph node metastasis.

Since the mid 1900s, D2 lymph node dissection has been the standard procedure worldwide for the management of EGC, particularly for submucosal tumors. Studies have shown that the rate of lymph node metastases for N1 is 9%-16%, 4%-6% for N2, and 0.3%-1% for N3[49,56,59,66]. Accordingly, if D2 surgery is performed in all patients with EGC, 70%-80% would undergo unnecessary lymph node evacuation, and analysis suggests that these patients do not necessarily benefit as a result[40]. Consequently, indications for standard D2 lymph node dissection should be reconsidered. Our study revealed that, for cancers originating in the middle third of the stomach, the lymph node metastasis rate at N2 was 5.1%, mainly station No. 9, whereas there were no metastases in stations No. 7 and No. 12. Similarly, the metastasis rate in lower third tumors was 2.5%, mainly station No. 7 and No. 14. There was one case of metastasis (1.3%) in each of these. In upper third cancers, the rate was 4.0%, predominately station No. 8, with one case of metastasis (4.0%). Thus, when performing D2 surgery, patients with station No. 7 and No. 12 lymph nodes in tumors from the middle third of the stomach, stations No. 1, 8, 9, 11, and 12 lymph nodes in those from the lower third, and stations No. 7, 9-11 lymph nodes in the upper third should not be referred for routine dissection. In tumors of the middle third of the stomach, there was only one

case of N3 lymph node metastasis in both stations No. 2 and No. 14, and the metastasis rate was 1.0%. There were no N3 and distal lymph node metastases in tumors from both the upper and the lower third of the stomach. The benefit of performing D3 dissection in EGC is extremely low, with a long operative time and relatively more complications, which adversely affect quality of life. Therefore, D3 dissection should be avoided in EGC. There were no distal lymph node metastases in our study, and thus expanded lymph node dissection was not required.

Investigating the correlation between pN1 and pN2 lymph node metastases and clinicopathological factors, we found no significant associations between nodal status at pN1 and pN2 and tumor diameter, morphological classification, tumor differentiation and lymphatic invasion. The lymph node metastasis rate (22.5%, 18/80) at N1 in patients with submucosal cancers was higher than in those with mucosal cancers (9.0%, 11/122), and the difference was significant ($\chi^2 = 7.144$, $P = 0.008$). A histological study performed by Asao *et al*[67] on 417 patients with gastric cancer who had routine gastrectomy plus D2 lymph node dissection, reported a rate of lymph node metastases in submucosal cancer of 1.3% (2/154), and in mucosal cancer distal to the perigastric part of 18% (17/96). These nodal metastases were mainly concentrated around the common hepatic artery and the coeliac trunk. In submucosal cancer, there was no nodal metastases elsewhere[67]. However, our study highlighted that tumors with N2 lymph node metastases were usually submucosal, with a diameter greater than 2.0 cm and were of the depressed subtype. Thus, we suggest that submucosal cancers, with a diameter greater than 2.0 cm, and of the depressed subtype are risk factors lymph node metastases. Patients with these factors should be identified during surgery.

There are limitations to this study. Firstly, it was retrospective, based on the examination of morphological samples after surgery. Prospective studies are needed to confirm whether our approach could be applied to endoscopic surgery based on biopsies. It is essential to define the acute T stage before surgery, in order to safely implement limited lymph node dissection. Although the accuracy of endoscopic ultrasonography is relatively high, overestimation or underestimation occurs[46]. Secondly, according to a previous Japanese study based on different depths of tumor invasion, mucosal cancer can be further subdivided into m1, m2, and m3 cancer, and submucosal cancer into sm1, sm2 and sm3 cancer. Moreover, there were obvious differences between the subgroups, with m1 and m2 cancers not usually associated with lymph node metastases, and the rate of nodal metastases in m3 cancers varies from 0% to 12.8%. At present, most endoscopic specialists agreed that all patients with mucosal cancers are suitable for EMR, while Hölscher *et al*[19] oppose this view. Unfortunately, our two hospitals do not routinely perform sub-classifications for mucosal and submucosal cancers, which we plan to redress in future studies. Thirdly, the number of patients in our study was lower than in the Japanese study. In a seminal study by Gotoda[46], 5000 patients with EGC were enrolled. Results from our study need to be confirmed in larger studies.

In summary, our research revealed a lymph node metastasis rate of 14.4% in patients with EGC, with a rate of 5.4% for mucosal tumors and 8.9% for submucosal tumors. The occurrence of lymph node metastasis in EGC is related to tumor location, depth of invasion, morphological classification and venous invasion, with depth of invasion identified as the only independent risk factor for nodal involvement. Lymph node metastases should be considered when deciding on the surgical management of EGC.

COMMENTS

Background

Five-year survival rates in early gastric cancer (EGC) tend to be greater than 90%, with lymph node status the most important prognostic factor. Recently, in order to reduce operative and post-operative complications, and to improve quality of life, less invasive surgical alternatives, such as endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), laparoscopy-assisted gastrectomy and limited surgery, are used for the treatment of EGC. Although there has been substantial research on the prediction of risk factors for lymph node metastases in EGC, no definitive criteria exist.

Research frontiers

The presence of lymph node metastases in EGC predicts a poor prognosis. In the area of the appropriate extent of lymphadenectomy in EGC, the research hotspot is to investigate the relationship between nodal involvement and clinicopathological factors, predict risk factors for lymph node metastases in EGC and determine the indications for local treatment in EGC, and limited surgery and the appropriate extent of lymphadenectomy.

Innovations and breakthroughs

The presence of lymph node metastases predicts a poor prognosis, univariate analysis confirmed a correlation between tumor location, depth of invasion, morphological classification, venous invasion and lymph node metastases in EGC in this study. The results suggest that the rate of lymph node metastases is markedly higher in those with cancers of the middle and lower third of the stomach, and those with submucosal tumors, who are therefore not suitable for endoscopic surgery. When performing D2 surgery, patients with station No. 7 and No. 12 lymph nodes in tumors from the middle third of the stomach, stations No. 1, 8, 9, 11, and 12 lymph nodes in those from the lower third, and stations No. 7, 9, 10, and 11 lymph nodes in the upper third should not be referred for routine dissection. D3 dissection should be avoided in EGC. There were no distal lymph node metastases in this study, and thus expanded lymph node dissection was not required. The authors suggest that submucosal cancers, with a diameter greater than 2.0 cm, and of the depressed subtype, are risk factors for lymph node metastases. Patients with these factors should be identified during surgery.

Applications

The depth of invasion is the only independent risk factor for lymph node metastases. Risk factors for metastases should be considered when choosing surgery for EGC.

Terminology

EGC: A lesion confined to the mucosa or the submucosa, irrespective of the presence of regional lymph node metastases; EMR: A technique used for providing accurate histological staging of superficial gastrointestinal neoplasms and providing a minimally invasive technique for removal of superficial malignancies; ESD: A technique for the resection of early gastrointestinal neoplasia, whose main advantage is that lesions can be resected without almost any size limit.

Peer review

The paper is good and complex. The authors thoroughly explained the problem of prediction of risk factors for lymph node metastasis in early gastric cancer.

They have described, in detail, the research results, as well as, the discussion. The conclusion was a logical consequence of the research results.

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BRIEF ARTICLE

Rectal gastrointestinal stromal tumors: Imaging features with clinical and pathological correlation

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Abstract

AIM: To investigate computed tomography (CT) and magnetic resonance imaging (MRI) manifestations of rectal gastrointestinal stromal tumors (GISTs) in order to enhance the recognition of these rare tumors.

METHODS: Fourteen patients with pathologically proven rectal GISTs were retrospectively reviewed. Patient histories were retrospectively reviewed for patient age, gender, presenting symptoms, endoscopic investigations, operation notes and pathologic slides. All tumors were evaluated for CD117, CD34 expression, and the tumors were stratified according to current criteria of the National Institutes of Health (NIH). In all cases the first pre-operation imaging findings (CT and MRI, n = 3; MRI only, n = 8; CT only, n = 3) were analyzed by two experienced radiologists by consensus, which include: tumor size, shape, CT density (hypodense, isodense and hyperdense), MRI signal intensity (hypointense, isointense and hyperintense), epicenter (intraluminal or extraluminal), margin (well-defined or ill-defined), internal component (presence of calcifications, necrosis, hemorrhage or ulceration), pattern and degree of enhancement, invasion into adjacent structures. After review of the radiologic studies, clinical and pathological findings were correlated with radiological findings.

RESULTS: The patients, 13 men and 1 woman, were aged 31-62 years (mean = 51.5 ± 10.7 years). The most common initial presentation was hematochezia (n = 6). The mean tumor diameter was 5.68 ± 2.64 cm (range 1.5-11.2 cm). Eight lesions were round or oval, and 6 lesions were irregular. Eleven lesions were well-defined and 3 had ill-defined margins. Ten tumors were extraluminal and 4 were intraluminal. The density and MR signal intensity of the solid component of the lesions were similar to that of muscle on unenhanced CT (n = 6) and T1-weighted images (n = 11), and hyperintense on T2-weighted MR images. Calcification was detected in 2 tumors. Following intravenous injection of contrast media, 3 lesions had mild enhancement and 11 lesions had moderate enhancement. Enhancement was homogenous in 3 lesions and heterogeneous in 11. In 1 of 11 patients who underwent both CT and MRI, the tumor was homogenous on CT scan and heterogeneous on MRI. Eight patients were classified as high risk according to the modified recurrent risk classification system of NIH.

CONCLUSION: Rectal GISTs usually manifest as large, well-circumscribed, exophytic masses with moderate and heterogeneous enhancement on CT and MRI. The invasion of adjacent organs, bowel obstruction and local adenopathy are uncommon.

Key words: Gastrointestinal stromal tumors; Rectum;

Computed tomography; Magnetic resonance imaging

Core tip: Rectal gastrointestinal stromal tumors (GISTs) are so rare that little information has been reported on their radiological features. This study describes the computed tomography (CT) and magnetic resonance imaging (MRI) features of rectal GISTs with clinical and pathological correlation, in order to better understand this rare disease. Rectal GISTs usually manifest as large, well-circumscribed, exophytic masses with moderate and heterogeneous enhancement on CT and MRI. The presence of invasion of adjacent organs, bowel obstruction and local adenopathy is uncommon.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs), which arise from the interstitial cell of Cajal or its precursor, the intestinal mesenchymal precursor cell, are the most common mesenchymal neoplasms of the gastrointestinal tract. They are defined by their expression of CD117 (KIT), a tyrosine kinase growth factor receptor, which distinguishes them from other mesenchymal neoplasms such as leiomyomas, leiomyosarcomas, schwannomas, and neurofibromas and which determines the appropriateness of CD117-inhibitor therapy. Throughout the length of the gastrointestinal tract, GISTs arise most commonly in the stomach (60%-70%) followed by the small bowel (20%-25%), however, GISTs in the rectum are extremely rare (5%)[1]. It was reported that GISTs account for 0.6% of all malignant rectal tumors[2]. The imaging features of rectal GISTs are unclear due to their rarity and have only been described in small series[3-6]. Surgical resection remains the initial treatment for localized rectal GISTs[7], familiarity of these imaging features may permit preoperative diagnosis and improve the surgical management of patients. The purpose of our study was to describe the computed tomography (CT) and magnetic resonance imaging (MRI) features of rectal GISTs with clinical and pathological correlation, in order to enhance the understanding of this rare disease.

MATERIALS AND METHODS

The database of the Department of Surgery at our hospital was reviewed in order to identify patients presenting between January 2000 and June 2012 with histologically and immunochemically confirmed GISTs arising in the rectum. Fourteen patients were identified during this period. Patient histories were retrospectively reviewed for patient age, gender, presenting symptoms, endoscopic investigations, and surgical notes. Pathologic slides of the specimens and mitotic activity (number of mitoses per 50 consecutive high-power fields) were reviewed by an experienced pathologist, and the tumors were stratified according to the current criteria of the National Institutes of Health (NIH)[8]. All tumors were evaluated for CD117 and CD34 expression.

In all cases, the first pre-operative CT or MRI was reviewed. Of the 14 patients, 3 underwent both CT and MRI examinations, 8 underwent only MRI examinations and 3 underwent only CT examinations. The CT and MRI technique varied somewhat due to the different imaging equipment and the retrospective nature of the study. However, intravenous contrast-enhanced images had been obtained for studies in all patients. A retrospective review of CT and MR images was implemented by two radiologists by consensus. The following imaging features of each mass were assessed: tumor size, shape, CT density (hypodense, isodense and hyperdense), MRI signal intensity (hypointense, isointense and hyperintense), epicenter (intraluminal or extraluminal), margin (well-defined or ill-defined), internal component (presence of calcifications, necrosis, hemorrhage or ulceration), pattern of enhancement (homogeneous or heterogeneous), and invasion into adjacent structures. The degree of enhancement of the lesion was assessed subjectively and categorized as follows: mild, when the enhancement was similar to that of adjacent muscle; moderate, when the enhancement was higher than that of muscle, but lower than that of blood vessels; and marked, when the enhancement was approaching that of blood vessels. High signal intensity in the necrosis on T1WI was defined as hemorrhage. The adjacent structures were also assessed for the presence of invasion. Findings on CT and MRI for abdominal adenopathy and hepatic metastasis were also evaluated. After review of the radiologic studies, surgical notes and excised specimens were correlated with radiological findings.

RESULTS

Clinical findings

Our patient cohort included 13 men and 1 woman with a mean age of 51.5 ± 10.7 years (range, 31-62 years), and the clinical findings are summarized in Table 1. Digital colorectal examination was performed in 7 patients. Colonoscopy showed a mass protruding from the rectal wall with intact overlying mucosa in 6 patients (Figure 1), while a central mucosal ulceration was revealed in 1 patient. Two of these 7 patients underwent additional endoscopic ultrasonography, which showed hypoechoic masses that were contiguous with the muscularis propria of the rectal wall (Figure 1). All masses were found in the lower part of the rectum, with a distance from the anal verge ranging from 2.0 to 5.5 cm. Twelve patients

Table 1 Summary of the presenting complaints of 14 patients with rectal gastrointestinal stromal tumors

Presenting symptoms or signs	*n*
Incidental finding on an imaging study	2
Change of defecation habit	2
Hematochezia	6
Pelvic pain	1
Narrow stools	1
Difficult defecation	1
Sense of anal falling inflation	1

underwent radical resection, including abdominoperineal resection (n = 9) and low anterior resection (n = 3) depending on the extent and localization of the tumors. The other 2 patients underwent transanal excision. At the time of surgical exploration, none of the patients had evidence of remote metastasis. Tumor rupture was not found during surgery. No lymph node metastases were identified pathologically in all 12 patients who underwent lymphadenectomy.

Pathological findings

Immunohistochemical staining showed that all tumors were positive for CD117 (14/14) (Figure 2), while 13 patients were positive for CD34 (13/14). The masses were solid and grayish-white or dark-red with areas of hemorrhage or necrosis on the cut sections (Figure 3), and showed a spindle cell pattern under light microscope (Figure 2). According to the current NIH classification scheme, 8 tumors (8/14) had high risk, 3 (3/14) had intermediate risk, 3 (3/14) had low risk for aggressive behavior.

Imaging findings

The diameter of the tumors ranged from 1.5 to 11.2 cm (mean, 5.68 ± 2.64 cm). The tumor was smaller than 5 cm in 4 patients and larger than 5 cm in 10 patients. Eight masses were either round (n = 2) or oval (n = 6), and 6 were irregular. The tumors showed definite intraluminal tumor growth in 4 patients, whereas extraluminal tumor growth was seen in the majority of our patients (10/14) (Figures 1-8). Eleven tumors showed a well-defined margin, and 3 tumors showed contour irregularity or blurring.

On unenhanced CT images, the density of the 6 tumors was 33-45 HU (mean, 38 HU) which was similar to that of muscle. Flecks of calcification were detected in 2 extraluminal tumors (Figures 4, 5). Following intravenous administration of contrast media, the majority (4/6) of rectal GISTs were moderately enhanced masses with areas of unenhanced low attenuation on CT scan. A homogeneous pattern of enhancement was less common and was present in 2 of the patients who underwent CT studies (Figure 6); in 1 patient who underwent both CT and MRI, the tumor was homogenous on CT scan, but demonstrated heterogeneous enhancement with foci of fluid signal on MR images. All the heterogeneous tumors showed areas of fluid density in keeping with cystic changes on cut sections.

In the 11 cases examined with MRI, the solid component of all the tumors was isointense to skeletal muscle on T1-weighted images and hyperintense on T2-weighted images. There were some intratumoral high-intensity foci on T1WI representing hemorrhage (n = 1) (Figure 3) and hyperintensity on T2WI corresponding to necrosis (n = 8) (Figure 7). Nine of these 11 tumors were heterogeneous, with non-enhancing components showing fluid signal. After intravenous administration of gadolinium, 7 lesions had moderate enhancement and 4 lesions had mild enhancement on fat-suppressed T1WI. Ulceration to the rectal lumen was seen in 1 patient (Figure 8). Bowel obstruction, abdominal adenopathy and remote metastasis were not seen in any of our patients.

DISCUSSION

Although an increasing amount of literature concerning GISTs has been published, there is little information reported on the radiological features of rectal GISTs due to their rarity. To our knowledge, this article is the largest study of rectal GISTs in the radiological literature.

Clinical characteristics

It was reported that rectal GISTs occur in adults between the fifth and sixth decades with a significant male predominance (71%)[9]. In this study, there was a marked male predominance with a male: female ratio of 13:1, and the mean age at onset was 51.5 years, in line with the pathological literature. The clinical manifestations of rectal GISTs depend on the location and size of the tumors and are often nonspecific[10-12]. A GIST can remain clinically silent and present late when the tumor is large. Two of our patients were asymptomatic and the tumors were incidental findings, perhaps due to their small size (less than 3.0 cm in maximum diameter) and tendency to grow exophytically. The exophytic growth pattern may also explain why rectal GISTs rarely cause intestinal obstruction even when they are large. Miettinen *et al*[9] reported that rectal bleeding was the most common symptom in patients with a large rectal GIST more than 5 cm. In this study, the most frequent symptom was also hematochezia.

Imaging features

Rectal GISTs generally manifest as large eccentric masses growing beyond the rectal wall[3-6]. Our series showed a similar growth pattern. Although larger tumors have a higher rate of malignancy, size does not predict benignity, and small GISTs have been known to behave in a malignant fashion[13-16]. Since most rectal GISTs arise within the muscularis propria of the intestinal wall, they most commonly have an exophytic growth pattern with the epicenter located well outside the rectum[17,18]. GISTs arising from the anterior rectal wall in male patients can even mimic tumors of prostatic origin on CT[19,20]. Thus,

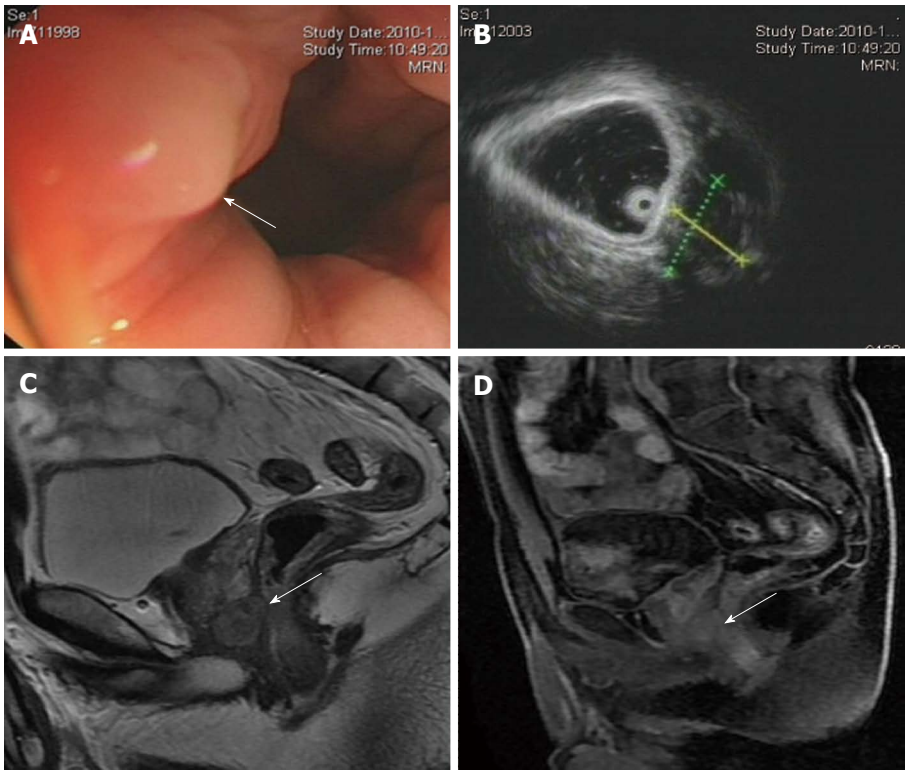

Figure 1 A 40-year-old man with a rectal gastrointestinal stromal tumor. A: Colonoscopy shows a mass protruding from the rectal wall with intact overlying mucosa (arrow); B: Endoscopic ultrasonography shows a well-defined hypoechoic mass located along the right anterior aspect of the rectal wall; C: Sagittal T2-weighted magnetic resonance imaging shows an oval, homogenous, hyperintense mass with a sharp margin bordering the anterior rectal wall. A small area of anatomical continuity between the tumor and the anterior rectal wall is observed (arrow); D: Postcontrast T1-weighted image shows a slightly homogenously enhancing mass (arrow).

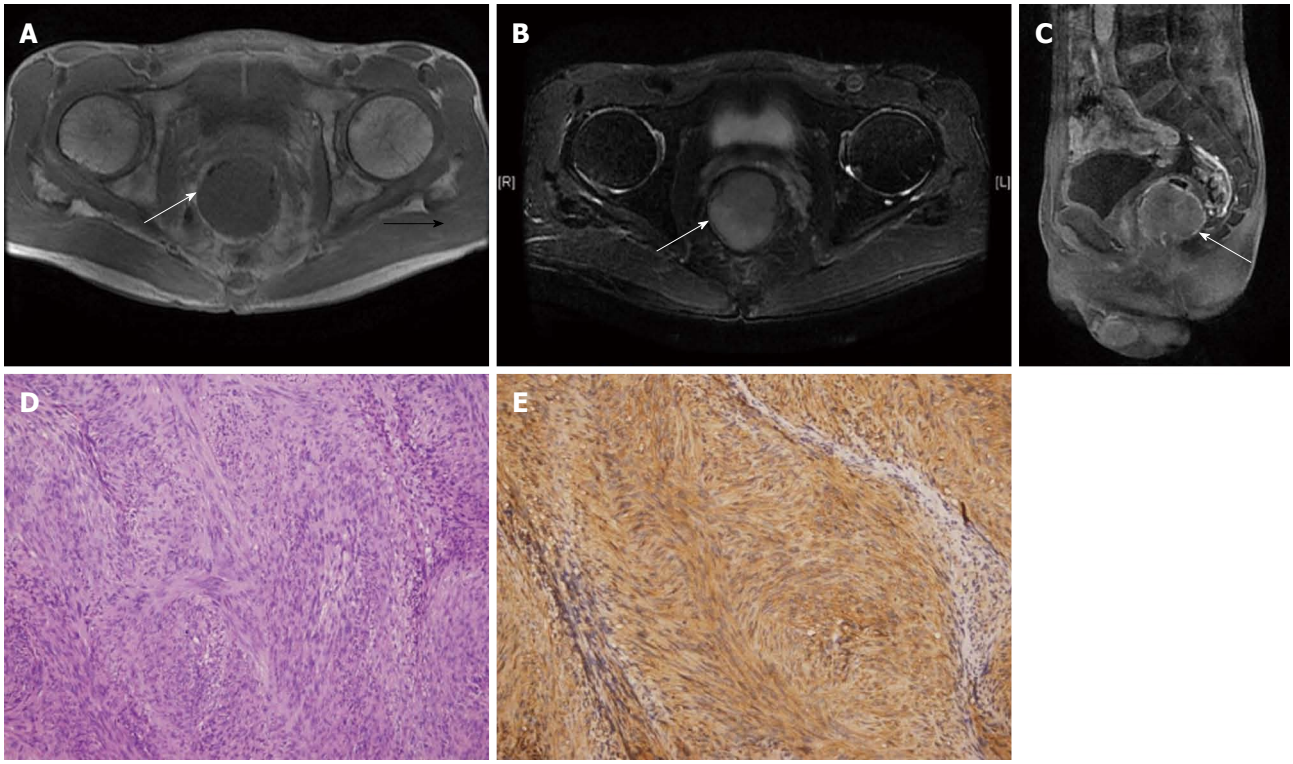

Figure 2 A 56-year-old man with a rectal gastrointestinal stromal tumor. A: Axial T1WI shows the lesion as a round, intraluminal, homogenous, hypointense mass with a sharp margin (arrow); B: It shows homogenous hyperintense on T2WI (arrow); C: Sagittal enhanced T1WI shows homogenous moderate enhancement (arrow); D: Photomicrograph shows fascicular proliferation of spindle-shaped tumor cells (hematoxylin and eosin, × 200); E: The tumor cells were strongly and diffusely positive for CD117 staining (immune-histochemistry, × 200).

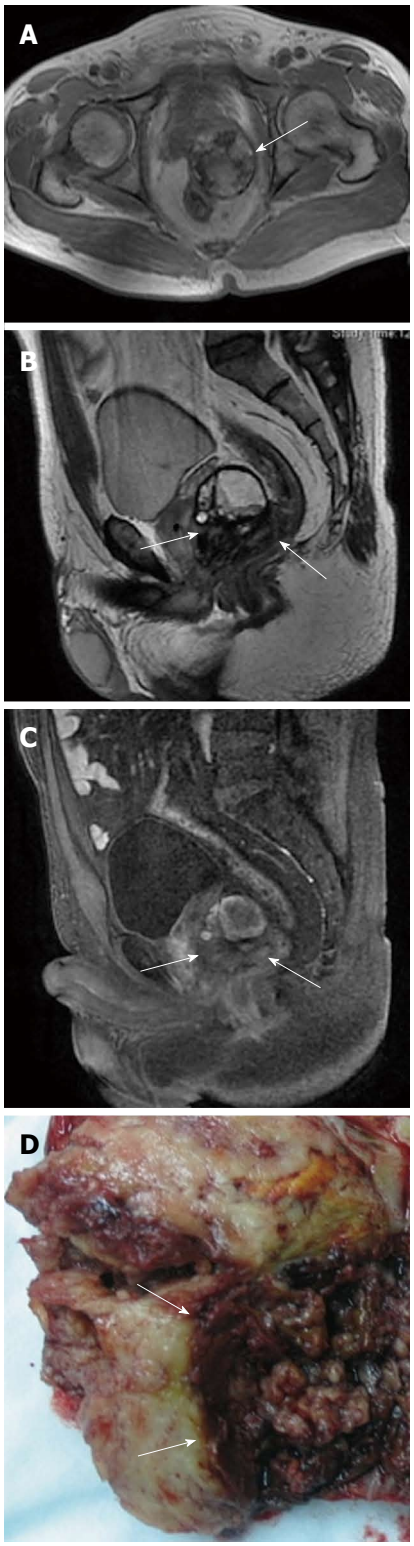

Figure 3 A 62-year-old man with a rectal gastrointestinal stromal tumor. A: The mass is located between the prostate and the anterior rectal wall and its epicenter is well outside the rectum. Axial T1-weighted MR image shows a high signal (arrow) within the mass, in keeping with hemorrhage; B: Sagittal T2WI shows a heterogenous mixed-intense mass with blurring contour (arrow); C: Postcontrast T1WI shows the solid component of the mass enhanced heterogeneously (arrow); D: Macroscopic cross section shows a pale yellow, tan and solid mass with hemorrhage (arrow).

the classic rule regarding determination of the organ of origin based on the location of the epicenter of a

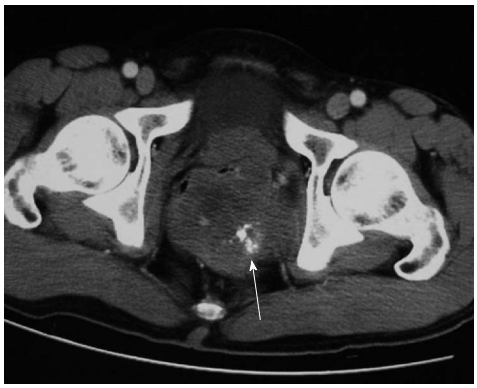

Figure 4 A 52-year-old man with a rectal gastrointestinal stromal tumor. The mass is located in the left posterior wall of the rectum with scattered calcification (arrow).

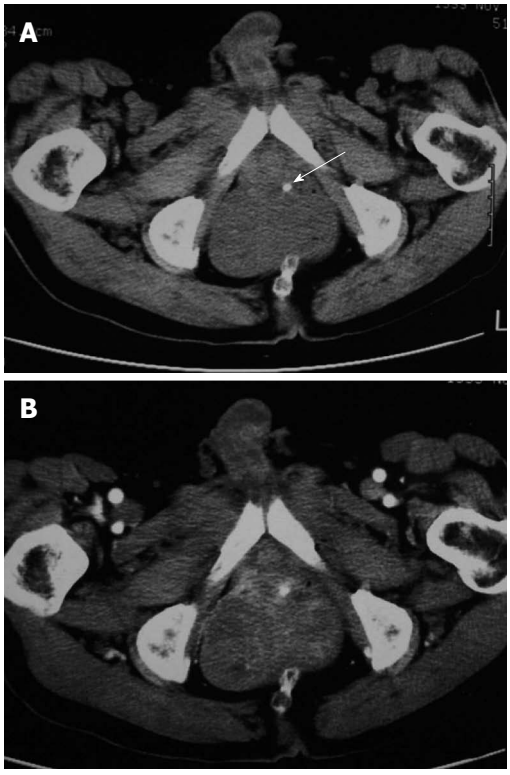

Figure 5 A 61-year-old man with a rectal gastrointestinal stromal tumor. A: The mass located in the left wall of the rectum with fleck of calcification at the tumor margin (arrow); B: The mass enhanced heterogeneously following intravenous administration of contrast media.

tumor is often not applicable. In such cases, enhanced MRI should be performed. MRI with direct multiplanar capability is definitely more useful in determining the exact tumor origin, delineating the spatial relation to adjacent structures, and outlining the pelvic floor surgical anatomy. In our study, tumor-rectal wall continuity was revealed in the sagittal plane, which suggested the diagnosis of a rectal mass. On the other hand, invasion of adjacent organs was better seen on MRI compared with CT examination.

In this study, most of the tumors were round or oval and smooth with well-defined margins. On unenhanced CT, rectal GISTs appear as isodense with normal muscle

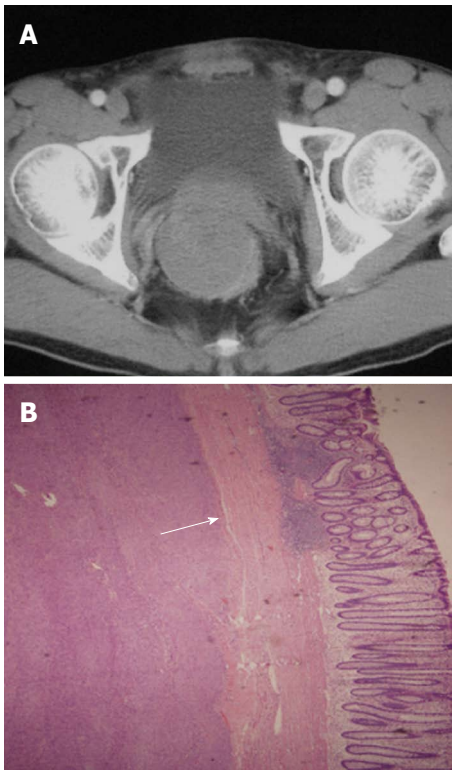

Figure 6 A 53-year-old man with a rectal gastrointestinal stromal tumor. A: Computed tomography scan shows a round intraluminal mass with a sharp margin; B: Photomicrograph shows the tumor originating from the muscularis propria (arrow) of the rectum (hematoxylin and eosin, × 20).

as the standard for comparison. On unenhanced MRI, rectal GISTs appear as isointense to skeletal muscle on T1-weighted images and hyperintense on T2-weighted images, and moderately or mildly enhanced on CT and/or MR studies. A heterogeneous pattern of enhancement is more common on CT and/or MR studies, and was present in 11 of our cases. Heterogeneity corresponds to intralesional necrosis or hemorrhage, which was confirmed on cut sections. It is likely that MRI may be superior to CT in detecting the internal component. Only one of our patients underwent both CT and MRI, and the tumor was homogenous on CT scan, but was heterogeneous with fluid signal on MR images.

Although calcification is not a usual clinicopathologic feature of GISTs, it has been reported in previous studies[21-23]. Most calcifications within GISTs are circumscribed and patchy. In our study, one of the two calcified tumors showed focal calcification and the other was mottled. Previous episodes of bleeding or tumor necrosis with cystic degeneration may cause calcification[24,25]. Because these tumors are submucosal, the overlying mucosa can be intact. However, overlying mucosal ulcerations are often present and cause bleeding, and are more common in high risk GISTs[26-28].

Differential diagnosis

Unfortunately, there is significant overlap between the imaging appearances of rectal GISTs and other rectal diseases, such as epithelial neoplasms, lymphoma or

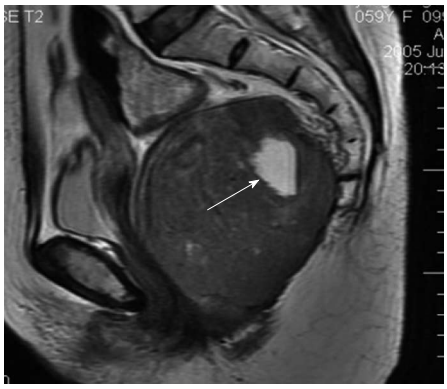

Figure 7 A 59-year-old woman with a rectal gastrointestinal stromal tumor. The mass is located between the uterus and the anterior rectal wall with focal fluid signal on T2WI corresponding to necrosis (arrow).

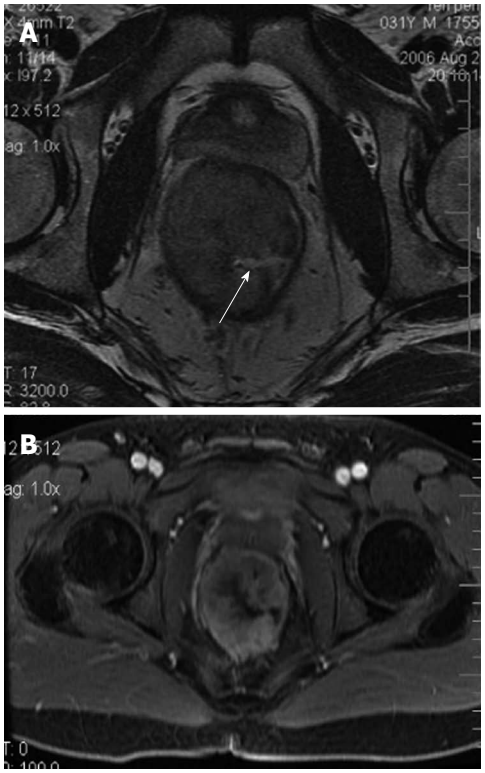

Figure 8 A 31-year-old man with a rectal gastrointestinal stromal tumor. A: Axial T2WI shows the lesion as an intraluminal mass with ulceration (arrow) of the rectal lumen; B: The mass is enhanced moderately heterogeneously following intravenous administration of gadolinium.

carcinoids. Most of these tumors may be differentiated from rectal GISTs by the presence of the following features: well-demarcated margins, prominent extraluminal location and no surrounding adenopathy, and the lack of bowel lumen constriction despite the large size of the rectal GIST. This behavior is unlike most cases of adenocarcinoma which has a propensity for luminal obstruction. The appearance of a smooth regular border is a feature that allows these tumors to be differentiated from malignant epithelial neoplasms. Signs of intratumoral degeneration, such as cystic change, hemorrhage, and calcification, should exclude lymphoma from the

differential diagnosis.

Due to the submucosal origin of the tumors, endoscopy is only of use when the tumor infiltrates the mucosa and can be detected[29]. In addition to endoscopy, endoscopic ultrasonography is a valuable technique in the diagnosis of these tumors because it can reliably distinguish intramural lesions from extrinsic compression. Enhanced MRI with an endoluminal coil has been performed to determine the tumor origin in some reported cases[30-32], but these procedures are invasive, and are rarely used in routine examinations.

Pathological features

CD117 is the most specific and important diagnostic molecular marker of GISTs. Most GISTs (more than 95%) express CD117, which can be detected immunohistochemically[9,33]. Although CD117-positive expression is very common in GISTs and a major defining characteristic of GISTs, it is not absolutely necessary for the diagnosis of GISTs. CD34 is also commonly expressed in GISTs, which is less specific compared with CD117 and is not considered a requirement for the diagnosis of GISTs either[34-36]. The review of pathology in our patients showed that the tumors were strongly positive for CD117 and in most cases also CD34, consistent with previous reports.

The pathologic differentiation and biologic behavior of GISTs have been continuing topics of controversy for many years. A small number of GISTs recur or metastasize despite a histologically benign appearance. Therefore, some authors support stratifying GISTs into very low-, low-, intermediate-, and high-risk categories rather than classifying them as benign or malignant. The NIH risk classification system, which consists of tumor size, mitotic count (number of mitoses per 50 consecutive high-power fields), anatomic location, and tumor rupture, is recommended as a valuable tool for estimating the clinical behavior of GISTs[8]. Rectal GISTs have a high-risk tendency[23] which was observed in the majority of our patients.

Attempts to predict the potential high-risk behavior of GISTs from their imaging features is difficult. It was reported that GISTs with irregular margins, size larger than 10 cm, central necrosis, ulceration, and heterogeneous contrast enhancement are normally regarded as denoting aggression[37,38]. These signs are mainly derived from a study population of stomach and small intestine GISTs and seldom from rectal GISTs. However, our series is small and no correlation between radiologic appearance and risk levels could be established with regard to the degree of necrosis, hemorrhage, ulceration, or contrast material enhancement.

There are several limitations in this study. The study was retrospective and the reviewers of the imaging studies knew that all patients had a pathologically confirmed rectal GIST, which may have increased the sensitivity for detecting each imaging sign. In addition, different CT and MRI equipment and techniques were used. However, these problems are simply unavoidable due to the rarity of this type of tumor, and this should not have significantly affected the imaging features studied.

In summary, we present the largest series of radiological studies of rectal GISTs to date. These tumors often present with hematochezia. They are normally seen as large, bulky, exophytic rectal masses with heterogenous enhancement on CT and MRI. Cross-sectional imaging, which allows better visualization of the origin of the mass, its internal components, and other organ involvement, is indicated for surgical planning.

COMMENTS

Background

Rectal gastrointestinal stromal tumors (GISTs) are very rare, which accounts for 0.6% of all rectal malignant tumors. Only a few reports concerning the radiological features of rectal GIST has been published due to its rarity. Surgical resection remains the mainstay of therapy for localized rectal GISTs, familiarity of their radiological features may permit preoperative diagnosis and improves surgical management of patients. Thus, it is very important to enhance the understanding of the imaging features of this rare tumor.

Research frontiers

The current imaging knowledge of rectal GISTs is based on a few case reports. Grassi *et al* reported a 4-cm mass in a 70-year-old man that showed marked, irregular, eccentric thickening of the lateral left wall of the lower third of the rectum. Hama *et al* reported a 9.8-cm mass in a 50-year-old man that was contiguous with the prostate and enhanced on both computed tomography (CT) and magnetic resonance imaging (MRI). Levy *et al* reported six anorectal gastrointestinal stromal tumors, and found that anorectal GISTs were typically large, well-demarcated anorectal masses containing hemorrhage.

Innovations and breakthroughs

This study contained a relative large cohort of cases of rectal GISTs confirmed by histology and immunochemistry. All the patients had a complete medical records including age, gender, presenting symptoms, endoscopic examinations, surgical notes and pre-operative cross-sectional imaging studies. The authors focused on the correlation of imaging features of this rare tumor with clinical and pathological characteristics.

Applications

Rectal GISTs are normally seen as large, bulky, exophytic rectal masses with heterogenous enhancement on CT and MRI. Cross-sectional imaging, which allows better visualization of the origin of the mass, its internal components, and other organ involvement, is indicated for surgical planning.

Terminology

GIST is the most common mesenchymal neoplasm of the gastrointestinal tract, which arises from the interstitial cell of Cajal or its precursor. The rectum is the rare primary site involving about 5% cases. Due to the submucosal origin of the rectal GISTs, the presence of the following features allows these tumors to be differentiated from malignant epithelial neoplasms: well-demarcated margins, prominent extraluminal location and no surrounding adenopathy, and the lack of bowel lumen constriction despite the large size of the tumor.

Peer review

The authors reported imaging features of rectal gastrointestinal stromal tumors with clinical and pathological correlation. The main strength of this study is the relatively large number (n = 14) of patients and broader spectrum of imaging findings in this rare tumor. The findings are instructive to both radiologists and physicians.

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BRIEF ARTICLE

Synchronous adenocarcinoma and gastrointestinal stromal tumors in the stomach

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Abstract

AIM: To review the clinicopathological characteristics of concurrent gastrointestinal stromal tumors (GISTs) and gastric adenocarcinoma.

METHODS: We retrospectively analyzed eight cases of synchronous adenocarcinoma and GIST in the stomach that had been surgically resected with curative intent between March 2003 and December 2008 in Xinhua hospital and Ruijin hospital. The adenocarcinoma was determined to be the primary tumor based on the histological features. The GIST cells were diffusely and strongly positive for CD34 and CD117.

RESULTS: The patients were six men and two women aged 47-80 years (average, 68.6 years). GIST was preoperatively detected in only one patient. The average sizes of the gastric adenocarcinomas and GISTs were 6.000 ± 2.6186 cm and 1.825 ± 1.4370 cm, respectively. All GISTs were very low- or low-risk lesions that were detected during evaluation, staging, operation or follow-up for gastric adenocarcinoma.

CONCLUSION: We hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in a simultaneous proliferation of different cell lines (epithelial and stromal cell).

Key words: Gastric adenocarcinoma; Gastrointestinal stromal tumor; Synchronous occurrence; Gastrectomy

Core tip: We retrospectively analyzed eight cases of synchronous adenocarcinoma and gastrointestinal stromal tumors (GISTs) in the stomach that had been surgically resected with curative intent between March 2003 and December 2008 in Xinhua hospital and Ruijin hospital. The average sizes of the gastric adenocarcinomas and GISTs were 6.000 ± 2.6186 cm and 1.825 ± 1.4370 cm, respectively. All GISTs were very low- or low-risk lesions that were detected during evaluation, staging, operation or follow-up for gastric adenocarcinoma. We hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in a simultaneous proliferation of different cell lines (epithelial and stromal cell).

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesen-

chymal tumors of the digestive tract and have various clinical and biological characteristics. The expression of c-Kit distinguishes GISTs from true leiomyomas, leiomyosarcomas and other mesenchymal tumors of the gastrointestinal tract[1-3]. The stomach (60%-70%) and small intestine (20%-30%) are the most common sites of GISTs[1,2]. GISTs are composed of spindle (70%) or epithelioid (30%) cells and are positive for c-Kit (CD117), CD34 and occasionally for actin, but almost always negative for desmin and S-100 protein[4], in contrast to other mesenchymal tumors. Benign GISTs are far more common than malignant ones (10%-30%)[1], and all GISTs are often found incidentally at surgery and excised in the same session[5].

Collision tumors of the stomach are uncommon; however, several cases have been reported. Most collision tumors of the stomach are composed of an adenocarcinoma intermixed with a gastric lymphoma[6-9]. Some are composed of an adenocarcinoma intermixed with a carcinoid tumor[8,10,11]. However, gastric collision tumors composed of a GIST and an adenocarcinoma are exceedingly rare. Ruka *et al*[12] found that 10% of their GIST patients had an associated non-GIST neoplasm, usually, a carcinoma. Furthermore, Maiorana *et al*[13] found that of 52 patients with gastric GISTs, six patients (11.5%) had an associated, second gastric tumor (five adenocarcinomas and one carcinoid tumor).

Most of these publications were reports of single cases. Here, we present a series of eight patients with synchronously occurring GISTs and gastric adenocarcinomas. The aim of this study was to evaluate the clinicopathological characteristics of GISTs occurring concomitantly with gastric adenocarcinomas and to provide an English literature review[14,15].

MATERIALS AND METHODS

Patients

We analyzed the clinicopathological findings in eight patients with CD117-immunopositive GISTs (six men and two women) who underwent surgery with a curative intent for a primary, resectable tumor without detectable metastases, between March 2003 and December 2008. Their clinicopathological data were supplemented by a review of all available medical and histopathological records from Xinhua hospital and Ruijin hospital. Selected were the patients with postoperative pathological diagnosis of primary gastric GIST, and patients who didn't received chemotherapy and Imatinib as adjuvant treatment. Excluded were patients who had synchronous gastrointestinal stromal tumors in stomach and extra-stomach neoplasms, patients with initial surgery perfomed in other hospital, and patients with the pathology diagnostic data insufficiency. None of the patients had a family history of gastrointestinal carcinoma or GIST. Prior to the commencement of the present study, our ethics committee deemed that approval of the committee and informed consent were not required for the retrospective analysis of clinical data.

Methods

GISTs were defined as primary spindle cell and/or epithelioid neoplasms of the tubular gastrointestinal tract with CD117 overexpression, with or without CD34 expression according to well-established criteria for the diagnosis of GISTs[16,17]. The risk of aggressive GISTs was assessed using criteria derived from the 2002 Fletcher classification. For each patient with a primary tumor and no metastatic disease at the time of diagnosis, a TGM system[18] was used for staging: T: T1, localized and < 5 cm; T2, localized and ≥ 5 cm; T3, contiguous organ invasion or peritoneal implants; T4, tumor rupture. G: G1, low grade; G2, high grade. M: M0, no metastases; M1, metastases present. The final staging system was determined as follows: Stages Ⅰ (< 5 cm) and Ⅱ (≥ 5 cm) are localized lesions with low histologic grade. Stage Ⅲ lesions are either high-grade tumors of any size or tumors with regional involvement (contiguous organ invasion or peritoneal implants). Stage ⅣA refers to patients with systemic metastases or unresectable tumor. Stage ⅣB is designated when tumor rupture has occurred despite resection of all macroscopic disease. The gastric adenocarcinomas were staged according to the TNM system devised by the International Union Against Cancer[19].

Representative hematoxylin and eosin-stained slides of archival tumor specimens were prepared from formalin-fixed, paraffin-embedded tissue blocks. To confirm the histogenesis of GISTs, immunohistochemistry (IHC) panels were obtained with the following markers: CD117, CD34, smooth muscle actin (SMA) and S-100. The IHC studies were performed using formalin-fixed, paraffin-embedded blocks and primary antibodies to the above markers, on a standard, automated, streptavidin-biotin peroxidase-detection system (EnVision™ Autostainer Visualization System; DakoCytomation, Glostrup, Denmark) equipped with a microwave antigen-retrieval step. Parallel positive controls were run for each antibody. A rabbit or mouse, universal, negative-control monoclonal antibody was used for each specific antibody.

Ethics committee approval

This study was approved by the Bioethics Committees at each hospital.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL). Descriptive data are presented as the mean ± SD.

RESULTS

The study consisted of eight patients, including six men (75%) and two women (25%). The median age of the patients at the time of presentation was 68.6 years (range, 47-80 years). The most common presenting features were

Table 1 Size and histological characteristics of gastrointestinal stromal tumors in eight patients

Patient No.	Age (yr)	Sex	Localization	Size (cm)	Growth pattern	Type	Grade	Staging	Surgical resection	Origin
1	47	M	Cardia	2.0	Extraluminal	Spindle	Very low	T1G1M0, Ⅰ	Complete	Subserous
2	80	M	Cardia	1.5	Extraluminal	Spindle	Very low	T1G1M0, Ⅰ	Complete	Subserous
3	60	M	Antrum	0.6	Extraluminal	Spindle	Very low	T1G1M0, Ⅰ	Complete	Subserous
4	67	F	Anterior wall	0.8	Extraluminal	Spindle	Very low	T1G1M0, Ⅰ	Complete	Subserous
5	78	M	Posterior wall	2.5	Extraluminal	Spindle	Low	T1G1M0, Ⅰ	Complete	Submucosal
6	78	M	Body	1.4	Extraluminal	Spindle	Low	T1G1M0, Ⅰ	Complete	Subserous
7	59	F	Anterior wall	0.8	Extraluminal	Spindle	Low	T1G1M0, Ⅰ	Complete	Subserous
8	80	M	Lesser curvature	5.0	Extraluminal	Spindle	Low	T2G1M0, Ⅱ	Complete	Subserous

M: Male; F: Female.

Table 2 Immunohistochemical characteristics of gastrointestinal stromal tumors in eight patients

Patient No.	CD117	CD34	SMA	VIM	S-100	DES
1	+	+	++	+	+	++
2	+	+	+	+	-	+
3	+	+	-	+	-	-
4	+	+	-	+	+	+
5	+	+	-	+	+	-
6	+	+	-	-	±	-
7	+	+	-	-	-	-
8	+	+	-	+	-	-

SMA: Smooth muscle actin; VIM: Vimentin; DES: Desmin.

abdominal discomfort (n = 3), gastrointestinal bleeding (n = 2), difficulty eating (n = 1), nausea and vomiting (n = 1) and weight loss (n = 1). Some patients had more than one of these symptoms. The median duration of disease was 1.5 mo (range, 0.5-6 mo). Total (n = 1) or subtotal gastrectomies (n = 7) were performed in the patients with gastric malignancy.

All patients underwent preoperative gastroscopy, which revealed an ulcerative lesion in four patients, a diffuse infiltrative lesion in two patients and an infiltrative ulcerative lesion in two patients. All lesions were diagnosed as adenocarcinomas on biopsy examination. Body computer tomography (CT) scans and chest images were available for review in all eight patients. In one patient, preoperative CT revealed a soft tissue lesion with a diameter of 5.0 cm in the lesser curvature, this lesion was considered a GIST.

All patients underwent simultaneous, radical resection of the gastric adenocarcinoma and GIST. In most cases, the stromal tumors were an incidental finding during operation. Detailed clinicopathological data for all GISTs are shown in Tables 1 and 2. According to the American Joint Committee on Cancer staging, 87.5% of patients had stage Ⅰ tumors, and 12.5% of patients had stage Ⅱ tumors. The mean GIST size was 1.825 ± 1.4370 cm (range, 0.6-5.0 cm). Seven GISTs were located in the serosal layer, and one was present in the muscular layer. All GISTs were of the spindle type, and were strongly and diffusely positive for CD117 and CD34. Six GISTs were also positive for vimentin (VIM) (75.0%), four for S-100 (50.0%), three for desmin (37.5%) and two for SMA (25.0%).

The synchronous gastric adenocarcinomas were located in different parts of the stomach (Table 3). The mean sizes of the primary adenocarcinomas were 6.000 ± 2.6186 cm (range, 2.0-8.0 cm). No patients had distant metastases at the time of diagnosis. Half of the tumors were poorly differentiated; in the case of the other half, differentiation was not recorded because there is no standard for pathological recording. One of the patients had early gastric cancer, and the other seven had advanced gastric cancers; two patients each had stage Ⅰ and Ⅱ disease, three had stage Ⅲ disease and one had stage Ⅳ disease.

DISCUSSION

The term gastric stromal tumor was originally coined by Mazur *et al*[20]. Most gastric stromal tumors exhibit variable differentiation; these tumors tend to originate from primitive mesenchymal cells[21], rather than from smooth muscle cells. GISTs are rare neoplasms that are most commonly located in the stomach (39%-60%), small bowel (30%-42%), colon-rectum (5%-10%) and esophagus (5%)[22]. The most common symptoms of the patients in the current study were abdominal masses, pain and bleeding[23,24]. The symptoms of these tumors depend on their size and location[24]. GISTs are generally found within the deeper stroma and the submucosa, and therefore, in most of the reported cases of synchronous gastric adenocarcinoma and GIST, the preoperative biopsy specimens have shown only adenocarcinoma. GISTs are often incidentally detected during imaging studies, operation or examination of the resected specimens[25].

In this report, only one case of GIST was detected preoperatively, on a CT scan; the others were found incidentally during or after operation, upon general pathological examination. Owing to the variability and specific characteristics of GISTs, the coexistence of these tumors with other gastric tumors should be considered in the treatment of gastric cancer. To avoid missed diagnosis or misdiagnosis of GISTs, imaging studies such as ultrasonography, CT and magnetic resonance imaging should be performed in patients in whom gastric cancer is detected on gastroscopy. Moreover, surgical exploration in these patients should be careful and comprehensive. If suspi-

Table 3 Clinical characteristics, treatments and outcomes of gastric adenocarcinoma in eight patients

Patient No.	Primary site	Size (cm)	TNM status	Surgical resection	Gross appearance	Histology	Clinical presentation
1	Cardia	8	T3N1M0, ⅢB	Radical operation	Ulcerative infiltrative type	The low differentiation adenocarcinoma	Difficulty in swallowing
2	Antrum	2	T1N0M0, ⅠA	Radical operation	Ulcerative	The low differentiation adenocarcinoma	Haematemesis
3	Antrum	8	T3N0M0, Ⅱ	Radical operation	Ulcerative	Adenocarcinoma	Black stool
4	Antrum	4	T3N1M0, ⅢA	Radical operation	Ulcerative	Adenocarcinoma	Abdominal discomfort
5	Cardia	6	T4N2M0, Ⅳ	Palliative gastrectomy	Ulcerative	Adenocarcinoma	Abdominal discomfort
6	The lesser curvature	10	T3N1M0, ⅢA	Radical operation	Infiltrative type	Tubular adenocarcinoma	Emaciation
7	The posterior wall	4	T2N1M0, Ⅱ	Radical operation	Infiltrative type	The low differentiation adenocarcinoma	Nausea, vomiting
8	Antrum	6	T2N0M0, ⅠB	Radical operation	Uplift ulcer type	The low differentiation adenocarcinoma	Abdominal discomfort

TNM: Tumor, nodes, metastasis-classification.

cious lesions are found, these should not be assumed to be metastases of gastric cancer. Physicians should consider the possibility of other types of tumors, radically resect the suspicious lesion, obtain frozen sections for pathological examination, determine the histological origin of the lesion and apply appropriate surgical techniques. In addition, any nodule on the walls of the digestive tract should be carefully assessed so that no small GIST is overlooked during the postoperative pathological examination.

Liszka *et al*[26] retrospectively analyzed the clinicopathological characteristics of 22 cases of concurrent GISTs and other tumors, including two cases of GISTs accompanied by gastric cancers, and 60 cases of only GISTs. They found that the risk of invasion was much lower and the tumor diameter was smaller in patients with concurrent GISTs and other tumors than in patients with only GISTs ($P < 0.05$), which is consistent with our results. These findings may be attributable to the following factors: the risk of malignant invasion of GISTs is relatively low, and the biological behavior of GISTs might have been inhibited by the gastric cancer. However, definitive evidence for this theory is lacking at present.

The morphology of GIST cells is usually spindle shaped (70%), epithelioid (20%) or mixed. GISTs are immunohistochemically positive for Kit expression (90%-95%) and often for Bcl-2 (80%), CD34 (70%), SMA (35%), S-100 (10%) and desmin (5%) expression. In this study, all the GISTs were strongly and diffusely positive for CD117 and CD34. Six GISTs were also positive for VIM (75.0%), four for S-100 (50.0%), three for desmin (37.5%) and two for SMA (25.0%). Fletcher *et al*[17] proposed a classification for malignancies that was based on tumor size and the number of mitotic divisions. According to this classification, all the tumors in our study were classified as low risk or very low risk; nevertheless, careful follow-up is mandatory.

Collision tumors rarely develop in the stomach. The frequency of secondary malignancies in GIST patients has been reported to be 4.5%-35% in different series[15,27-33]. The most common GIST-associated malignancies were gastrointestinal carcinomas (47%), prostate cancer (9%), lymphoma/leukemia (7%) and breast cancer (7%)[27]. Single case reports have described the occurrence of adenocarcinoma admixed with gastric lymphoma, carcinoid tumor, leiomyosarcoma[13,34-37] or rhabdomyosarcoma[35,36], as well as adenoma admixed with a sarcomatous stromal component[38]. Thus far, only a few case reports of gastric collision tumors consisting of adenocarcinoma and leiomyoma have been documented[39,40]. Rare cases of concurrent presentation of gastric adenocarcinoma and GIST have been reported in the literature[13,15,24,37,41-46]. Maiorana *et al*[13] found that 6 of 52 (11.5%) patients with gastric GISTs had an associated second gastric tumor (five adenocarcinomas and one carcinoid tumor), which considering the restriction of both tumor types to the stomach, indicates a high incidence. In 2000, Maiorana *et al*[13] found that GISTs were the most common non-epithelial tumor that could simultaneously occur with epithelial tumors (carcinomas and carcinoid tumors); they also reported five cases of GIST combined with gastric cancer.

The admixture of gastric epithelial and stromal tumors raises the question of whether such an occurrence is a simple incidental association or whether the two lesions are connected by a causal relationship. Some researchers claim that this simultaneous presentation is coincidental; however, others hypothesize that some unknown carcinogens induce the simultaneous proliferation and oncogenesis of epithelial and stromal cells[13-15,24,41,42,44,45]. Theoretically, genetic mutations could play an important role in the pathogenesis of gastric synchronous tumors; however, no available evidence supports a common genetic mutation underlying gastric adenocarcinoma and GIST[27,47,48]. Another hypothesis suggested in experimental models is that the same unknown carcinogenic agent may interact with two adjacent tissues, causing the simultaneous development of tumors of different histological types[13,41,49-52]. Cohen *et al*[51] reported that exposure to both acetylsalicylic acid and nitrosoguanidine causes synchronous development of both gastric cancer and leiomyosarcoma. An interesting hypothesis is that a single

carcinogenic agent may interact with two neighboring tissues, inducing the development of tumors of different histological types in the same organ[51]. *Helicobacter pylori* has been found to be related to the pathogenesis of gastric carcinoma and mucosa-associated lymphoid tumor[53,54] or GIST[24]. Liu *et al*[42] hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in the simultaneous proliferation of different cell lines (epithelial and stromal cells).

Our study has some limitations: it was retrospective and the number of patients was small. This limited the validity of significant statistical evidence that can be extrapolated from our study. In addition, our study does not answer the question of whether a causal relationship exists between gastric adenocarcinoma and GIST, since we did not perform genetic and molecular ancillary tests. This study, however, highlights an interesting observation of a possible association between gastric adenocarcinoma and GIST that should encourage further statistically validated and genetically based studies.

In conclusion, the synchronous occurrence of GISTs and other gastrointestinal malignancies is more common than it has been considered. The concomitant GIST is usually discovered incidentally during endoscopy, imaging study or operation performed because of the other malignancy. We have reported eight cases of GIST combined with gastric cancer. All the GISTs were positive for CD117 and CD34. The cause of the association between GISTs and adenocarcinoma is difficult to determine. In the majority of cases, this association is most likely coincidental. Surgical excision is the mainstay of therapy, and further research is required for explaining this simultaneous tumor development.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors of the digestive tract and have various clinical and biological characteristics. The expression of c-Kit distinguishes GISTs from true leiomyomas, leiomyosarcomas and other mesenchymal tumors of the gastrointestinal tract.

Research frontiers

GISTs are generally found within the deeper stroma and the submucosa, and therefore, in most of the reported cases of synchronous gastric adenocarcinoma and GIST, the preoperative biopsy specimens have shown only adenocarcinoma. GISTs are often incidentally detected during imaging studies, operation or examination of the resected specimens.

Innovations and breakthroughs

The authors hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in a simultaneous proliferation of different cell lines (epithelial and stromal cell).

Applications

In the majority of cases, this association is most likely coincidental. Surgical excision is the mainstay of therapy, and further research is required for explaining this simultaneous tumor development.

Peer review

Synchronous gastric adenocarcinoma and GIST were not common but can be found sometimes in practice. Usually, intraoperatively serendipitous GISTs are of very low risk and with their max diameters less than 1 cm. Carefully preoperative imaging evaluation might be helpful to find the > 1 cm GIST. This topic is interesting and lack of relevant literatures, especially the data of the epidemiological incidence. If possible, the author should report the data of its proportion in the consecutive series.

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BRIEF ARTICLE

Risk factors for proton pump inhibitor refractoriness in Chinese patients with non-erosive reflux disease

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Author contributions: Yu BP designed and supervised the study; Han Z instructed the study; Niu XP conceived and designed the study, collected and compiled the patient data, performed the scale survey, wrote the manuscript and gave final approval of the version to be published; Wang YD and Liu SF performed the scale survey and the questionnaire on patient general status and lifestyle; He CY compiled the data and performed the statistical analysis; Wu WC and Zhang GZ collected the cases and enrolled the patients.

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Abstract

AIM: To analyze risk factors for refractoriness to proton pump inhibitors (PPIs) in patients with non-erosive reflux disease (NERD).

METHODS: A total of 256 NERD patients treated with the PPI esomeprazole were enrolled. They were classified into symptom-free and residual symptoms groups according to Quality of Life in Reflux and Dyspepsia (QolRad) scale. All subjects completed questionnaires on psychological status (self-rating anxiety scale; self-rating depression scale) and quality of life scale (Short Form 36). Multivariate analysis was used to determine the predictive factors for PPI responses.

RESULTS: According to QolRad, 97 patients were confirmed to have residual reflux symptoms, and the remaining 159 patients were considered symptom free. There were no significant differences between the two groups in lifestyle factors (smoking and alcohol consumption), age, *Helicobacter pylori* infection, and hiatal hernia. There were significant differences between the two groups in relation to sex, psychological distress including anxiety and depression, body mass index (BMI), and irritable bowel syndrome (IBS) ($P < 0.05$). Logistic regression analysis found that BMI < 23, comorbid IBS, anxiety, and depression were major risk factors for PPI resistance. Symptomatic patients had a lower quality of life compared with symptom-free patients.

CONCLUSION: Some NERD patients are refractory to PPIs and have lower quality of life. Residual symptoms are associated with psychological distress, intestinal disorders, and low BMI.

Key words: Risk factors; Refractoriness; Proton pump inhibitors; Non-erosive reflux disease

Core tip: Non-erosive reflux disease (NERD) is significantly more refractory to proton pump inhibitor (PPI) treatment than erosive esophagitis is, although the reason is unclear at present. Here, we investigated the risk factors for refractoriness to PPI treatment in patients with NERD. Our results demonstrate that some NERD patients are refractory to standard doses of PPIs and have a lower quality of life. Residual symptoms are associated with psychological distress, intestinal disorders, and low body mass index. Recognition of this might hold the key to improving long-term management of NERD.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is caused by abnormal reflux of gastric contents into the esophagus and is characterized by specific symptoms such as heartburn and acid regurgitation. An epidemiological survey has found that GERD is a common condition with a prevalence of 10%-20% in Western Europe and North America[1]. The prevalence of GERD in China is lower than that in Western countries, but appears to be increasing[2,3]. However, only about one-third to one-half of patients with GERD has endoscopically positive findings such as erosions and ulcers, whereas others with GERD symptoms have no obvious mucosal breaks during endoscopic examination. Therefore, GERD includes erosive esophagitis (EE) and endoscopy-negative reflux disease, which is also known as non-erosive reflux disease (NERD)[4,5]. At present, the most effective drug therapy for GERD is proton-pump inhibitors (PPIs)[6]. PPI treatment results in sustained acid reduction for symptom control in the majority of patients. However, 17%-32% of patients with GERD in primary care trials have experienced persistent, troublesome heartburn or regurgitation despite standard-dose PPI treatment, and the majority of them have even experienced refractory symptoms at higher doses[7-10]. Studies have shown that EE and NERD have different responses to PPIs because their pathogenesis is distinct[11-13]. In addition, NERD is significantly more refractory than EE to PPI treatment[14,15]. However, a recent meta-analysis has reported that NERD has the same response rate to PPIs as EE has, and the previously reported low response rate in patients with NERD was likely the result of inclusion of patients with upper gastrointestinal symptoms who did not have reflux disease[16,17].

PPI failure has become a common clinical dilemma in gastrointestinal clinics and has been increasingly encountered at the primary care level as well. It is likely to be an expensive clinical problem because patients tend to utilize health care resources repeatedly, such as clinic visits, diagnostic studies, and prescription medication. A previous observation has shown that non-acid reflux contributes to poor effectiveness of PPIs in both NERD and EE patients[18]. However, most previous studies regarding PPI responses and the natural course of EE and NERD were based on Western countries; there have been few reports from Eastern Asian countries, including China[14,19]. In addition, the risk factors that affect the response of patients with NERD to PPIs are unclear at present[20,21]. To the best of our knowledge, there has been no report on the risk factors for response to PPIs in patients with NERD in China. Here, we investigated the risk factors for refractoriness to PPI treatment in patients with NERD and propose a potential treatment strategy for them.

MATERIALS AND METHODS

Patient selection

Patients with NERD receiving PPI (esomeprazole) maintenance treatment were consecutively enrolled from May 2008 to August 2010. We enrolled 256 patients with reflux symptoms who were assessed by a locally validated GERD questionnaire, the Chinese GerdQ[22]. All patients were positive for ambulatory 24-h esophageal pH monitoring (DeMeester score > 14.27). All patients had undergone endoscopy at his/her first visit to exclude erosive reflux disease. All patients with NERD recruited for our study were using standard-dose esomeprazole for at least 6 mo.

Exclusion criteria

According to the recent Rome III Criteria, patients with functional heartburn whose typical symptoms were associated with neither abnormal pH testing nor a positive symptom index were excluded[23,24]. Patients were excluded if there was a history of gastrointestinal surgery, Barrett's esophagus, peptic ulcer, or gastroduodenal cancer, and if they could not accurately express their condition or were unwilling to accept the scale survey.

Assessments

The patients' medical records were screened for gastrointestinal morbidity, years since the first episode, and comorbidity (unclear what to deliver). Information was obtained regarding age, sex, smoking, alcohol use, *Helicobacter pylori* (*H. pylori*) infection, body mass index (BMI), comorbid irritable bowel syndrome (IBS), and hiatal hernia. The BMI was categorized using 23 and 25 kg/m^2 as a cut-off point in accordance with the WHO recommendation for Asia. IBS was diagnosed using a questionnaire based on the Rome Ⅲ Criteria. All patients were asked to complete the following questionnaires.

Quality of life in reflux and dyspepsia

The reflux version of quality of life in reflux and dyspepsia (QolRad) is a disease-specific instrument, including 25 items combined into five dimensions: emotional distress, sleep disturbance, vitality, food/drink problems, and physical/social functioning. The recall period refers to the last week. QolRad outcome has been shown to reflect treatment response and impact of symptoms[25]. A 7-point scale was used to assess item severity or frequency (1 = a great deal/all of the time; 2 = a lot/most; 3 = a moderate amount/quite a lot; 4 = some; 5 = a little; 6 = hardly any; 7 = none). The lower the scores were, the more severe the impact on daily functioning. Patients scoring ⩾ 6 on all dimensions were considered symptom free, and those scoring < 6 on at least one dimension as having residual symptoms.

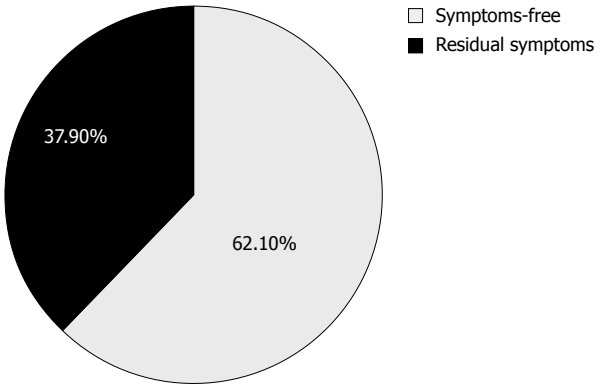

Figure 1 Effectiveness of proton pump inhibitor in 256 patients with non-erosive reflux disease.

Table 1 Self-rating anxiety scale and self-rating depression scale scores in the two groups of patients

Group	*n*	SAS score	SDS score	SAS%	SDS%
Residual symptoms	97	42.68 ± 6.21	52.36 ± 6.93	46.39% (45/97)	50.52% (49/97)
Symptom-free	159	31.17 ± 6.15	43.13 ± 5.27	10.06% (16/159)	8.18% (13/159)
Control	52	30.74 ± 8.18	35.32 ± 6.71	0.00% (0/20)	1.90% (1/52)

Self-rating anxiety scale (SAS) grades: $F = 104.54$, $P < 0.001$; Self-rating depression scale (SDS) grades: $F = 143.91$, $P < 0.001$; SAS%: $\chi^2 = 52.30$, $P < 0.001$; SDS%: $\chi^2 = 79.58$, $P < 0.001$.

Zung self-rating anxiety scale and Zung self-rating depression scale

The scores of the 20 items in the self-rating depression scale (SDS) and self-rating anxiety scale (SAS) were added and multiplied by 1.25. The nearest integer was taken as the standard score. An SDS standard score ⩾ 53 indicated the presence of depression. An SAS standard score ⩾ 50 indicated the presence of anxiety[26].

Quality of life scale (Short Form 36, SF-36)

This 36-question survey measured generic quality of life in eight dimensions[27]: physical functioning (PF), role limitations-physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role limitations-emotional (RE), and mental health (MH). Summary physical component score (PCS) and mental component score (MCS) were also calculated from patient responses. Raw scores were converted to a scale of 0 to 100, with higher scores indicating higher levels of health or wellbeing.

Data analysis

The ages of the patients were categorized into deciles. Descriptive statistics (mean and SD) and additional analyses were calculated using SPSS version 14.0. Mean values were compared using Student's *t* test and analysis of variance, and two-sided *P* values of 0.05 were considered statistically significant. Ninety-five percent confidence intervals and two-tailed *P* values were calculated for the ORs. Multivariate logistic regression analysis was carried out using determinants with *P* values ⩽ 0.1.

Table 2 Lifestyle characteristics of two groups of patients

Variables	Residual symptoms (*n* = 97)	Symptom-free (*n* = 159)	t/χ^2	*P* value
Age (yr)	59.3 ± 11.2	57.1 ± 12.7	1.28	0.203
Sex (male/female)	38/59	97/62	0.92	0.016
Alcohol consumption	19%	26%	0.44	0.509
Smoking	37%	32%	9.93	0.062
Helicobacer pylori positive	59%	62%	11.52	< 0.001
BMI (kg/m^2)			6.19	0.045
⩾ 25	37	86		
23–24.9	30	38		
< 23	30	35		
Comorbid IBS	37%	12%	36.44	< 0.001
Hiatal hernia	9%	8%	1.75	0.186

BMI: Body mass index; IBS: Irritable bowel syndrome.

RESULTS

A total of 256 patients with NERD on PPI treatment were included in the study (mean age 58 years, 53% male). Residual reflux symptoms were investigated by analyzing their impact on QOLRad score. They were divided into the residual symptoms (*n* = 97) and symptom-free (*n* = 159) groups according to QolRad scores (Figure 1). Scores for SAS and SDS were significantly higher in the residual symptoms than symptom-free group (Table 1).

Demographics, clinical and laboratory findings is summarized in Table 2. We found that there was no significant difference between the two groups with regard to age, smoking, alcohol consumption, *H. pylori* infection, and hiatal hernia. A significant sex difference was observed in the residual symptoms and symptom-free groups. There were more women than men in the residual symptoms than symptom-free group ($P < 0.05$). There were significantly more patients in the residual symptoms group with BMI < 23 and comorbid IBS than in the symptom-free group (Table 2). Multivariate analysis of patient characteristics indicated that the risk factors for residual symptoms were: BMI < 23, comorbid IBS, and mental health problems (anxiety or depression) (Table 3). The SF-36 scores indicated that symptomatic patients had a lower quality of life than those who were symptom free (Table 4).

DISCUSSION

Patients with NERD experience typical GERD symptoms caused by acid and non-acid reflux, but they do not have visible esophageal injury[28,29]. NERD is a more common type of GERD in Asian than in Western populations[30]. Patients with NERD are a heterogeneous group including various subpopulations with different mechanisms for their main symptoms: reflux of acidic and non-acidic

Table 3 Multivariate logistic regression of patient characteristics (n = 256)

Variables	β	SE	Z	*P* value	B	OR	95%CI
Women	-0.14	0.10	6.25	< 0.001	-0.19	0.66	0.31-0.89
BMI < 23	0.09	0.03	14.12	< 0.001	0.32	1.56	1.37-2.81
Comorbid IBS	0.21	0.06	12.30	< 0.006	0.37	1.33	1.26-2.55
SDS score	0.46	0.29	5.95	< 0.026	0.53	1.56	1.13-2.39
SAS score	1.05	0.32	9.26	< 0.001	0.85	2.17	1.57-2.76

BMI: Body mass index; IBS: Irritable bowel syndrome; SDS: Self-rating depression scale; SAS: Self-rating anxiety scale.

Table 4 Social functioning-36 scores in residual symptoms and symptom-free groups

Dimensionality	Residual symptoms	Symptom-free	Control	F	*P* value
PF	91.31 ± 8.73	92.03 ± 7.62	92.31 ± 8.26	0.81	0.3926
RP	80.01 ± 19.12[1]	89.93 ± 18.93	94.10 ± 12.15	3.90	0.0117
BP	59.00 ± 10.06[1]	86.22 ± 11.13	89.31 ± 14.81	31.24	< 0.0001
GH	61.25 ± 16.12[1]	85.28 ± 15.26	83.15 ± 11.32	30.27	< 0.0001
VT	64.46 ± 17.92[1]	81.30 ± 19.21	90.61 ± 20.13	12.93	< 0.0001
SF	61.53 ± 11.46[1]	79.23 ± 19.73	80.16 ± 17.23	10.25	< 0.0001
RE	57.64 ± 10.11[2]	68.17 ± 23.55	82.72 ± 18.19	11.75	< 0.0001
MH	50.96 ± 13.13[2]	66.21 ± 12.46	89.15 ± 16.28	23.29	< 0.0001

[1]Significant difference between residual symptoms and symptom-free groups and control group; [2]Significant difference among the three groups. PF: Physical functioning; RP: Role limitations-physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role limitations-emotional; MH: Mental health.

gastric contents, mucosal hypersensitivity, intraesophageal distension by gas, intraduodenal infusion of fat, muscle contractions, and psychological abnormalities. Some studies have reported that the proportion of patients with NERD that responds to a standard dose of PPI is 20%-30%, which is lower than the proportion of patients with EE[15,17]. Some NERD patients even use high doses of PPI but cannot completely control the symptoms. For a long time, ineffective drug maintenance treatment has become a common problem in primary care[31,32]. These patients tend to utilize repeatedly healthcare resources, such as frequent consultations, referrals, diagnostic tests, and repeat prescriptions, which consume a large amount of medical resources. These patients also bear physical suffering and economic pressure, which seriously affect their quality of life[33]. Several studies have demonstrated that the proportion of patients with NERD that respond to a standard dose of PPI is 60%-70%, which is lower than that of patients with EE[34]. A recent study has suggested that, in well-defined NERD patients, the estimated complete symptom response rate after PPI therapy is comparable to the response rate in patients with EE. The previously reported low response rate in studies with patients classified as NERD was probably the result of inclusion of patients with upper gastrointestinal symptoms who did not have reflux disease. In the present study, we found that only 62.1% of patients with NERD treated with PPI were symptom free, and 37.9% of patients had residual symptoms and had a significantly lower quality of life.

The reason why NERD is more refractory than EE to PPIs is unclear at present. Previous observations have shown that the pathogenesis of NERD is associated with age, sex, lifestyle, *H. pylori* infection, BMI, comorbid IBS, and hiatal hernia[31]. It has also been shown that patients with NERD often have more psychological problems than those with EE[35,36]. A large number of epidemiological investigations have found that anxiety, depression, and chronic stress can lead to NERD[37]. Conventional treatment for NERD depends on PPI applications, but it cannot resolve the underlying psychological problems. Patients with NERD are often not satisfied with the treatment. Zerbib *et al*[38] have reported that a no-reflux pattern demonstrated by 24-h pH-impedance monitoring is associated with response to PPIs in patients with GERD symptoms. In contrast, absence of esophagitis, presence of functional digestive disorders, and BMI ⩽ 25 kg/m^2 are strongly associated with PPI failure.

To date, there has been no report on the risk factors that affect the response of NERD patients to PPI therapy in China. In the present study, we found that there was no significant difference between the symptom-free and residual symptoms groups with regard to age, smoking, alcohol consumption, *H. pylori* infection, and hiatal hernia. However, symptomatic patients differed from symptom-free patients in relation to sex, BMI, comorbid IBS, and psychological distress. These variables were analyzed by multivariate analysis and we found that anxiety, depression, comorbid IBS, and BMI < 23 were independent risk factors associated with residual symptoms, but sex was not a risk factor for residual symptoms. We used the modified BMI criteria as proposed by the WPRO, which considers the smaller body frame of Asians and provides a more accurate reflection of body fat stores, thus avoiding a false perception of not being overweight[39].

In conclusion, our results demonstrate that some NERD patients are refractory to standard doses of PPIs and have a lower quality of life. Residual symptoms are associated with psychological distress, intestinal disorders, and low BMI. Recognition of this might hold the key to improving long-term management of NERD.

COMMENTS

Background

Non-erosive reflux disease (NERD) is significantly more refractory than erosive esophagitis to proton pump inhibitor (PPI) treatment, although the reason is unclear at present.

Research frontiers

Here, the authors report a study of the risk factors for PPI refractoriness in Chinese patients with NERD. The majority of studied cases were sporadic.

Innovations and breakthroughs

Although a previous observation has shown that non-acid reflux contributes to poor effectiveness of PPIs in NERD patients, there has been no report on the risk factors for response to PPIs in patients with NERD in China. Here, the authors investigated the risk factors for refractoriness to PPI treatment in patients

with NERD and propose a potential treatment strategy for them.

Applications

The authors found that some NERD patients were refractory to standard doses of PPIs and had a lower quality of life. Residual symptoms were associated with psychological distress, intestinal disorders, and low body mass index. Recognition of this might hold the key to improving long-term management of NERD.

Terminology

There is no specific, unique terminology that will not be familiar to the majority of readers.

Peer review

This was a qualitative study with an original approach to establishing the risk factors for refractoriness to PPIs in patients with NERD. This is an important problem in the treatment of these patients. The study was well designed and the results are clearly described.

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BRIEF ARTICLE

Expression of huCdc7 in colorectal cancer

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Abstract

AIM: To detect the expression of huCdc7 in colorectal cancer.

METHODS: The mRNA and protein expression of huCdc7 in 39 colorectal cancer tissue specimens and matched tumor-adjacent normal colorectal tissue specimens was detected by reverse transcription-polymerase chain reaction and immunohistochemistry, respectively.

RESULTS: The relative expression level of huCdc7 mRNA in colorectal cancer was significantly higher than that in tumor-adjacent normal colorectal tissues (0.03675 ± 1.00 *vs* 0.01199 ± 0.44, $P < 0.05$). huCdc7-positive cells displayed brown granules in the nucleus. Tumor tissues contained many huCdc7-positive cells, whereas normal colorectal tissues contained very few positive cells.

CONCLUSION: huCdc7 may play an important role in the development and progression of colorectal cancer.

Key words: huCdc7; Semiquantitative reverse transcription-polymerase chain reaction; Colorectal cancer

Core tip: huCdc7 is ubiquitously expressed in human tissues and can regulate DNA replication initiation. Abnormal expression or excessive activation of huCdc7 can promote excessive cell proliferation and cause tumorigenesis. Colorectal cancer is a common digestive tract tumor with a gradually increasing incidence. We found that huCdc7 may play an important role in the development and progression of colorectal cancer.

Chen HJ, Zhu Z, Wang XL, Feng QL, Wu Q, Xu ZP, Wu J, Yu XF, Qian HL, Lu Q. Expression of huCdc7 in colorectal cancer. *World J Gastroenterol* 2013; 19(20): 3130-3133 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3130.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3130

INTRODUCTION

huCdc7 is ubiquitously expressed in human tissues and can regulate DNA replication initiation. Abnormal expression or excessive activation of huCdc7 can promote excessive cell proliferation and cause tumorigenesis. Colorectal cancer is a common digestive tract tumor with a gradually increasing incidence. This study aimed to detect the expression of huCdc7 in colorectal cancer to provide a basis for the diagnosis and treatment of this malignancy.

MATERIALS AND METHODS

Specimen collection

Tissue specimens were collected from 39 patients with colorectal cancer who were surgically treated at our hospital. Colorectal cancer tissue specimens and matched

tumor-adjacent normal colorectal tissue specimens (5 cm away from tumor tissue) were placed in liquid nitrogen immediately after surgical specimens were removed. The final diagnosis of colorectal cancer and tumor-adjacent normal colorectal tissues was made pathologically.

Main reagents

M-MLV reverse transcriptase (Promega, United States), Taq DNA polymerase (Takara, Japan), and dNTPs (Takara, Japan) were obtained commercially. The primers for the amplification of a 525-bp CDC7 gene fragment were: forward, 5'-GCT CAG CAG GAA AGG TGT TC-3' and reverse, 5'-AGT TTG ATT GGG GCA CTT TG-3'. The primers for amplification of a 420-bp glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene fragment were: forward, 5'-GTC AGT GGT GGA CCT GAC CT- 3' and reverse, 5'-AGG GGT CTA CAT GGC AAC TG-3'.

Reverse transcription-polymerase chain reaction

Preparation of total RNA: Total RNA was extracted using TRIzol reagent (GIBCO/BRL, United States). All centrifuge tubes, plastic and glasswares and water used for RNA extraction were treated to create an RNAse-free environment. A homogenizer was baked at 200 ℃ for 4 h to remove RNAse and then cooled. Tissue samples were frozen in liquid nitrogen, pulverized into powder, and placed in the homogenizer containing TRIzol reagent. After homogenization for several minutes, the homogenized sample was transferred to an RNAse-free centrifuge tube. Following the addition of chloroform, the tube was centrifuged at 4 ℃. The upper aqueous phase was transferred to an RNAse-free centrifuge tube, and isopropanol was added to precipitate the RNA. The tube was centrifuged at 4 ℃, and the pellet was washed twice with 75% ethanol and dissolved in RNAse-free deionized water. The purity of extracted RNA was assessed by measuring the *A*260/*A*280 ratio (1.7-2.0) using an ultraviolet spectrophotometer. 3-morpholinopropanesulfonic acid-formaldehyde denaturing agarose gel electrophoresis was performed to check if the extracted RNA was degraded. To remove the contamination of genomic DNA, RNA sample was treated with RNAse-free DNA enzyme I (Ambion, United States).

cDNA synthesis: Two micrograms of total RNA were mixed with 1 μL of random primers (50 pmol/L, 1 μL) and 15 μL of diethylpyrocarbonate-treated water, placed at 70 ℃ for 3 min and then immediately placed on ice for 5 min. The following components were then added: 5 μL of 5 × buffer, 1 μL of 10 mmol/L dNTP, 1 μL of M-MLV reverse transcriptase (200 U/μL), and 0.75 μL of RNAse inhibitor (40 U/μL, TaKaRa). The mixture was incubated at 42 ℃ for 2 h.

Semiquantitative reverse transcription-polymerase chain reaction: The *huCdc7* gene was amplified using cDNA as the template. GAPDH was used as a control. Polymerase chain reaction (PCR) conditions were as follows: denaturation at 94 ℃ for 3 min; 35 cycles (25 cycles for GAPDH) of denaturation at 94 ℃ for 30 s, annealing at 58.5 ℃ for 30 s, and extension at 72 ℃ for 40 s; and a final extension at 72 ℃ for 5 min. PCR products were resolved by 2% agarose gel electrophoresis. Band densities were analyzed using the FR-980 bio-electrophoresis image analysis system.

Real-time PCR: The CFX96 real-time PCR detection system (BIO-RAD, United States) was used to detect the expression of genes of interest in tumor tissues and matched tumor-adjacent normal tissues. PCR reaction was performed in a 20-μL system consisting of 10 μL of SYBR Premix EX Taq, 0.4 μL of each primer (10 μm), 2 μL of DNA, and 7.2 μL of ddH_2O. GAPDH was used as an internal control. The experiment was repeated three times to ensure the reliability of the results. The expression level of the *huCdc7* gene was calculated using the following formulas: Cdc7 ΔCt = mean Cdc7Ct - mean GAPDHCt, and Cdc7 ΔΔCt = Cdc7 ΔCt cancer tissue - Cdc7 ΔCt tumor-adjacent tissue. Relative expression level of the *huCdc7* gene was calculated using the $2^{-\text{Cdc7 }\Delta\Delta\text{Ct}}$ method.

Immunohistochemsitry

Streptavidin-peroxidase immunohistochemical staining was performed using a commercial kit according to the manufacturer's instructions.

Statistical analysis

Statistical analysis were performed using SPSS 10.0 software. Means between two groups were compared using the *t* test. *P* values < 0.05 were considered statistically significant.

RESULTS

huCdc7 mRNA expression

The relative expression levels of huCdc7 mRNA in colorectal cancer and tumor-adjacent normal colorectal tissues were 0.03675 ± 1.00 and 0.01199 ± 0.44, respectively. Statistical analysis indicated that the expression level of huCdc7 mRNA was significantly higher in colorectal cancer than in tumor-adjacent normal colorectal tissues ($P < 0.05$) (Figure 1).

huCdc7 protein expression

huCdc7-positive cells displayed brown granules in the nucleus. Tumor tissues contained many huCdc7-positive cells, whereas normal colorectal tissues contained very few positive cells (Figure 2).

DISCUSSION

Cdc7 is a serine/threonine kinase, and huCdc7 is expressed in all human tissues[1]. By forming complex with other molecules in the nucleus, huCdc7 can phosphorylate and activate chromosome-binding minichromosome

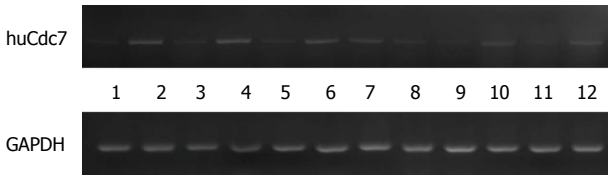

Figure 1 Semiquantitative reverse transcription-polymerase chain reaction determination of huCdc7 and glyceraldehyde-3-phosphate dehydrogenase mRNA expression in colorectal cancer (1, 3, 5, 7, 9 and 11) and tumor-adjacent normal colorectal tissues (2, 4, 6, 8, 10 and 12). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

maintenance complex (MCM) proteins. The MCM family has multiple members, including MCM2, MCM4 and MCM6. huCdc7 has the strongest ability to phosphorylate MCM2[2]. On one hand, MCMs function as helicase, a component of cell cycle initiation complex[3]. On the other hand, MCMs can act as important regulatory factors for S phase checkpoints to control cell cycle progression.

It is still unclear how huCdc7 mediates these processes. The expression of huCdc7 is tightly controlled by some factors and auxiliary proteins in normal cell cycle and maintained in a dynamic equilibrium state. In tumor cells, huCdc7 is abnormally expressed and excessively activated due to cell cycle disturbances. Hess *et al*[4] found that huCdc7 was overexpressed in tumor cells and excessive expression of huCdc7 promoted excessive MCM2 activation and abnormal proliferation of tumor cells. In addition, they found that huCdc7 was overexpressed in metastatic tumor cells, suggesting that tumor metastasis may be closely related to abnormal high huCdc7 expression. A previous study has revealed that CDC7 kinase is a predictor of survival and a novel therapeutic target in epithelial ovarian carcinoma[5]. Similar findings have also been reported in some studies on lymphoma[6].

In this study, we utilized semi-quantitative reverse transcription-PCR and immunohistochemistry to determine the expression of huCdc7 in colorectal cancer and tumor-adjacent normal colorectal tissues. We found that huCdc7 mRNA expression was significantly higher in colorectal cancer than in normal colorectal tissues. huCdc7 is a conservative serine/threonine kinase that is indispensable for DNA replication initiation. Abnormal high expression of huCdc7 will promote DNA replication, cause abnormal cell proliferation, and thereby lead to the occurrence of tumors.

To investigate the relationship between huCdc7 and tumor development and progression, Montagnoli *et al*[7] used the siRNA interference technology to suppress high huCdc7 expression in tumor cells. They found that the phosphorylation levels of MCM2 activation sites were greatly decreased, DNA replication initiation in tumor cells was restrained, and tumor cell growth slowed down. In addition, some researchers believe that high huCdc7 expression in tumor cells is related to p53 inactivation[8].

A study on ovarian cancer revealed that CDC7 kinase can predict survival and serve as a target for cancer treatment. Kim *et al*[9] found that siRNA-mediated inhibition

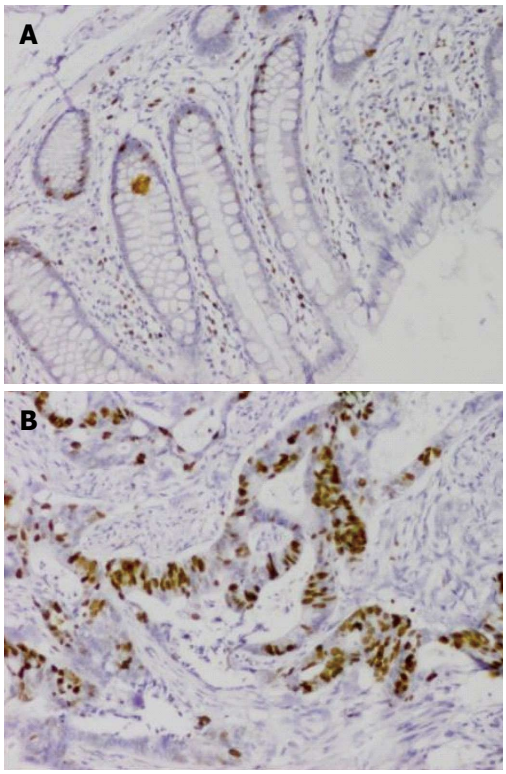

Figure 2 Immunohistochemical staining of huCdc7 in normal colorectal tissues (A) and colorectal cancer (B) (magnification, × 100).

of huCdc7 reduced the phosphorylation level of the N-terminus of MCM4. As a result, Cdc 45 could not be positioned to the chromosome and replication initiation complex could not form. This inhibited, to a certain extent, tumor cell proliferation. Thus, inhibition of huCdc7 activity can effectively inhibit tumor cell growth and promote tumor cell apoptosis, without damage to normal cells. A recent study has identified PHA-767491 as an inhibitor of huCdc7[10], which may be used to down-regulate abnormal huCdc7 expression to inhibit abnormal DNA replication and cell cycle progression in tumors.

Small molecule compounds that can interfere with the activity of CDC7 have been developed and proved to be effective in inhibiting tumor growth in animal models. Unprecedented attention has been paid to the inhibition of CDC7 kinase activity for suppressing DNA replication in tumor cells and inducing tumor cell apoptosis. Further elucidation of the role of huCdc7 in colorectal cancer may improve the treatment of this malignancy.

COMMENTS

Background

huCdc7 is ubiquitously expressed in human tissues and can regulate DNA replication initiation. Abnormal expression or excessive activation of huCdc7 can promote excessive cell proliferation and cause tumorigenesis. Colorectal cancer is a common digestive tract tumor and has a gradually increasing incidence.

Research frontiers

In this study, tissue specimens were collected from 39 surgically treated patients with colorectal cancer. Colorectal cancer tissue specimens and matched tumor-adjacent normal colorectal tissue specimens (5 cm away from tumor tis-

sue) were placed in liquid nitrogen immediately after surgical specimens were removed. The final diagnosis of colorectal cancer and tumor-adjacent normal colorectal tissues was made pathologically.

Innovations and breakthroughs

The relative expression level of huCdc7 mRNA in colorectal cancer was significantly higher than that in tumor-adjacent normal colorectal tissue. huCdc7-positive cells displayed brown granules in the nucleus. Tumor tissue contained many huCdc7-positive cells, whereas normal colorectal tissue contained very few positive cells.

Applications

The authors found that huCdc7 may play an important role in the development and progression of colorectal cancer.

Peer review

In the manuscript, the authors detected the expression of huCdc7 in colorectal cancer. The data is interesting. The manuscript is short, but informative.

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BRIEF ARTICLE

Nonalcoholic fatty liver disease and microvascular complications in type 2 diabetes

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Abstract

AIM: To evaluate the correlation between nonalcoholic fatty liver disease (NAFLD) and microvascular complications in type 2 diabetes mellitus (T2DM).

METHODS: Data were obtained from 1217 inpatients with T2DM (757 females, 460 males; aged 63.39 ± 12.28 years). NAFLD was diagnosed by hepatic ultrasonography. Diabetic nephropathy (DN), diabetic peripheral neuropathy (DPN), and diabetic retinopathy (DR) were diagnosed according to their respective criteria. The prevalence of NAFLD and the independent correlations of clinical characteristics with NAFLD were determined by cross-tabulation and logistic regression, respectively.

RESULTS: Approximately 61% of inpatients with T2DM in Qingdao, China had NAFLD, which decreased significantly with increase in age and prolonged course of diabetes. The prevalence of NAFLD in patients presenting with DN, DPN and DR was 49.4%, 57.2% and 54.9%, respectively. These rates were significantly lower than those of patients without DN, DPN and DR (65.9%, 65.6% and 66.1%, respectively, $P < 0.05$). Participants with NAFLD had greater body weight, waist circumference (WC), body mass index (BMI), fasting blood glucose (FBG), hemoglobin A1c, alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, blood pressure, as well as triglyceride (TG) levels and lower high-density lipoprotein (HDL) concentration than those without NAFLD ($P < 0.05$). NAFLD was positively correlated with BMI, WC, TG, FBG, diastolic blood pressure, and systolic blood pressure but negatively correlated with the duration of diabetes, DR, DPN, DN, and HDL.

CONCLUSION: Despite the benign nature of NAFLD, efforts should be directed toward early diagnosis, intensive blood glucose and blood pressure control, and effective dyslipidemia correction.

Key words: Nonalcoholic fatty liver disease; Type 2 diabetes mellitus; Diabetic nephropathy; Diabetic retinopathy; Diabetic neuropathy

Core tip: Nonalcoholic fatty liver disease (NAFLD) and diabetic microangiopathy complications represent important burdens for patients with type 2 diabetes mellitus (T2DM). However based on the finding that the prevalence of NAFLD was negatively correlated with age and duration of T2DM, we suggest that NAFLD is benign process and efforts should be directed at strengthening early diagnosis, intensive blood glucose

and pressure control, and effective dyslipidemia correction to prevent and minimize occurrence of NAFLD.

Lv WS, Sun RX, Gao YY, Wen JP, Pan RF, Li L, Wang J, Xian YX, Cao CX, Zheng M. Nonalcoholic fatty liver disease and microvascular complications in type 2 diabetes. *World J Gastroenterol* 2013; 19(20): 3134-3142 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3134.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3134

INTRODUCTION

Diabetes is an independent risk factor for the development of nonalcoholic fatty liver disease (NAFLD) and progression to advanced liver disease, including fibrosis, cirrhosis, and hepatocellular carcinoma[1]. Cross-sectional studies have reported that the prevalence of NAFLD in patients with type 2 diabetes mellitus (T2DM) ranges from 42.1% to 75.2% in China[2,3]. With the rising incidence and prevalence of T2DM in China[4], a close estimate of the prevalence of NAFLD, as well as its clinical risk factors, is important for predicting the number of patients that require monitoring for more advanced liver diseases, or those who may benefit from future disease-modifying agents.

Targher *et al*[5] have reported that most individuals with NAFLD are older, more likely to be male, and have a longer duration of diabetes than those without NAFLD. By contrast, Williamson *et al*[6] have reported that participants with definite steatosis (grade 3) are significantly younger and have shorter duration of diabetes than the combined normal/probably normal groups (grades 0-2). Zhou *et al*[3] have indicated that the prevalence of NAFLD in men aged < 50 years is higher than that in women. However, this finding is contrary for patients > 50 years; that is, the prevalence of NAFLD is higher in women[3]. Thus, further studies must be conducted to ascertain the differences in the prevalence of NAFLD between the sexes and the relationship between age, duration of diabetes, and prevalence of NAFLD.

The present cross-sectional study determined the prevalence and some risk factors for NAFLD and evaluated its correlations with microvascular complications in a large cohort of inpatients with T2DM in Qingdao, China. Differences in the prevalence of NAFLD between men and women, as well as the clinical and biochemical characteristics of the condition, are discussed.

MATERIALS AND METHODS

A total of 1217 T2DM patients (757 women and 460 men) participated in this study. All patients were hospitalized between January 2008 and January 2012 at the Department of Internal Medicine, Affiliated Hospital of Medical College, Qingdao University, China. Most participants abstained from alcohol consumption (n = 1107; 91%) or drank minimally (alcohol consumption < 20 g/d; n = 110; 9%). Patient information such as sex, birth date, duration of diabetes, daily alcohol consumption, smoking status, and medications (including hepatotoxic drugs such as glucocorticoids, amiodarone, methotrexate, or antineoplastic drugs) were obtained by the questionnaire method. The heights and body weights (BWs) of the patients were measured (*i.e.*, without wearing a heavy coat and shoes). Body mass index (BMI) was calculated by dividing the BW (kg) by the square of the height (m). Waist circumference (WC) was measured in a standing position at the level of the umbilicus. Fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), fasting serum triglyceride (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were determined using standardized methods in our laboratory. Serology for viral hepatitis B and C was also assessed in all participants. Hepatic ultrasonography scanning was performed on patients after an overnight fast by assigned and experienced radiologists, who were blinded to the health conditions of the patients. The liver was graded for markers of hepatic steatosis by using established criteria: a bright hepatic echo pattern (compared with the echo response of the right kidney); increased attenuation of the echo beam; and presence of focal fatty sparing[6]. Participants manifesting symptoms of hepatic steatosis or showing abnormal blood tests of liver function tests were further investigated for other parameters such as antinuclear antibody, antismooth muscle antibody, antimitochondrial antibody, and ferritin.

This study was approved by the Ethics Committee of the Affiliated Hospital Medical College, Qingdao University, and all participants provided written informed consent.

Definition of NAFLD

NAFLD was defined as the presence of definite hepatic steatosis on ultrasound scan (*i.e.*, grade 3) in the absence of a secondary cause for hepatic steatosis. Secondary causes were defined as: alcohol consumption ≥ 14 U/wk or participant report of alcohol excess; use of hepatotoxic medication (glucocorticoids, isoniazid, methotrexate, amiodarone, and tamoxifen) within 6 mo prior to the study; positive hepatitis B or C serology; ferritin concentration ≥ 1000 mg/L (milder hyperferritinemia can be associated with obesity, insulin resistance, and NAFLD); clinically significant positive immunology titers (antismooth muscle antibody titer ≥ 1:160 or antimitochondrial antibody titer ≥ 1:40); or previous diagnosis of a persistent secondary cause for chronic liver disease according to their medical records. Patients were excluded from calculations on the prevalence of NAFLD if their data on the above-mentioned measures were missing, such that a secondary cause could not be excluded.

Diabetic microvascular complications included dia-

Table 1 Baseline characteristics of the study participants, according to nonalcoholic fatty liver disease status

Variables	Without NAFLD	With NAFLD	*P* value
n	475	742	
Sex (female/male)	287/188	470/272	> 0.05
Age (yr)	65.11 ± 11.70	62.29 ± 12.52	< 0.05
Diabetes duration (yr)	11.31 ± 7.10	8.47 ± 6.87	< 0.05
Height (cm)	163.99 ± 8.49	164.20 ± 8.24	> 0.05
BW (kg)	65.54 ± 9.76	74.32 ± 11.64	< 0.05
WC (cm)	88.75 ± 8.72	96.76 ± 10.32	< 0.05
BMI (kg/m^2)	24.33 ± 2.87	27.49 ± 3.37	< 0.05
Current smokers (%)	17.7	14.3	> 0.05
SBP (mmHg)	131.43 ± 17.93	141.56 ± 16.15	< 0.05
DBP (mmHg)	76.94 ± 8.87	83.61 ± 9.57	< 0.05
FBG (mmol/L)	7.72 ± 3.18	8.97 ± 3.20	< 0.05
HbA1c (%)	8.45 ± 2.44	8.91 ± 2.47	< 0.05
ALT (mmol/L)	17.16 ± 8.19	25.72 ± 13.01	< 0.05
AST (mmol/L)	18.23 ± 6.74	22.34 ± 9.00	< 0.05
GGT (mmol/L)	19.47 ± 16.32	25.05 ± 17.94	< 0.05
TG (mmol/L)	1.45 ± 1.06	2.12 ± 1.43	< 0.05
TC (mmol/L)	4.99 ± 1.41	5.04 ± 1.22	> 0.05
LDL (mmol/L)	2.95 ± 0.92	2.95 ± 0.82	> 0.05
HDL (mmol/L)	1.24 ± 0.29	1.17 ± 0.28	< 0.05

Data are expressed as mean ± SD or proportions of the entire cohort of participants. BW: Body weight; WC: Waist circumference; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; TG: Triglyceride; TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

betic nephropathy (DN), diabetic peripheral neuropathy (DPN), and diabetic retinopathy (DR). DN was diagnosed by a positive persistent proteinuria for at least three consecutive readings per year, serum creatinine > 130 μmol/L, and/or glomerular filtration rate < 60 mL/min. DPN was diagnosed in the presence of persistent numbness, paresthesia, loss of hearing of the tuning fork and sense of vibration, and failure to elicit a knee and/or ankle jerk. DR was diagnosed in the presence of retinal hemorrhage, exudates, and macular edema[7].

Statistical analysis

Analyses were conducted using SPSS version 17.0. Data are presented as means ± SD or proportions, where appropriate. Skewed variables (*e.g.*, TG and HDL) were logarithmically transformed, and genomic means are presented. Independent *t* and χ^2 tests were used to compare the differences in means or proportions between different subgroups. The independent associations of variables with NAFLD were determined by a binary logistic regression. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and biological characteristics of study population

The mean demographic and clinical characteristics of the patients were as follows: age, 63.39 ± 12.28 years; duration of diabetes, 9.58 ± 7.09 years; BW, 70.9 ± 11.75 kg; WC, 93.63 ± 10.48 cm; BMI, 26.26 ± 3.54 kg/m^2; FBG, 8.48 ± 3.25 mmol/L; HbA1c, 8.73% ± 2.47%; TG, 1.85 ± 1.33 mmol/L; total cholesterol (TC), 5.0 ± 1.29 mmol/L; HDL, 1.20 ± 0.29 mmol/L; and LDL, 2.95 ± 0.86 mmol/L.

The baseline characteristics of the study participants according to their NAFLD status are presented in Table 1. The prevalence of NAFLD in patients with T2DM was 61%, and no statistical difference was indicated between men and women (59.1% *vs* 62.1%, $P > 0.05$). Participants with NAFLD had higher BW, WC, BMI, FBG, HbA1c, ALT, AST, GGT, blood pressure and TG levels, and lower HDL concentration than those without NAFLD ($P < 0.05$). Meanwhile, no statistical difference was observed in the smoking history, height, plasma TC, and LDL levels between the groups. However, participants diagnosed with NAFLD were younger and had shorter duration of diabetes than those without NAFLD ($P < 0.05$).

Prevalence of NAFLD in T2DM patients by age range and duration of diabetes

Patients were divided into three groups according to age: < 50, 50-60, and > 60 years. The prevalence rates of NAFLD for the three age groups were 73.4%, 61.6%, and 57.8%, respectively, indicating that the prevalence of NAFLD decreased significantly as age increased (Figure 1A, $P < 0.05$). This trend persisted even when patients were classified by sex (74.5%, 57.5%, and 54.2% in men and 72.3%, 65.1%, and 59.6% in women, $P < 0.05$). Although the prevalence of NAFLD in women was higher than that in men < 50 years and the reverse was evident at > 50 years, no significant difference was found in the prevalence of NAFLD between men and women at different ages (Figure 1A, $P > 0.05$).

When patients were divided into three groups according to duration of diabetes, that is, < 5 years, 5-10 years, and > 10 years, the prevalence rates of NAFLD were 74.9%, 65.5%, and 50.7%, respectively. This indicates that the prevalence of NAFLD decreased significantly as diabetes was prolonged (Figure 1B, $P < 0.05$). This trend persisted even when patients were classified by sex (72.3%, 65.6%, and 47.1% in men and 76.5%, 65.4%, and 52.6% in females, respectively, $P < 0.05$). No significant difference was indicated in the prevalence rate of NAFLD between men and women with different durations of diabetes (Figure 1B, $P > 0.05$).

Prevalence of NAFLD in T2DM with or without microangiopathic complications

The incidence of NAFLD was found to correlate negatively with DN, as shown in Figure 2A (r = -0.154, $P < 0.05$). The general prevalence of NAFLD in T2DM with and without DN was 49.4% and 65.9%, respectively ($P < 0.05$). This trend persisted even when patients were stratified by sex (r = -0.13 in men, r = -0.17 in women,

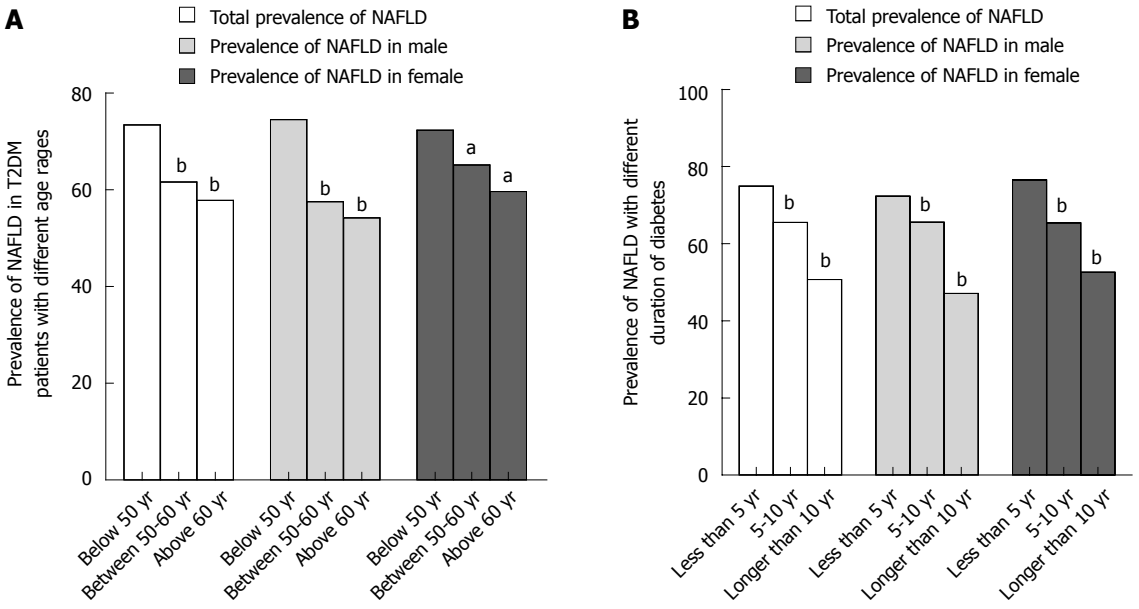

Figure 1 Prevalence of nonalcoholic fatty liver disease in type 2 diabetes mellitus patients according to different age ranges and durations of diabetes. A: Prevalence of nonalcoholic fatty liver disease (NAFLD) in patients aged < 50, 50-60, and > 60 years was 73.4%, 61.6%, and 57.8%, respectively, indicating that the prevalence of NAFLD decreased significantly with increasing age ($^{b}P < 0.01$ *vs* group below 50 years). In men (74.5%, 57.5%, and 54.2%, respectively, $^{b}P < 0.01$ *vs* group below 50 years) and women (72.3%, 65.1%, and 59.6%, respectively, $^{a}P < 0.05$ *vs* group below 50 years), the prevalence of NAFLD decreased significantly with increasing age. The prevalence of NAFLD in men was higher than in women (74.5% and 72.3%, respectively, $P > 0.05$) in the < 50 years age group; however, the reverse was evident in the > 50 years age group (57.5% and 65.1%, respectively, in the 50-60 years age group, and 54.2% and 59.6%, in the > 60 years age group, both $P > 0.05$); B: Prevalence of NAFLD, according to duration of diabetes, was 74.9%, 65.5%, and 50.7% for < 5, 5-10, and > 10 years, respectively. Prevalence of NAFLD decreased significantly with prolonged course of diabetes ($^{b}P < 0.01$ *vs* group less than 5 years). This trend persisted even when patients were stratified by sex (72.3%, 65.6%, and 47.1%, respectively, in men, and 76.5%, 65.4%, and 52.6% in women, $^{b}P < 0.01$ *vs* group less than 5 years). There were no significant differences in the prevalence of NAFLD between men and women with different durations of diabetes ($P > 0.05$). T2DM: Type 2 diabetes mellitus.

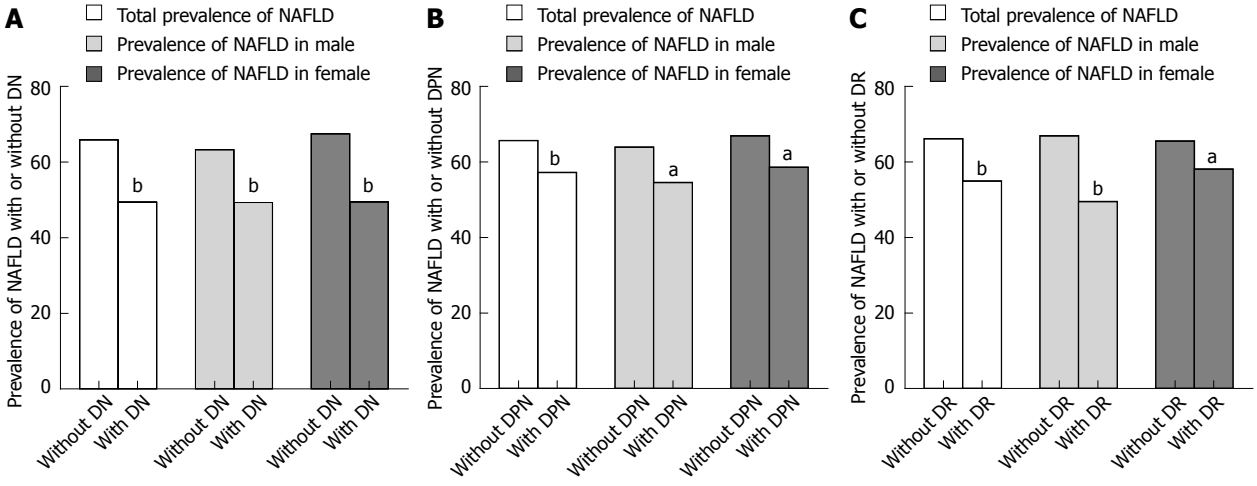

Figure 2 Prevalence of nonalcoholic fatty liver disease in T2DM with or without microangiopathy complications. A: Incidence of nonalcoholic fatty liver disease (NAFLD) negatively correlated with DN (r = -0.154, $P < 0.05$). This trend persisted even when patients were stratified by sex (r = -0.13 in men, r = -0.17 in women, both $P < 0.05$). Prevalence of NAFLD in T2DM with and without DN was 49.4% and 65.9%, respectively ($^{b}P < 0.01$ *vs* group without DN). Prevalence of NAFLD in men without DN was significantly higher than in patients with DN (63.3% and 49.3%, respectively, $^{b}P < 0.01$ *vs* the group without DN). Prevalence of NAFLD in women without DN was significantly higher than in patients with DN (67.5% and 49.5%, respectively, $^{b}P < 0.01$ *vs* the group without DN); B: Incidence of NAFLD negatively correlated with DPN (r = -0.086, $P < 0.05$). This trend persisted even when patients were stratified by sex (r = -0.095 in men, r = -0.084 in women, both $P < 0.05$). The general prevalence of NAFLD in T2DM with and without DPN was 57.2% and 65.6%, respectively ($^{b}P < 0.01$ *vs* the group without DPN). Prevalence of NAFLD in men without DPN was significantly higher than in patients with DPN (63.9% and 54.5%, respectively, $^{a}P < 0.05$ *vs* the group without DPN). Prevalence of NAFLD in women without DPN was also significantly higher than that in patients with DPN (66.9% and 58.6%, respectively, $^{a}P < 0.05$ *vs* the group without DPN); C: Incidence of NAFLD negatively correlated with DR (r = -0.114, $P < 0.05$). This trend persisted even when patients were stratified by sex (r = -0.176 in men, r = -0.076 in women, both $P < 0.05$). Prevalence of NAFLD in T2DM with and without DR was 54.9% and 66.1%, respectively ($^{b}P < 0.01$ *vs* the group without DR). Furthermore, the Prevalence of NAFLD in men without DR was significantly higher than that in patients with DR (66.9% and 49.5%, respectively, $^{b}P < 0.01$ *vs* the group without DR). Prevalence of NAFLD in women without DR was also significantly higher than that in patients with DR (65.5% and 58.1%, respectively, $^{a}P < 0.05$ *vs* the group without DR).

Table 2 Risk factors for nonalcoholic fatty liver disease

Variables	β	SE	*P* value	OR	95%CI
Total patients with T2DM					
logBMI	16.33	2.24	0.00	1.238×10^7	154915.06-9.897×10^8
logWC	5.57	2.57	0.03	261.45	1.69-40422.03
logTG	2.05	0.34	0.00	7.79	3.99-15.19
FBG	0.079	0.03	0.01	1.08	1.02-1.15
DBP	0.03	0.01	0.00	1.03	1.01-1.05
SBP	0.03	0.01	0.00	1.03	1.02-1.04
Duration of diabetes	-0.05	0.01	0.00	0.96	0.93-0.98
DR	-0.035	0.17	0.04	0.71	0.51-0.99
DPN	-0.38	0.17	0.03	0.69	0.50-0.95
DN	-1.15	0.19	0.00	0.32	0.22-0.46
logHDL	-1.76	0.84	0.04	0.17	0.03-0.89
Male patients with T2DM					
logBMI	24.53	3.37	0.00	4.52×10^{10}	6.128×10^7-3.334×10^{13}
logTG	3.63	0.62	0.00	37.88	11.34-126.61
DBP	0.08	0.02	0.00	1.09	1.05-1.12
Duration of diabetes	-0.07	0.02	0.00	0.94	0.90-0.98
logHDL	-4.92	1.72	0.00	0.01	0.00-0.21
Female patients with T2DM					
logBMI	16.91	2.05	0.00	2.21×10^7	395927.04-1.23×10^9
logTG	1.25	0.42	0.00	3.48	1.54-7.90
FBG	0.2	0.04	0.00	1.22	1.13-1.31
SBP	0.04	0.01	0.00	1.04	1.03-1.05
Duration of diabetes	-0.04	0.01	0.00	0.96	0.93-0.98
DPN	-0.44	0.21	0.04	0.65	0.43-0.98
DR	-0.49	0.21	0.02	0.61	0.41-0.93
DN	-1.23	0.23	0.00	0.29	0.19-0.46

T2DM: Type 2 diabetes mellitus; WC: Waist circumference; TG: Triglyceride; BMI: Body mass index; FBG: Fasting blood glucose; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HDL: High-density lipoprotein; DR: Diabetic retinopathy; DPN: Diabetic peripheral neuropathy; DN: Diabetic nephropathy.

both $P < 0.05$). The prevalence of NAFLD in men without DN was significantly higher than that of patients with DN (63.3% and 49.3%, respectively, $P < 0.05$). Similarly, the prevalence of NAFLD in women without DN is significantly higher than that of patients with DN (67.5% and 49.5%, respectively, $P < 0.05$).

The incidence of NAFLD was found to correlate negatively with DPN, as shown in Figure 2B (r = -0.086, $P < 0.05$). The general prevalence of NAFLD in T2DM with and without DPN was 57.2% and 65.6%, respectively ($P < 0.05$). This trend also persisted when patients were stratified by sex (r = -0.095 in men, r = -0.084 in women, both $P < 0.05$). The prevalence of NAFLD in men without DPN was significantly higher than that of patients with DPN (63.9% and 54.5%, respectively, $P < 0.05$). Similar results were found in women (66.9% and 58.6%, respectively, $P < 0.05$).

The incidence of NAFLD was negatively correlated with DR, as shown in Figure 2C (r = -0.114, $P < 0.05$). The general prevalence of NAFLD in T2DM with and without DR was 54.9% and 66.1%, respectively ($P < 0.05$). This trend persisted even when patients were stratified by sex (r = -0.176 in men, r = -0.076 in women, both $P < 0.05$). The prevalence of NAFLD in men without DR was significantly higher than that of patients with DR (66.9% and 49.5%, respectively, $P < 0.05$). Similar results were determined in women (65.5% and 58.1%, respectively, $P < 0.05$).

Risk factors for the development of NAFLD in patients with T2DM

With NAFLD as the dependent variable, the variables that were independently associated with NAFLD in T2DM patients were identified by binary stepwise logistic regression. Independent predictors of NAFLD were BMI (OR for log BMI: 1.238×10^7; 95%CI: 154915.06-9.897×10^7), WC (OR for log WC: 261.44; 95%CI: 1.691-4.422.03), TG (OR for log TG: 7.79; 95%CI: 3.99-15.19), FBG (OR = 1.08; 95%CI: 1.02-1.15), diastolic blood pressure (DBP) (OR = 1.03; 95%CI: 1.01-1.05), systolic blood pressure (SBP) (OR = 1.03; 95%CI: 1.021-1.04), duration of diabetes (OR = 0.96; 95%CI: 0.93-0.98), DR (OR = 0.71; 95%CI: 0.51-0.99), DPN (OR = 0.69; 95%CI: 0.50-0.95), DN (OR = 0.32; 95%CI: 0.22-0.46), and HDL (OR for log HDL: 0.17; 95%CI: 0.03-0.89) (Table 2).

However, the risk factors for NAFLD in men and women were not identical. Independent predictors of NAFLD in men were BMI (OR for log BMI: 4.52×10^{10}; 95%CI: 6.12×10^7-3.33×10^{13}), TG (OR for log TG: 37.88; 95%CI: 11.34-126.61), DBP (OR = 1.09; 95%CI: 1.05-1.12), duration of diabetes (OR = 0.94; 95%CI: 0.90-0.98), and HDL (OR: for log HDL: 0.01; 95%CI: 0-0.21) (Table 2). Independent predictors of NAFLD in women were BMI (OR for log BMI: 2.21×10^7; 95%CI: 395 927.04-1.23×10^9), TG (OR for log TG: 3.48; 95%CI: 1.54-7.90), FBG (OR = 1.22; 95%CI: 1.13-1.31), SBP (OR = 1.04; 95%CI: 1.03-1.05), duration of diabetes (OR = 0.96; 95%CI: 0.93-0.98), DPN (OR = 0.65; 95%CI: 0.43-0.98), DR (OR = 0.61; 95%CI: 0.41-0.93), and DN (OR = 0.29; 95%CI: 0.19-0.46) (Table 2).

DISCUSSION

NAFLD represents an important burden of disease for patients with T2DM; however, the magnitude of the problem is currently unknown. In this study, the prevalence of NAFLD in hospitalized Chinese T2DM patients was calculated at 61%. This prevalence is lower than that reported by Lu *et al*[2] in 2009 (75.18%) but markedly higher compared with that reported by Zhou *et al*[3] conducted 5 years ago (42.1%).

A limitation of this study was that the diagnosis of NAFLD was based on ultrasound imaging. The patients did not undergo liver biopsy and histological examination, which is the gold standard technique for identifying steatosis. The sensitivity of ultrasonography in detecting

steatosis varies between 60% and 94% and is dependent on the degree of steatosis. In particular, sensitivity is significantly low when the degree of steatosis is < 30%[5,8]. Furthermore, some participants with normal ultrasound scans may have undiagnosed hepatic fibrosis and thus be considered a severe case of NAFLD[6]. Therefore, the cases that were potentially misclassified by ultrasonography might have resulted in an underestimated prevalence of NAFLD. This limitation might have attenuated the magnitude of our effect measures to null. The findings of this study may then be considered conservative estimates of the prevalence of NAFLD in hospitalized Chinese T2DM patients.

This study revealed that the prevalence of NAFLD in male hospitalized T2DM patients was 59.1%, which was slightly lower than that in female patients (62.1%), but without statistical significance. When patients were divided into three groups according to their age (*i.e.*, < 50, 50-60, and > 60 years), the prevalence of NAFLD in women was higher than in men aged < 50 years, whereas the reverse was evident when age was > 50 years. However, no significant difference was indicated in the prevalence of NAFLD between male and female patients at different ages. This finding differs from that of Zhou *et al*[3], who reported that the prevalence of fatty liver in patients < 50 years old was significantly higher in men than women; however, the reverse was evident when participants were older than 50 years. In the present study, several male patients with NFALD, especially those aged < 50 years, were excluded for overdrinking. With this factor considered, the prevalence of NAFLD in male hospitalized T2DM patients may be significantly higher than that of female patients.

In our study, patients were assessed according to three age groups. The prevalence rates of NAFLD significantly decreased as age increased. This trend persisted even when the patients were stratified by sex. These observations are inconsistent with those of Targher *et al*[5], who reported that the prevalence of NAFLD increased with age (*i.e.*, 65.4% among participants aged 40-59 years and 74.6% among those aged ≥ 60 years; $P < 0.001$). By contrast, Lu *et al*[2] found that the prevalence of NAFLD in Chinese T2DM patients did not increase with age. In particular, T2DM patients in China aged 40-59 years are more prone to NAFLD. These findings are consistent with those published in a review by Duvnjak *et al*[9], which reports that the highest prevalence of NAFLD occurs in those aged 40-60 years. A potential explanation for these observations is that middle-aged persons are often too busy to participate in physical exercise and are more likely to dine outside of the home, whereas older and retired persons may have more time for physical exercise and devote more attention to their lifestyle. These behaviors among middle-aged persons may be one reason why Chinese patients with T2DM are more vulnerable to NAFLD, considering that the prevalence of NAFLD does not increase with age[2].

Patients were also assessed according to the duration of diabetes (*i.e.*, < 5, 5-10, and > 10 years). The prevalence rates of NAFLD decreased significantly with the prolonged course of diabetes. This trend persisted even when patients were stratified by sex. No significant difference was observed in the prevalence of NAFLD between male and female patients with different durations of diabetes. These findings contradict those of Banerjee *et al*[10], who found that a longer duration of T2DM was significantly associated with NAFLD. The association of a shorter duration of diabetes with liver disease has been previously described. A possible explanation for this association may be that a greater degree of hyperinsulinemia in early T2DM increases the uptake of free fatty acids (FFAs) by hepatocytes[6]. Popović *et al*[11] presented a significant negative correlation between the duration of diabetes and fasting insulinemia as well as insulin resistance assessed by homeostatic model assessment-insulin resistance index. Increased fasting insulinemia and hepatic insulin resistance may trigger a reduced catabolism of lipoproteins rich in TGs and an increased hepatic very LDL production via changes in the rate of apolipoprotein B synthesis and degradation, as well as *de novo* lipogenesis or increased FFA flux from adipose tissue into the liver[12].

In this study, we demonstrated that the general prevalence of diabetic microangiopathy complications (*i.e.*, DN, DPN and DR) increased significantly with age increased (data not shown). This trend persisted even when the patients were stratified by sex. When the patients were stratified by duration of T2DM, the prevalence rates of diabetic microangiopathy complications increased significantly with prolonged duration of T2DM (data not shown). In previous reports, the age and duration of diabetes were common risk factors for the three microvascular complications of T2DM[13-17]. Thus, chronic microvascular diabetic complications increase gradually with prolonged duration of T2DM, and the clinical symptoms tend to worsen progressively. Consequently, these patients need to pay more attention to their lifestyle and drug therapy. Therefore, the incidence of NAFLD does not increase with a prolonged duration of T2DM.

The development of NAFLD is a multifaceted cascade of physiological and biochemical events, including genetic, environmental, metabolic, and stress-related factors; the exact risk factors for NAFLD have not been clearly identified[18]. On the basis of the univariate analysis results, patients diagnosed with NAFLD were significantly younger and had a shorter duration of diabetes than those without NAFLD. The BW, WC, BMI, SBP, DBP, FBG, HbA1c, and TG were significantly higher, and HDL cholesterol was significantly lower in patients with NAFLD (Table 1, $P < 0.05$). Multivariate analysis identified the following as independent pre-

dictors of NAFLD: BMI, WC, TG, FBG, DBP, SBP, duration of diabetes, DR, DPN, DN, and HDL. This observation is consistent with the findings reported in literature that the prevalence of NAFLD is closely associated with obesity, dyslipidemia, hypertension, and glucose intolerance, a cluster of metabolic disorders that is now recognized as metabolic syndrome[10,18,19]. However, the negative correlations between the prevalence of NAFLD and the duration of diabetes, DR, DPN, and DN identified in this study are inconsistent with the data reported in previous studies[10]. Given the negative correlations between the prevalence of NAFLD as well as the duration of diabetes and diabetic chronic microvascular complications, more attention should be given to lifestyle and drug therapy, particularly in patients presenting an aggravation in any of the clinical symptoms mentioned above.

Despite the difference in the independent risk factors for NAFLD between men (Table 2) and women (Table 2), both positively correlated with BMI, TG, and hypertension and a negative correlation with the duration of diabetes. In men, the prevalence of NAFLD positively correlated with DBP but negatively with HDL (Table 2). In women, the prevalence of NAFLD positively correlated with FBG and SBP but negatively with DPN, DR and DN (Table 2). According to a previous study, SBP represents the most prevalent type in obese women, being a stronger predictor of cardiovascular disease than DBP[20]. Therefore, the regulation of SBP is more critical for women.

Although most patients with NAFLD presented with non-progressive bland steatosis, few others developed a histological subtype of nonalcoholic steatohepatitis (NASH), which can develop to cirrhosis, hepatocellular carcinoma, and liver-cancer-related death[9,21,22], especially for NASH patients with T2DM[21,23,24]. Thus, specialists have considered that NAFLD may not be a completely benign disorder[9,25]. On the contrary, we consider NAFLD a benign condition because of the negative relationship between the prevalence of NAFLD with age and duration of T2DM. No treatment to alleviate NAFLD or prevent its progression has been scientifically proven. However, various therapeutic alternatives aimed at interfering with the risk factors involved in the pathogenesis of this disorder have been applied to prevent the progression of NAFLD to end-stage liver disease. The most important therapeutic measure is to increase insulin sensitivity by motivating patients to change their lifestyle habits, particularly by diet modification and participation in physical activities to lose weight[26,27].

Tushuizen *et al*[28] reported that after 44 wk of exenatide therapy, patients experienced a 73% reduction in hepatic fat content (*i.e.*, from a baseline of 15.8% to 4.3%), as evaluated by proton magnetic resonance spectroscopy. This finding is consistent with that reported by Ding *et al*[29] on the reduced hepatic lipid content in exenatide-treated ob/ob or obese mice compared with placebo-treated mice. Lazo *et al*[30] reported that a 12-mo intensive lifestyle intervention in patients with T2DM significantly reduced steatosis and NAFLD. In addition, Lo *et al*[24] have reported that diet-induced NASH fibrosis is exacerbated by diabetes and attenuated by insulin therapy. On the basis of these findings, we believe that NAFLD is reversible, and patients may benefit from its early diagnosis and treatment[31].

In conclusion, the results of this study suggest that NAFLD is extremely common in people with T2DM, positively correlates with BMI, WC, TG, FBG, DBP, and SBP, and negatively correlates with the duration of diabetes, DR, DPN, DN, and HDL. Given that hepatic fat content can be reversed with lifestyle changes and drugs, NAFLD should be included in future preventive public health initiatives, and the affected individuals should be motivated to adopt a healthier lifestyle[27]. Patients with T2DM should be always assessed for NAFLD to ensure early diagnosis and entry into proper and thorough medical care. Future efforts should be directed toward strengthening early diagnosis, intensive blood glucose and blood pressure control, and effective dyslipidemia correction to prevent and minimize the occurrence of NAFLD.

COMMENTS

Background

Diabetes is an independent risk factor for the development of nonalcoholic fatty liver disease (NAFLD) and its progression to more advanced liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma. NAFLD and diabetic microangiopathy complications represent important burdens of disease for patients with type 2 diabetes mellitus (T2DM), Previous studies have shown that the prevalence of NAFLD and microangiopathy complications in patients with T2DM significantly correlate with age and duration of T2DM. However, few studies have focused on the relationship between NAFLD and diabetic microangiopathy complications.

Research frontiers

Many studies have reported that individuals with NAFLD are older, more likely to be male, and have a longer duration of diabetes than those without NAFLD. However, recently, conflicting results have shown that participants with definite steatosis are significantly younger and have shorter durations of diabetes than the combined normal/probably normal groups. Further studies are required to ascertain the differences in the prevalence of NAFLD between the sexes, and the relationships between age, duration of diabetes, and prevalence of NAFLD.

Innovations and breakthroughs

The relatively larger cross-sectional study of inpatients with T2DM in Qingdao, China showed that the prevalence of NAFLD was 61%. However, based on the finding that the prevalence of NAFLD was negatively correlated with age and duration of T2DM, the authors believe that NAFLD is a benign process.

Applications

Our results suggest that patients with T2DM should be always assessed for NAFLD to ensure early diagnosis and entry into proper and thorough medical care. Future efforts should be directed at strengthening early diagnosis, intensive blood glucose and pressure control, and effective dyslipidemia correction to prevent and minimize the occurrence of NAFLD.

Terminology

NAFLD is defined as the presence of definite hepatic steatosis on ultrasound scan in the absence of a secondary cause for hepatic steatosis. Diabetic microvascular complications included diabetic nephropathy (DN), diabetic peripheral neuropathy (DPN), and diabetic retinopathy (DR). DN is defined by increased urinary albumin excretion in the absence of urinary tract infection or other renal abnormalities. DPN is diagnosed in the presence of persistent numbness, par-

esthesia, loss of hearing of the tuning fork and sense of vibration, and failure to elicit a knee and/or ankle jerk. DR is diagnosed in the presence of retinal hemorrhages, exudates, and macular edema.

Peer review

This was a good descriptive study in which the authors evaluated the correlation between NAFLD and microvascular complications in Chinese T2DM patients. The results are interesting and suggest that NAFLD is likely a benign process and efforts should be directed at early diagnosis, intensive blood glucose and blood pressure control, and effective dyslipidemia correction, which is of clinical interest.

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BRIEF ARTICLE

Value of circulating cell-free DNA in diagnosis of hepatocelluar carcinoma

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Abstract

AIM: To investigate the value of combined detection of circulating cell-free DNA (cfDNA), α-fetal protein (AFP) and α L-fucosidase (AFU) for diagnosis of hepatocellular carcinoma (HCC).

METHODS: Serum samples from 39 HCC patients and 45 normal controls were collected. Branched DNA (bDNA) was used to detect the level of cfDNA, and a receiver operating characteristic curve was employed to evaluate the diagnostic sensitivity, specificity, accuracy, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio and Youden index, and to assess the diagnostic efficiency and their correlations with the clinicopathological features. AFP and AFU were detected by chemiluminescence and colorimetry, respectively. The significance of combined detection of the three biomarkers was discussed.

RESULTS: cfDNA level was increased in 22 of the 39 HCC samples and in 2 of the 45 normal controls. cfDNA level in HCC samples was significantly higher than that in normal controls ($P < 0.05$). There were significant differences in sex and extra- and intrahepatic metastasis ($P < 0.05$). There was no significant correlation between cfDNA, AFP and AFU in the detection of HCC. The sensitivity of combined detection of cfDNA with one marker (AFP or AFU) and cfDNA with two markers (AFP and AFU) was 71.8%, 87.2% and 89.7% *vs* 56.4%, 53.8% and 66.7% for cfDNA, AFP and AFU used alone, respectively, the difference being statistically significant ($P < 0.05$).

CONCLUSION: Quantitative analysis of cfDNA is sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both could improve the diagnostic sensitivity for HCC.

Key words: Alu; Branched DNA; Circulating cell free DNA; Diagnosis; Hepatocellular carcinoma

Core tip: Various techniques are available to quantify serum circulating free cell DNA (cfDNA), some of these techniques require abstraction and purification as there is only a very small amount of cfDNA in the serum. This study investigates the value of combined detection of cfDNA, α-fetal protein (AFP) and α L-fucosidase (AFU) for diagnosis of hepatocellular carcinoma. Quantitative analysis of cfDNA is sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both could improve the diagnostic sensitivity for hepatocellular carcinoma.

Chen K, Zhang H, Zhang LN, Ju SQ, Qi J, Huang DF, Li F, Wei Q, Zhang J. Value of circulating cell-free DNA in diagnosis of hepatocelluar carcinoma. *World J Gastroenterol* 2013; 19(20): 3143-3149 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3143.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3143

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, with an incidence which ranks third and mortality which ranks second in all malignant tumors. The one-year survival in patients with HCC without surgical ablation is less than 30%, and the 5-year recurrence rate is about 80%, even in patients who undergo radical resection. Therefore, the key to HCC treatment is early detection and diagnosis[1,2]. Existing biomarkers for the diagnosis of HCC are alpha-fetal protein (AFP), γ-glutamyltranspeptidase isoenzymes Ⅱ (GGT-Ⅱ), α L-fucosidase (AFU) and acidic isoferritin[3], of which AFP remains the most specific marker due to its recognized effect in the diagnosis of HCC and assessment of the curative effect, prognosis and recurrence. However, not all HCC patients are diagnosed by AFP alone as the false negative rate of AFP is about 30%. Markers like AFU and GGT-Ⅱ have assisted in diagnosing patients with HCC whose AFP levels are negative, but they can not replace AFP in the diagnosis of HCC. Therefore, there is an urgent need to find a new biomarker which is superior or at least equivalent to AFP for the diagnosis of HCC.

Cell-free fetal DNA (cfDNA) has been extensively studied over the past few decades. Supported by theory and observation, two major sources of cfDNA have been postulated: first, fragmented DNA released as a consequence of cell death (apoptotic/necrotic) and, second, active metabolic secretion of DNA from cells. Considerable research effort has been made on the use of cfDNA as a biomarker in cancer diagnosis. cfDNA from malignancies exhibits characteristic changes such as mutations, deletions, methylations and microsatellite aberrations which are distinct from those in benign conditions, and thus may be useful in the diagnosis of cancer[4].

This study was designed to validate a bDNA-based Alu assay for quantifying human cfDNA in blood, and explore the feasibility of Alu quantification for HCC screening.

MATERIALS AND METHODS

Patients and samples

Included in this study were 39 patients randomly selected from those who had been diagnosed with HCC by clinical and radiographic evaluations at the Affiliated Hospital of Nantong University (Nantong, China) between May and October 2011. They included 32 males and 7 females ranging in age from 40 to 85 years. Forty-five healthy volunteers (36 males and 9 females; age range 28-83 years) who were negative for physical indices following health examination and documented to have normal liver biochemistry served as controls. Fresh blood was collected in serum separator tubes (Vacuette, Austria) coated with a clot activator and barrier gel, and EDTA tubes (Kangshi, China) for plasma, were centrifuged at 1600 × *g* for 10 min, and then microcentrifuged at 16000 × *g*. The separated serum was immediately stored at -80 ℃. The samples tested were diluted using the standard human genomic DNA qualification kit according to the manufacturer's instructions, with an initial concentration of 2.73×10^5.

Methods

Quantitation of cfDNA: Ten μL diluted serum was added to lysis solution to a final volume of 100 μL containing 50%Lysis Mixture (Panomics) and 1 g/L proteinase K. Target gene probe sets containing 250, 250 and 250 fmol of capture extender probe (CE), blocking probe and label extender probe (LE) were added to the blood lysate. The lysate was then transferred to an assay well in a 96-well plate (Panomics, Redwood City, CA, United States) covalently coated with capture probe (CP) oligo, incubated at 55 ℃ for 1 h, washed 3 times with 200 μL wash buffer (0.1 × standard 4 saline citrate containing 0.3 g/L lithium lauryl sulfate), sequentially hybridized with 100 μL 1:1000 dilution of bDNA preamplifier (amplifier) (Panomics) at 55 ℃ for 30 min and then with 100 μL (50 fmol) 3'-alkaline phosphatase-conjugated Label Probe oligo (Panomics) at 50 ℃ for 30 min, and then washed three times. After the final wash, the alkaline phosphatase substrate dioxetane (Panomics) was added to the wells and incubated at room temperature for 5-10 min to detect luminescent signals using an Lmax microtiter plate luminometer (a molecular device). The reagent for the QuantiGene 2.0 DNA Assay was provided by Professor Zhang Lurong from Florida State University, United States.

In the same 96-well-plate, samples with a known amount of Human Genomic DNA were added as follows: 0, 50, 100, 200 and 400 ng/mL, respectively. A standard curve was mapped out by the computer. The standard Human Genomic DNA solution was obtained from Promega, Madison, United States.

Quantitation of AFP: The concentration of AFP in the serum samples from HCC patients was measured manually with the ARCHITECT AFP Reagent Kit (Abbott, Chicago, IL, United States) according to the calibrator dose response curve by the chemiluminescence method using an Abbott 2000 immunoassay instrument.

Quantitation of AFU: The activity of AFU in the serum samples from HCC patients was measured manually with a Shanghai Institute of Naval Medicine Biotechnology Center Kit (Shanghai, China) by a colorimetric method using a BA-88 semi-automatic biochemical analyzer.

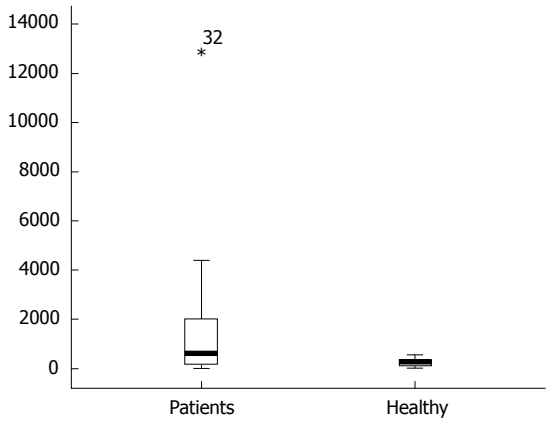

Figure 1 Circulating cell-free DNA levels in 39 hepatocellular carcinoma patients and 45 normal controls.

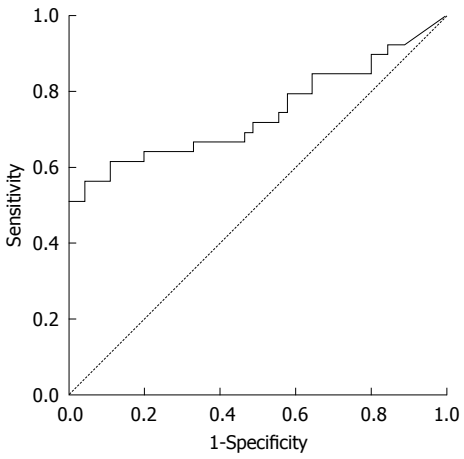

Figure 2 Receiver operating characteristic curve of serum circulating cell-free DNA in 39 hepatocellular carcinoma patients and 45 normal controls.

Interpretation of the results

Reference value of cfDNA: The content of cfDNA in each sample was calculated according to the standard curve. A receiver operating characteristic (ROC) curve was drawn to select the Youden's index[5], and the highest sensitivity and specificity (Youden's index = sensitivity + specificity-1) were selected as the cut-off values. A value greater than or equal to the cut-off value was regarded as positive, and smaller values were regarded as negative.

Reference value of AFP: According to the clinical diagnosis and staging criteria of HCC of the Chinese Anti-Cancer Association, a content of AFP greater than or equal to 400 ng/mL is considered positive, and a content smaller than this value is negative, excluding cases of pregnancy, embryonic tumors of the reproductive system, active liver disease and metastatic liver cancer.

Reference value of AFU: According to the reagent kit manual, the activity of serum AFU = (A determination tube-A control tube)/average A standard tube × 400, a value greater than 650 μmol/L per hour is considered positive, 550-650 μmol/L per hour is suspicious, and less than 550 μmol/L per hour is negative.

Statistical analysis

Statistical analysis of the data was performed using the SPSS 13.0 software package. As the results of the concentrations of cfDNA, AFP and AFU in each group were normally distributed, they were presented as the median (range of detection). Measurement data were based on mean ± SD. Correlations between the three indices were evaluated with Pearson's correlation coefficient. The rates of interclass were determined using the χ^2 test. A P value less than 0.05 was considered statistically significant.

RESULTS

Results of quantitative detection of cfDNA

The content of serum cfDNA in the 39 HCC samples ranged from 12866.8 ng/mL to 0 ng/mL, with a median of 557.59 ng/mL compared with 569.2963 ng/mL, 0 ng/mL and 240.07 ng/mL in the 45 normal control samples, and a significant difference was observed between them ($P < 0.05$) (Figure 1). According to the ROC curve (Figure 2), the Youden index was maximal when the concentration of cfDNA was 509.9774 ng/mL, therefore 509.9774 ng/mL was defined as the positive value.

The ROC area under the curve (AUC) was 0.742 ± 0.058, therefore a sum score of the classifier at 509.9774 ng/mL was chosen as the optimal cut-off, as it had the highest Youden's index of 0.520. At this cut-off value, the sensitivity was 56.4%, the specificity was 95.6%, the accuracy was 77.4%, the positive predictive value was 91.7%, the negative predictive value was 71.7%, the positive likelihood ratio was 12.692 and the negative likelihood ratio was 0.048.

Correlations between clinicopathological features and cfDNA expression

Correlations between clinicopathological features and serum cfDNA level were observed, suggesting that serum cfDNA level in HCC patients was correlated with intrahepatic and extrahepatic metastasis and gender (Table 1).

Results of quantitative detection of AFP and AFU

The content of serum AFP in HCC samples ranged from > 10000 ng/mL to 3.92 ng/mL, with a median of 392.07 ng/mL, compared with 526.10 ng/mL, 1.31 ng/mL and 6.02 ng/mL in the normal control samples, and a significant difference was observed between them ($P < 0.05$).

The content of serum AFU in HCC samples ranged from 2366 ng/mL to 362 ng/mL, with a median of 744 ng/mL compared with 792 ng/mL, 121 ng/mL and 431 ng/mL in the normal control samples, and a significant difference was observed between them ($P < 0.05$).

Correlation analysis between cfDNA, AFP and AFU

Scatter point diagrams were mapped out to analyze correla-

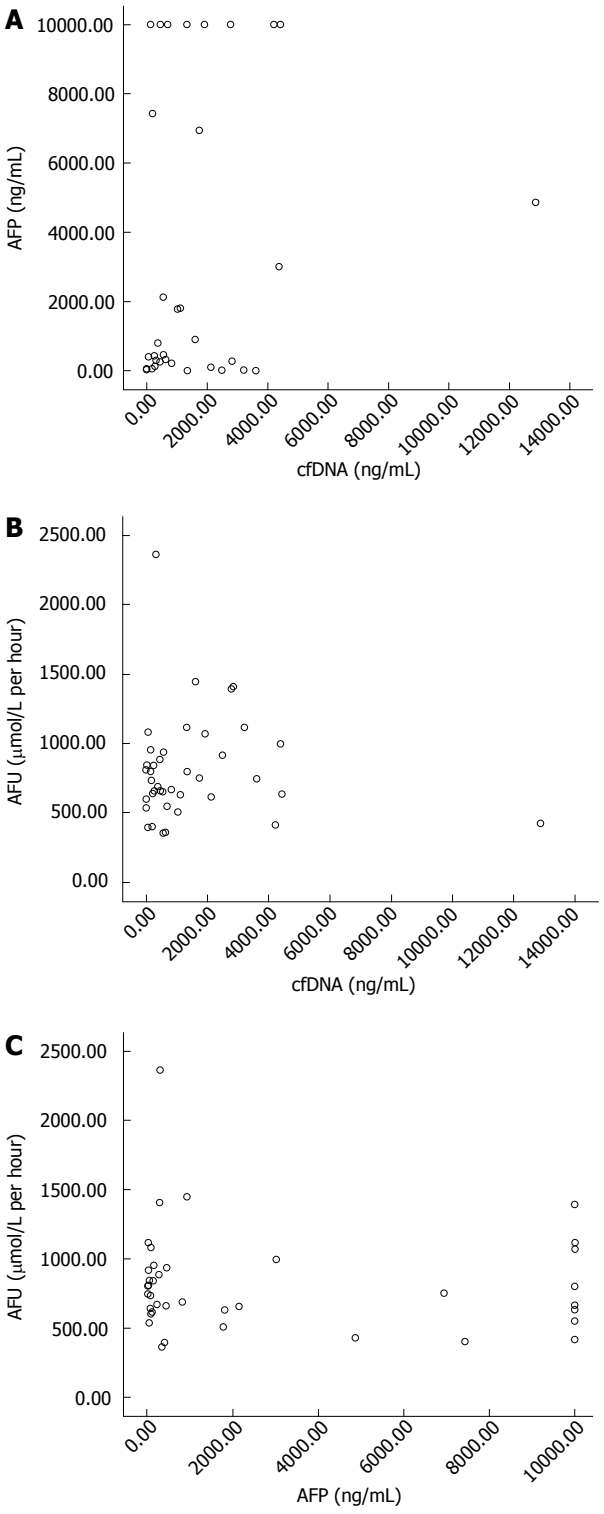

Figure 3 Correlation coefficient. A: r = 0.243 showing no significant correlation between circulating cell-free DNA and α-fetal protein; B: r = -0.0735 showing no significant correlation between circulating cell-free DNA and α L-fucosidase; C: r = -0.085 showing no significant correlation between α-fetal protein and α L-fucosidase.

tions between the three indices using SPSS13.0 (Figure 3).

Detection of cfDNA in combination with AFP and AFU

Compared with the diagnostic efficiency of cfDNA, AFP or AFU alone, combined detection improved the diagnostic efficiency of HCC to some extent (Table 2).

Table 1 Relationship between expression of circulating cell-free DNA and clinicopathological features of 39 hepatocellular carcinoma patients

Observation index	No.	No. of positive cfDNA (%)	*P* value
Age (yr)			
≤ 60	26	16 (61.5)	0.453255
> 60	13	6 (46.2)	
Sex			
Male	32	19 (59.4)	0.024449
Female	7	3 (42.9)	
HBsAg			
Positive	31	16 (51.6)	0.080857
Negative	8	6 (75)	
Intrahepatic and extrahepatic metastasis			
Positive	5	4 (80)	0.000242
Negative	34	18 (52.9)	
Cirrhosis of liver			
Positive	20	10 (50)	0.831179
Negative	19	12 (63.2)	

cfDNA: Circulating cell-free DNA.

DISCUSSION

Circulating free cell DNA is the fragmentation of nucleic acids in body fluids, and has been described in the serum of healthy persons and patients with a variety of diseases, including systemic lupus erythematosus (SLE), diabetes, cerebral stroke, rheumatoid arthritis, myocardial infarction, pulmonary embolism, preeclampsia, Whipple's disease and malignant tumors[6-10]. Investigations have detected increased cfDNA in the plasma of patients with severe injuries, organ failure and multiple organ dysfunction syndromes. Elevated levels of cfDNA were also found in patients who had undergone organ transplantation[11]. Fetal cfDNA has been detected in maternal plasma and has been used for the prenatal diagnosis of fetal abnormalities[12]. Blunt traumatic injury and burn injury can also cause cfDNA release into the blood circulation[13]. Many studies have reported the detection of cfDNA in the serum of patients with various malignant and benign tumors. It is hypothesized that cfDNA may originate from lymphocytes and other nucleated cells in healthy individuals, however, the origin of cfDNA in malignant tumors is not fully understood[14].

Previous studies have suggested the use of serum cfDNA in the diagnosis of HCC. One study showed that cfDNA level was significantly higher in HBV carriers than in normal controls[15]. Ren *et al*[16] suggested that cfDNA level correlated inversely with HCC prognosis, and was attributable to HBV infection in most cases, suggesting that cfDNA may be a predictive marker for the prognosis of HBV-related HCC. In HCV-related HCC patients, serum cfDNA levels were positively correlated with the increased expression of several inflammatory cytokine genes, suggesting that serum cfDNA is associated with local inflammation. cfDNA is a potential non-inva-

Table 2 Diagnostic value of circulating cell-free DNA, α-fetal protein and α L-fucosidase for hepatocellular carcinoma

Groups	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
cfDNA	56.4[c,e]	95.6[c,e]	77.4	91.7[c,e]	71.7
	(22/39)	(43/45)	(65/84)	(22/24)	(43/60)
AFP	53.8[c,e]	91.1[c,e]	73.8	84.0[e]	69.5[c,e]
	(21/39)	(41/45)	(62/84)	(21/25)	(41/59)
AFU	66.7[e]	75.6	71.4	70.3	72.3
	(26/39)	(34/45)	(60/84)	(26/37)	(34/47)
cfDNA + AFP	71.8[e]	86.7[e]	79.8	82.4	78
	(28/39)	(39/45)	(67/84)	(28/34)	(39/50)
cfDNA + AFU	87.2	71.1	78.6	72.3	86.5
	(34/39)	(32/45)	(66/84)	(34/47)	(32/37)
cfDNA + AFP + AFU	89.7[a]	64.4[a]	76.2	68.6	87.9
	(35/39)	(29/45)	(64/84)	(35/51)	(29/33)

[a]$P < 0.05$ *vs* group circulating cell-free DNA (cfDNA) + α-fetal protein (AFP); [c]$P < 0.05$ *vs* group cfDNA + α L-fucosidase (AFU); [e]$P < 0.05$ *vs* group cfDNA + AFP + AFU.

sive cancer biomarker as it is easily isolated from the circulation[17]. Positive correlations were observed between cfDNA level, aspartate aminotransferase (AST) level and the number of leukocytes and neutrophils in HCV-related HCC patients, but not in HCV carriers[18]. The level of cfDNA in serum can function as a predictor of overall survival in distant organs after curative hepatectomy in patients with HCV-related HCC[19]. In our study, serum cfDNA level was significantly higher in HCC patients than in normal controls; patients with intrahepatic and extrahepatic metastasis had a significantly higher cfDNA level; and male patients had higher cfDNA levels as compared with female patients. Therefore, it is clear that serum cfDNA level is associated with intrahepatic and extrahepatic metastasis and gender in HCC patients, but is not closely associated with age, HbsAg or liver cirrhosis. The significance of serum cfDNA in the clinical diagnosis of HCC is as follows: First, serum cfDNA level is positively correlated with the degree of differentiation and metastasis and negatively correlated with the prognosis of HCC patients, suggesting that cfDNA may be useful in monitoring the pathogenic condition of HCC. Second, serum cfDNA level is relevant to the survival of HCC patients after hepatectomy, suggesting that cfDNA may be a useful marker for surgical or non-surgical treatment decision making.

Various techniques are available to quantify serum cfDNA, some of these techniques require abstraction and purification as there is only a very small amount of cfDNA in the serum, and these technologies have failed to reach expectations. The bDNA amplification-based Alu assay is a new technology to quantify serum cfDNA directly and does not require extraction of cfDNA from the serum. The Alu family is the largest of the short interspersed elements (SINEs). Each Alu sequence is approximately 300 bp with a high homology of 70%-98%[20,21]. The sequence AGCT at the 170 degree position can be recognized and cut by the restriction enzyme Alu. Recent studies[22,23] on the Alu sequence showed that it had a unique structure and physiological function, and was associated with a variety of malignant tumors and other human diseases such as gene deletion or duplication of homologous recombination, gene rearrangement of chromosome translocation, and change in transcriptional level. The results of our study suggest that the bDNA technique may be a useful approach in the diagnosis of HCC, as amplification of the hybridization signal is needed instead of amplification of the target sequence. In addition, it is simple, economical, time saving and reproducible.

AFP is the most commonly used biomarker for HCC screening, and a continuous elevation in AFP is considered a risk factor for HCC. AFP has been widely used in HCC survey, diagnosis, evaluation of treatment effects and prediction of recurrence[24,25]. AFP can also be used to monitor treatment and prognosis. Vigilance is required when AFP is higher than 400 μg/L or rises continuously. The survival period is very short in patients with an AFP value > 500 μg/L and bilirubin > 2 mg/L. A rapid increase in AFP often suggests the occurrence of HCC metastasis. Postoperative elevation of AFP > 200 μg/L often indicates incomplete resection of HCC tissue, or the occurrence of metastasis. AFP can also be used in other cancers, such as testicular cancer, teratoma, gastric carcinoma, and pancreatic carcinoma. However, not all HCC cases are diagnosed by AFP alone, as the false negative rate is approximately 30%. False positive AFP results are also seen in some patients with non malignant conditions such as viral hepatitis and cirrhosis. The level of AFP can also increase continuously in the blood or urine of pregnant women, but usually does not exceed 300 μg/L.

AFU is a lysosomal acid hydrolase found extensively in human tissues, with higher activity in the liver and kidney. Some researchers[26] believe that increased serum AFU in HCC patients is associated with an increase in its synthesis, while others reported that hepatoma cells can produce a type of inhibitor to reduce substrate hydrolysis, resulting in substrate accumulation and a compensatory increase in AFU, which is an effective marker of HCC[27,28]. In the present study, the sensitivity of AFU, cfDNA and AFP was 66.7% 56.4% and 53.8%, respec-

tively; the specificity of AFU was only 75.6% compared with 95.6% for cfDNA and AFP, and 91.1% for AFP; the accuracy of cfDNA, AFP and AFU was 77.4%, 73.8% and 71.4%, respectively, with no significant differences between them. Although the sensitivity of AFU was the highest of the three, its specificity was very low. In contrast, the sensitivity of cfDNA and AFP was lower than that of AFU, and their specificity was relatively high. There were no significant correlations between cfDNA, AFP and AFU. The specificity of detection of cfDNA combined with AFP or AFU was lower. However, the sensitivity improved significantly, and the accuracy was similar. The sensitivity of combined detection with the three items was slightly improved, while the specificity was decreased. Taking into account the economic burden of HCC treatment, we believe that combined detection of any two of the three biomarkers is better than combined detection of all three biomarkers in the diagnosis of HCC. In our opinion, cfDNA in combination with AFP or AFU may effectively improve the clinical detection of HCC.

In conclusion, cfDNA for HCC detection has a high sensitivity and specificity, and the clinical significance is similar to AFP, and cfDNA is not correlated with AFP and AFU. The combined detection of cfDNA with one or two markers can significantly improve the diagnostic rate of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Existing biomarkers for the diagnosis of HCC are α-fetal protein (AFP), γ-glutamyltranspeptidase isoenzymes Ⅱ (GGT-Ⅱ), α L-fucosidase (AFU) and acidic isoferritin, of which AFP remains the most specific marker for the diagnosis of HCC. However, not all HCC patients are diagnosed using AFP alone. Circulating free cell DNA (cfDNA) has been extensively studied over the past few decades, and has a high sensitivity and specificity in the detection of HCC.

Research frontiers

cfDNA results from the fragmentation of nucleic acids in body fluids, and has been described in the serum of healthy persons and patients with a variety of diseases. This research was designed to investigate the value of cfDNA in the diagnosis of HCC, determine correlations between cfDNA and clinicopathological features, and explore the value of combined detection of cfDNA, AFP and AFU in the clinical diagnosis of HCC.

Innovations and breakthroughs

Various techniques are available to quantify serum cfDNA, some of these techniques require abstraction and purification as there is only a very small amount of cfDNA in the serum, and these technologies have failed to reach expectations. The bDNA amplification-based Alu assay is a new technology to quantify serum cfDNA directly and does not require the extraction of cfDNA from serum.

Applications

The results of this study suggest that the quantitative analysis of cfDNA is sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both could improve the diagnostic sensitivity for HCC.

Terminology

cfDNA: cfDNA results from the fragmentation of nucleic acids in body fluids, and has been described in the serum of healthy persons and patients with a variety of diseases. Alu: The Alu family of short (~300 bp) interspersed elements is one of the most successful mobile genetic elements, having increased to a copy number in excess of one million in primate genomes in the last 65 million years.

Peer review

The study provides some new information. However, a brief mention regarding the importance of studying circulating DNA and its pathophysiological relevance in cancer needs to be highlighted. The authors should be encouraged to present the observed levels of DNA in healthy controls and patients. This will facilitate the readers to understand the significance of the observed differences in both groups studied. The clinical translational potential of cfDNA as a diagnostic/prognostic biomarker for HCC is not addressed in detail, and needs to be elaborated.

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BRIEF ARTICLE

Protective effects of two *Lactobacillus plantarum* strains in hyperlipidemic mice

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Abstract

AIM: To investigate the effects of *Lactobacillus plantarum* (*L. plantarum*) CAI6 and *L. plantarum* SC4 on hyperlipidemic mice.

METHODS: Male Kunming mice were fed a high-cholesterol diet for 28 d to construct hyperlipidemic models. Hyperlipidemic mice and normal mice were assigned to 3 groups which were separately treated with *L. plantarum* CAI6, *L. plantarum* SC4, and physiological saline through oral gavage for 28 d. Total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were measured by commercially available enzyme kits. FACS Calibur flow cytometry was used to examine hepatic and renal nuclear factor-erythroid 2-related factor 2 (Nrf2) expression. The morphology of livers was checked by hematoxylin and eosin staining and optical microscope observation.

RESULTS: Compared with normal mice, hyperlipidemic mice possessed significantly higher TC (3.50 ± 0.43 *vs* 2.89 ± 0.36, $P < 0.01$), TG (1.76 ± 0.07 *vs* 1.10 ± 0.16, $P < 0.01$), and LDL-C (1.72 ± 0.20 *vs* 0.82 ± 0.10, $P < 0.01$) levels, resulting in an increase of atherogenic index (AI) (2.34 ± 1.60 *vs* 0.93 ± 0.55, $P < 0.05$) and LDL-C/HDL-C ratio (1.43 ± 0.12 *vs* 0.51 ± 0.16, $P < 0.05$). After treatment with *L. plantarum* CAI6/*L. plantarum* SC4, TG (1.43 ± 0.27/1.54 ± 0.10 *vs* 1.76 ± 0.07, $P < 0.01$/$P < 0.05$) and LDL-C (1.42 ± 0.07/1.47 ± 0.12 *vs* 1.72 ± 0.20, $P < 0.01$/$P < 0.01$) in hyperlipidemic mice significantly decreased. In addition, TC, HDL-C, AI, and LDL-C/HDL-C ratio were all positively changed. Meanwhile, the treatment markedly alleviated hepatic steatosis and significantly stimulated Nrf2 expression (73.79 ± 0.80/72.96 ± 1.22 *vs* 54.94 ± 1.84, $P < 0.01$/$P < 0.01$) in hepatocytes of hyperlipidemic mice.

CONCLUSION: *L. plantarum* CAI6 and *L. plantarum* SC4 may protect against cardiovascular disease by lipid metabolism regulation and Nrf2-induced antioxidative defense in hyperlipidemic mice.

Key words: *Lactobacillus plantarum*; Hypolipidemic; Nuclear factor erythroid 2-related factor 2; Metabolic syndrome; Hepatic steatosis; Cardiovascular disease

Core tip: Protective effects of *Lactobacillus plantarum* (*L. plantarum*) CAI6 and *L. plantarum* SC4 strains on hyperlipidemic mice were found, including regulating lipid metabolism, alleviating hepatic steatosis and reducing cardiovascular disease (CVD) risk. The mechanism of intracorporal antioxidation of Lactobacillus strains may be related to stimulation of nuclear factor-

erythroid 2-related factor 2 (Nrf2) expression. Hypolipidemic effect and Nrf2-induced antioxidative defense may contribute to the reduction of CVD risk. We suggest that food fermented by the strains be used as part of the diet to relieve lipid metabolism related metabolic syndrome and to reduce the risk of CVD.

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INTRODUCTION

Metabolic syndrome is a cluster of cardiovascular and type 2 diabetic risk factors[1]. The International Diabetes Federation defines metabolic syndrome as central obesity plus any two of raised triglycerides (TG), reduced high-density lipoprotein cholesterol (HDL-C), raised blood pressure, and raised fasting plasma glucose[2]. Disturbances of lipid homeostasis lead to metabolic syndrome and increase the risk of cardiovascular disease. A 1% increase in plasma cholesterol levels can increase the risk of coronary events up to 3%[3]. Elevated low-density lipoprotein cholesterol (LDL-C) accompanied with insufficient HDL-C is a risk predictor of atherosclerosis[4]. Hypercholesterolemia stimulates formation of free radicals, decreases activity of antioxidant enzymes, and eventually leads to elevated lipid peroxides including oxidized low density lipoprotein (Ox-LDL), which is an independent atherogenic factor[5].

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor that binds to antioxidant response elements (ARE) in the promoter regions of many antioxidative enzymes and phase Ⅱ detoxifying enzymes[6]. Oxidative stress results in accumulation of Nrf2 in the cytoplasm and initiation of transcription of target genes after translocating into the nucleus[7]. Inhibition of LDL oxidation can protect against oxidative stress linked atherosclerosis. Belghitha found that bacterial levan was beneficial for antiarteriosclerosis hypolipidemic and antioxidant effects[8]. We suppose that both exogenous antioxidants and endogenous antioxidant enzymes can strengthen antioxidative defense. Therefore, antioxidant defense triggered by improving Nrf2 expression can be envisaged as a promising strategy in reducing the incidence of atherosclerosis.

Some strains of *Lactobacillus* have been studied as probiotics. They may protect against mutagens and carcinogen exposure, gastrointestinal disease, skin disorders, yeast infections, urinary tract infection, diabetes[9], and immune dysfunction[10]. Hypolipidemic effects of LAB were shown in mice[11,12] and rabbits[13]. Antioxidative activity of LAB, a potential application valued in prevention of atherosclerosis, was also reported *in vitro*[14] and in *vivo*[15]. Extracorporeal antioxidative activity of *Lactobacillus casei* KCTC 3260 may be caused by chelating metal ions[14]. However, an intracorporal antioxidative mechanism of LAB has not been defined. In the present study, we investigated the change in Nrf2 expression in mice after the application of *Lactobacillus plantarum* (*L. plantarum*) CAI6 or *L. plantarum* SC4 to discuss the possible mechanism.

Probiotic strains may have acid or bile salt tolerance to colonize the gastrointestinal tract of the host. Several tests have been taken to screen strains tolerating low pH and high concentration of cholate[16]. Cells have been coated with sodium alginate to increase viscosity and stability of strains[17].

The primary aim of this study was to investigate the effects of *L. plantarum* CAI6 and *L. plantarum* CS4 on alleviation of metabolic syndrome and prevention of cardiovascular disease (CVD) in high-cholesterol fed mice.

MATERIALS AND METHODS

Microbial cultures

L. plantarum CAI6 (GenBank accession number: KC470704) and *L. plantarum* SC4 (GenBank accession number: KC470705) were isolated from Chinese pickled cabbage and raw milk, respectively. The two strains could survive in MRS medium (pH 3.0) supplemented with 0.3% sodium taurocholate (date not shown). The strains were cultured in MRS broth and incubated at 37 ℃ for 24 h. Each activated culture was centrifuged and diluted with 0.9% saline water to obtain a preparation of 2.0×10^9 cfu/mL. 0.1% (w/v) sodium alginate was added to make the strains have better acid resistance, bile resistance, and stability in digestive tract.

Animals

Thirty-Six adult male Kunming mice with a weight of 20 ± 2 g were obtained from the Academy of Military Medical Sciences (Beijing, China). The mice were housed in a controlled animal room at 20 ± 2 ℃ and 60% relative humidity with 12 h light/dark photoperiod. Diet and water could be accessed freely. The care and handling of the animals were in compliance with the internationally accepted standard guidelines for use of animals, and was approved by Science and Technology Committee of Yanshan University on Animal Care and Use.

Experimental protocol

Half of the mice were fed a high-cholesterol diet (75%basic diet, 10%lard, 10%soybean meal, 5%egg yolk) for a period of up to 28 d to construct a hyperlipidemic model (HM). For comparison, the other mice were maintained on standard laboratory diet for 28 d to develop a normal model (NM). Then the mice of HM and NM were randomly assigned to three groups ($n = 6$), respectively.

Group Ⅰ (NM): normal mice fed a standard laboratory diet and physiological saline (10 mL/kg); Group Ⅱ (NM/CAI6): normal mice fed a standard laboratory diet and *L. plantarum* CAI6 (10 mL/kg); Group Ⅲ (NM/SC4): normal mice fed a standard laboratory diet and *L. plan-*

Table 1 Effects on body weight, diet intake, liver index, and serum lipid levels

Group	NM	NM/CAI6	NM/SC4	HM	HM/CAI6	HM/SC4
Body weight, food intake, and liver index of normal model and hyperlipidemic model mice after treatment for 30 d						
IBW (g)	26.53 ± 2.96	25.47 ± 2.71	27.03 ± 2.09	33.17 ± 1.44[b]	31.32 ± 1.06[b]	30.78 ± 1.66[b]
FBW (g)	33.43 ± 4.78[d]	28.50 ± 3.56[d]	32.30 ± 2.73[d]	39.05 ± 2.50[b]	36.68 ± 2.22	38.08 ± 1.87[a]
TFI (g)	44.76 ± 6.94	47.07 ± 4.04	47.05 ± 2.51	52.29 ± 11.97	51.09 ± 6.34	53.32 ± 13.14
[1]LI (%)	4.11 ± 0.30	4.08 ± 0.15	4.10 ± 0.49	4.65 ± 0.50	4.41 ± 0.58	4.54 ± 0.64
Serum lipid levels in the different groups of mice						
TC (mmol/L)	2.89 ± 0.36[d]	2.60 ± 0.24[c]	2.85 ± 0.28[a]	3.50 ± 0.43[b]	3.16 ± 0.35	3.27 ± 0.36
TG (mmol/L)	1.10 ± 0.16[d]	1.06 ± 0.15[d]	1.08 ± 0.13[d]	1.76 ± 0.07[b]	1.43 ± 0.27[d]	1.54 ± 0.10[a]
HDL-C (mmol/L)	1.62 ± 0.52	1.76 ± 0.48[a]	1.67 ± 0.14	1.20 ± 0.42	1.36 ± 0.55	1.31 ± 0.55
LDL-C (mmol/L)	0.82 ± 0.10[d]	0.75 ± 0.07	0.81 ± 0.09	1.72 ± 0.20[b]	1.42 ± 0.07[d]	1.47 ± 0.12[d]
[2]AI	0.93 ± 0.55[a]	0.57 ± 0.41[d]	0.71 ± 0.09[d]	2.34 ± 1.60[a]	1.65 ± 0.99	1.94 ± 1.47
LDL-C/HDL-C	0.51 ± 0.16[a]	0.43 ± 0.19[d]	0.49 ± 0.04[d]	1.43 ± 0.12[a]	1.04 ± 0.16	1.12 ± 0.14

Data represent mean ± SD (n = 6 for each group). [1]LI: Weight of liver (mg)/body weight (g), [2]AI = (TC-HDL-C)/HDL-C, [a]P < 0.05 *vs* NM group, [b]P < 0.01 *vs* NM group, [d]P < 0.01 *vs* HM group. IBW: Initial body weight; FBW: Final body weight; TFI: Total feed intake; LI: Liver index; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; AI: Atherogenic index; NM: Normal diet group; HM: High-cholesterol diet group; NM/CAI6: Normal diet with *Lactobacillus plantarum* (*L. plantarum*) CAI6 group; NM/SC4: Normal diet with *L. plantarum* SC4 group; HM/CAI6: High-cholesterol diet with *L. plantarum* CAI6 group; HM/SC4: High-cholesterol diet with *L. plantarum* SC4 group.

tarum SC4 (10 mL/kg); Group Ⅳ (HM): hyperlipidemic mice fed a high-cholesterol diet and physiological saline (10 mL/kg); Group Ⅴ (HM/CAI6): hyperlipidemic mice fed a high-cholesterol diet and *L. plantarum* CAI6 (10 mL/kg); and Group Ⅵ (HM/SC4): hyperlipidemic mice fed a high-cholesterol diet and *L. plantarum* SC4 (10 mL/kg).

The mice were treated by intragastric administration for 28 d before being anesthetized with chloral hydrate and sacrificed. Food and water consumption and body weight were recorded daily. Blood samples were drawn from the ophthalmic venous plexus. After centrifugation (2000 rmp, 10 min, 4 ℃), the serum samples were collected and stored at -20 ℃. The liver and kidney were excised, rinsed in ice-cold physiological saline, weighed, and then stored at -20 ℃.

Serum lipids analysis

Serum TC, TG, HDL-C, and LDL-C levels were measured using commercially available enzyme kits (Beihuakangtai clinical reagent Ltd, Beijing, CHN). The TC level was measured with an enzymatic method based on the conversion to a chromogen with maximum absorption at 500 nm by cholesterol ester hydrolase, cholesterol oxidase, and peroxidase[18]. The determination of TG was based on an enzymatic method coupled with lipase, glycerokinase, glycerol oxidase and peroxidase[19]. The HDL-C level was assayed by the same enzymatic method based on specific precipitation of VLDL-C and LDL-C in the presence of phosphotungstic acid. The LDL-C value was calculated by following formula: LDL-C = TC-HDL-C-TG/5[20].

Morphology of liver

Fresh livers of mice were fixed with 4% paraformaldehyde for 24 h, dehydrated gradually in a graded series of ethanol, clarified in xylene, and embedded in paraffin wax. Hematoxylin and eosin stained liver was observed by an optical microscope (X5Z-G, Chongqing Optical and Electrical Instrument Co., Ltd, CHN).

Determination of Nrf2 expression in liver and kidney

Liver and kidney tissues suspensions were prepared in ice-cold 0.1 mol/L PBS. Antibodies were diluted in 0.1 mol/L PBS containing 0.1%NaN_3. 10^6 cells/mL were incubated with primary anti-Nrf2 antibodies for 30 min on ice, mixed with 200 μL of 4 % paraformaldehyde, and incubated for 30 min at 4 ℃. After washing twice with 0.1 mol/L PBS containing 0.1%NaN_3, the supernatant was discarded by centrifugation. 3%BSA, 0.1%NaN_3 and 10% saponin (Sigma Co., United States) were added to cell pellets and blended for 15 min, then mixed with 500 μL PBS (pH 7.4). After removal of the supernatant, 0.5 μg of secondary FITC-conjugated rabbit anti-mouse antibody were added and incubated on ice for 60 min. Finally, the cells were resuspended in 1 mL of 0.1 mol/L PBS. The hepatocytes and nephrocytes were scanned using a FACSCalibur (Becton-Dickinson, United States) respectively, and fluorescence of Nrf2 positive cells were quantified. Nonspecific binding of secondary antibody was excluded by incubating the cells only with the FITC-labelled secondary antibody. For reproducibility, the experiment was repeated three times. The software BD CellQuest Pro (Becton Dickinson Biosciences, United States) was used and the data were calculated by fluorescence intensity formula [I = Log (x-mode) × 340].

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using SPSS 13.0 software. Differences between the groups were analyzed by One-Way ANOVA followed by Duncan's multiple range tests. Statistical significance was considered at the P < 0.05 level.

RESULTS

Effects on body weight, diet intake, and liver index

As shown in Table 1, the mice subjected to a high cholesterol diet had a significant increase in body weight (BW)

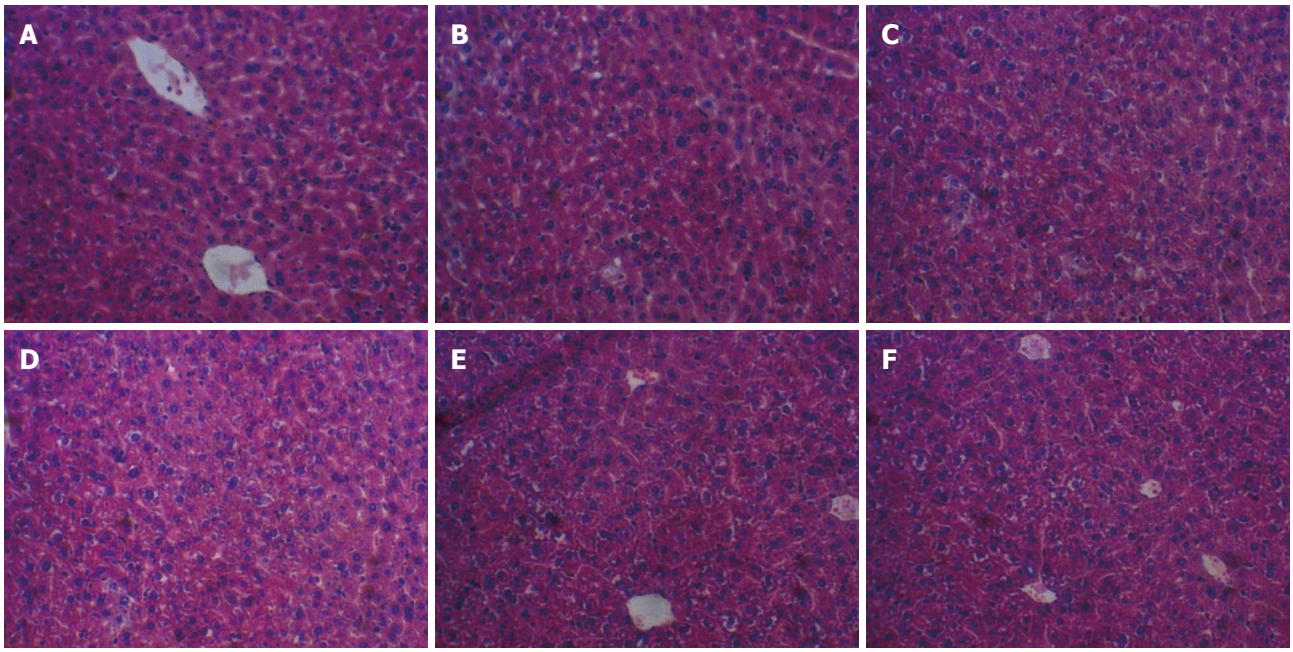

Figure 1 Effects of *Lactobacillus plantarum* SC4 or *Lactobacillus plantarum* CAI6 on liver morphology in mice fed high-cholesterol diet. Tissues were stained with hematoxylin and eosin (20 × 10). A: High-cholesterol diet group; B: Normal diet group; C: Normal diet with *Lactobacillus plantarum* (*L. plantarum*) CAI6 group; D: Normal diet with *L. plantarum* SC4 group; E: High-cholesterol diet with *L. plantarum* CAI6 group; F: High-cholesterol diet with *L. plantarum* SC4 group.

compared with mice on a normal diet ($P < 0.01$). In HM mice, oral administration of CAI6 or SC4 daily did not have any significant effect on BW, while CAI6-treated mice and SC4-treated mice had a significant ($P < 0.01$ and $P < 0.05$, respectively) increase *vs* the NM group. In NM mice, CAI6 or SC4 had no significant effect on BW compared with NM mice receiving neither CAI6 or SC4. None of the groups showed a significant difference in total dietary intake or liver index. However, an increase in liver index in the HM group compared to the NM group was found, and CAI6 and CS4 decreased this.

Effect on plasma lipid profiles

Effect of the treatment on serum lipid levels was shown in Table 1. The TC, TG and LDL-C levels of mice in the HM group showed a significant increase ($P < 0.01$) compared with the NM group. Meanwhile, HDL-C in the HM group decreased by 26% compared to the NM group. Moreover, a significantly increased ($P < 0.05$) atherogenic index (AI) accompanied with a significantly reduced ($P < 0.05$) LDL-C/HDL-C ratio was observed in the HM group. The HM mice orally administrated with strains (CAI6/SC4) showed different degrees of decline in serum TG (-19%, $P < 0.01$/-13%, $P < 0.05$), TC (-10%/-7%), LDL-C (-17%, $P < 0.01$/-15%, $P < 0.01$) levels, and an increase in HDL-C (+13%/+9%) level as compared to the HM group that did not receive CAI6 or SC4. As a result, those mice had a lower AI (-29%/-17%) and a higher LDL-C/HDL-C ratio (+23%/+11%) than the HM group receiving no treatment. As for the NM mice, the administration had no significant effect on serum TC, TG, HDL-C and LDL-C levels, but could reduce AI (-39%/-24%) and increase LDL-C/HDL-C ratio (+21%/+5%).

Hepatic morphology

The livers of the HM group were larger compared with those in the NM group and became beige. The hepatic cells with clear cytoplasm, nucleus, nucleolus and central vein (A) in the NM, NM/CAI6 and NM/SC4 group, showed normal histology in Figure 1A-C[21]. The liver sections of the HM mice exhibited massive fatty changes and severe steatosis with cytoplasmic vacuoles confirmed by histopathological examination in Figure 1D. In addition, the size of lipid droplets in the HM/CAI6 and NM/SC4 groups were remarkably smaller than those of the HM group (Figure 1E, F), suggesting that the *L. plantarum* strains could reduce the build-up of lipid droplets and keep hepatocytes normal.

Effect on Nrf2 levels

Nrf2 expression in liver and kidney tissues was shown in Figure 2. Liver/kidney Nrf2 levels were significantly higher (+26%, $P < 0.01$/+38%, $P < 0.01$) in the HM group than the NM group. Liver Nrf2 increased significantly in the HM/CAI6 (+34%, $P < 0.01$) and HM/SC4 (+33%, $P < 0.01$) groups respectively, compared with the HM group. A significant increase in liver Nrf2 level was also found in the NM/CAI6 (+9%, $P < 0.05$) and NM/SC4 (+10%, $P < 0.05$) groups as compared to the NM group. Contrary to the situation in the liver, the HM/CAI6 or HM/SC4 groups showed no significant increase in kidney Nrf2 level.

DISCUSSION

The mice in the HM group possessed raised TG, reduced HDL-C, and elevated BW resulting in a high risk of developing metabolic syndrome. Accumulation of TG and

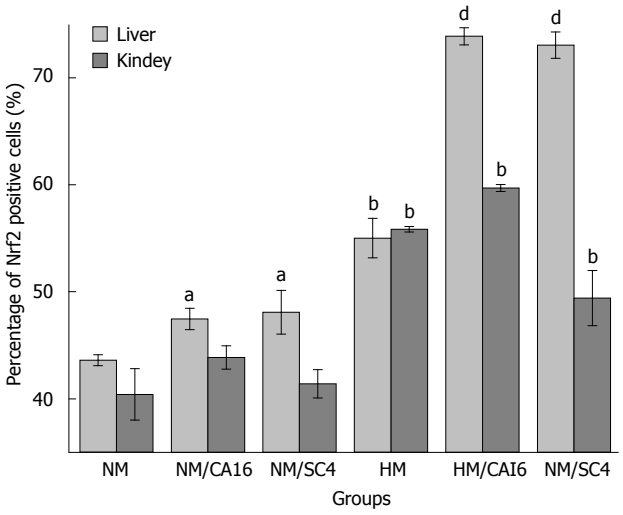

Figure 2 Effect of *Lactobacillus plantarum* SC4 or *Lactobacillus plantarum* CAI6 on liver and kidney nuclear factor-erythroid 2-related factor 2 expression in mice fed high-cholesterol diet. Results were expressed as mean ± SD (n = 6). $^{a}P < 0.05$ *vs* normal diet (NM) group, $^{b}P < 0.01$ *vs* NM group, $^{d}P < 0.01$ *vs* high-cholesterol diet (HM) group. NM/CAI6: Normal diet with *Lactobacillus plantarum* (*L. plantarum*) CAI6 group; NM/SC4: Normal diet with *L. plantarum* SC4 group; HM/CAI6: High-cholesterol diet with *L. plantarum* CAI6 group; HM/SC4: High-cholesterol diet with *L. plantarum* SC4 group; NM: Normal diet group; HM: High-cholesterol diet group.

TC in the bloodstream has been proven related to atherosclerosis, and by extension, the risk of coronary heart disease and stroke. Elevated concentrations of oxidized LDL-C are associated with artherosclerotic plaque formation on the walls of arteries, but increased HDL-C levels may reduce the risk due to the ability of HDL transporting cholesterol back to the liver for excretion or to other tissues[22]. Therefore, the HM mice had an increased risk of CVD. The results showed that both AI, an indicator for severity of atherosclerosis[23], and LDL-C/HDL-C, a valuable tool to evaluate coronary heart disease (CHD) risk[24], were increased in HM mice, which is in agreement with the above view.

Serum TG, TC and LDL-C levels decreased in the HM/CAI6 and HM/SC4 groups when compared with the HM group while serum HDL-C level increased revealing that *L. plantarum* CAI6 and *L. plantarum* SC4 exerted a hypolipidemic effect and could relieve lipid related metabolic syndrome. AI and LDL-C/HDL-C ratios were also lower in the HM/CAI6 and HM/SC4 groups, suggesting that *L. plantarum* CAI6 and *L. plantarum* SC4 reduced the risk of CVD. Non-HDL-C, representing the total amount of cholesterol in the potentially atherogenic lipoproteins, was regarded as a risk predictor of cardiovascular outcomes on drug treatment[25]. After strains (CAI6/SC4) treatment, non-HDL-C decreased by 22% (1.80 *vs* 2.30) and 15% (1.96 *vs* 2.30) respectively. Additionally, NM mice treated with strains (CAI6/SC4) had positive changes in AI (-39%/-24%) and LDL-C/HDL-C ratio (-16%/-4%). So, we suppose that the strains have preventive and therapeutic effects for CVD.

The liver is the hub of fat synthesis and transportation. The liver can use fatty acids hydrolyzed from fat in food to synthetize cholesterol and TG. High fat intake of HM mice disequilibrated lipid metabolism, resulting in accumulation of TG in liver and an increased increment of the liver index, and hepatic steatosis occurred[26]. The result of liver tectology proved that the *L. plantarum* strains had important potential in alleviating hepatic steatosis attributed to mediation of lipid metabolism and had protective effects on hepatic structure.

Oxidative stress is thought to be linked to atherogenesis[27]. Therefore, much attention has been paid to the antioxidant hypothesis in prevention and treatment of CVD[28,29]. In recent times, antioxidant activity of many hypolipidemic substances has been detected and the activities of SOD, CAT and GSH-Px are usually used as test standards[15,30]. To suffer ROS-mediated injury, cells initiate the Nrf2/ARE signaling pathway stimulated by Nrf2. Giovanni. E reported that the pathway plays an important role in vascular homeostasis and the defense of endothelial and smooth muscle cells against sustained oxidative stress associated with diseases such as atherosclerosis and preeclampsia[31]. Our results showed that liver and kidney Nrf2 expression was significantly ($P < 0.01$) increased in the HM group compared with the NM group, revealing that a cholesterol-rich diet may bring about remarkable modifications in antioxidant defense mechanisms *in vivo*. Furthermore, oral administration of *L. plantarum* CAI6 and *L. plantarum* SC4 both could significantly ($P < 0.01$) increase liver Nrf2 levels compared with the HM group. So we envisage that spontaneous and strain-stimulated complex mechanisms conjointly promote Nrf2 expression in the liver. Accumulation of Nrf2 in the liver may initiate anti-oxidative stress and result in inhibition of Ox-LDL production, reducing the risk of atherosclerosis. Combining all the analyses above, the anti-CVD mechanism of the strains may be a regulation of lipid metabolism to alleviate metabolic syndrome and a stimulation of Nrf2 expression to initiate antioxidant defense. The actual mechanisms which account for strain-stimulated Nrf2 expression and the transcription and expression of antioxidase genes in Nrf2/ARE pathway need further study.

Dietary management has been shown to be an effective way to alleviate metabolic syndrome[32,33]. Some LAB-fermented probiotic products are commercially available[34]. Jeun *et al*[11] found that live *L. plantarum* was more effective than dead *L. plantarum* in reduction of plasma lipid levels and supposed that the active compounds may be derived from metabolic activity of live *L. plantarum*. Adding fermented foods seems like a good way to increase the number of live bacteria in a diet. LAB-fermented food, including both live LAB and active metabolin, seems the best form to apply LAB in our daily lives. The strains *L. plantarum* CAI6 and *L. plantarum* SC4, which can be regarded as probiotics for their beneficial impacts, were isolated from milk and pickle, respectively. Therefore, *L. plantarum* CAI6-fermented milk or *L. plantarum* SC4-fermented pickle may also exert those effects.

We suppose that the strains-fermented food may be used as a dietary approach to alleviate metabolic syndrome and prevent CVD.

COMMENTS

Background

Hyperlipidemia, an integral part of metabolic syndrome, is a major risk factor for cardiovascular diseases (CVD). Oxidation of low-density lipoprotein (LDL) cholesterol is linked to atherogenesis, and antioxidant drugs become a therapeutic approach. *Lactobacillus* regarded as probiotics have been proven to possess hypolipidemic and antioxidant effects. A probiotic diet, a kind of dietary management, for example fermented food, is a promising way to prevent and treat CVD.

Research frontiers

CVD remains a major public health problem. The relationship between cardiovascular risk and serum lipid and related factors has been extensively studied to find valuable predictors of cardiovascular risk. Oxidative modification of LDL plays an important role in the genesis of arteriosclerosis. Based on the antioxidant hypothesis that suboptimal levels of principal antioxidant micronutrients are hitherto underrated risk factors for CVD, much research has focussed on the effects of antioxidant substances on CVD. Some *Lactobacillus* strains are probiotics, so novel *Lactobacillus* strains potentially used as probiotics have been continuously selected, and their applications have been investigated. Dietary approaches to alleviate metabolic syndrome have been shown in many studies.

Innovations and breakthroughs

Recent research has highlighted the antioxidant hypothesis in prevention and treatment of CVD and demonstrated the antioxidant ability of *Lactobacillus* strains. However, the antioxidative mechanism of *Lactobacillus in vivo* has not been known. In the study, the authors investigated the effects of *Lactobacillus plantarum* (*L. plantarum*) CAI6 and *L. plantarum* SC4 on nuclear factor-erythroid 2-related factor 2 (Nrf2) expression in hyperlipidemic mice. This is the first study to report the mechanism of intracorporal antioxidation of *Lactobacillus* strains may be the stimulation of Nrf2 expression. Furthermore, these studies suggest that *L. plantarum* CAI6 and *L. plantarum* SC4 could reduce the risk of CVD by a hypolipidemic effect and Nrf2-induced antioxidative defense.

Applications

As *L. plantarum* CAI6 and *L. plantarum* SC4 have shown obviously protective effects on hyperlipidemic mice, food fermented by the strains may be used as dietary management to alleviate disorders of lipid metabolism related metabolic syndrome and to reduce the risk of CVD.

Terminology

Metabolic syndrome is a combination of medical disorders, such as disturbances of glucose and lipid homeostasis, which increase the risk of developing CVD. Coronary heart disease, a type of CVD, is a narrowing or blockage of the coronary arteries, usually caused by atherosclerosis in a condition where an artery wall thickens as a result of the accumulation of fatty materials. Nrf2 is a transcription factor that regulates the expression of anti-oxidative stress enzyme genes. Stimulating the Nrf2 related signal pathway has been studied for treating diseases caused by oxidative stress.

Peer review

This manuscript is worth publishing.

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CASE REPORT

Small undifferentiated intramucosal gastric cancer with lymph-node metastasis: Case report

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Abstract

It has been reported recently that small undifferentiated intramucosal early gastric cancer (EGC) < 20 mm in size without any lymphovascular involvement or ulcerative findings had virtually no risk of lymph-node (LN) metastasis. Consequently, the indications for endoscopic resection were expanded to include such undifferentiated EGC lesions. We describe herein a case of a small undifferentiated intramucosal EGC < 20 mm in size without lymphovascular involvement or ulcerative findings that involved lymph-node metastasis. A 57-year-old female underwent pylorus preserving gastrectomy as standard treatment for an undifferentiated EGC 15 mm in size without any ulcerative finding. The surgical specimen revealed a signet-ring cell carcinoma with a moderately to poorly differentiated adenocarcinoma limited to the mucosa that was 15 mm in size with no lymphovascular involvement or ulcerative findings. This case involved LN metastasis, however, and the lesion was diagnosed as pathological stage ⅡA (T1N2M0) according to the Japanese Classification of Gastric Carcinoma.

Key words: Early gastric cancer; Endoscopic submucosal dissection; Expanded indications; Lymph-node metastasis; Undifferentiated type

Core tip: Herein is described rare case of small undifferentiated intramucosal early gastric cancer (EGC) < 20 mm in size with lymph-node (LN) metastasis. It has been reported recently that small undifferentiated intramucosal EGC < 20 mm in size without lymphovascular involvement or ulcerative findings had virtually no risk of LN metastasis. Therefore, such small undifferentiated EGCs have become candidates for endoscopic resection. It was within this context we experienced the present case involving a small undifferentiated intramucosal EGC < 20 mm in size without lymphovascular involvement or ulcerative findings that evidenced LN metastasis.

Odagaki T, Suzuki H, Oda I, Yoshinaga S, Nonaka S, Katai H, Taniguchi H, Kushima R, Saito Y. Small undifferentiated intramucosal gastric cancer with lymph-node metastasis: Case report. *World J Gastroenterol* 2013; 19(20): 3157-3160 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3157.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3157

INTRODUCTION

Endoscopic resection (ER) is generally indicated for early gastric cancer (EGC) with no risk of lymph-node (LN) metastasis. Hirasawa *et al*[1] reported there was virtually no risk of LN metastasis in undifferentiated EGCs < 20 mm

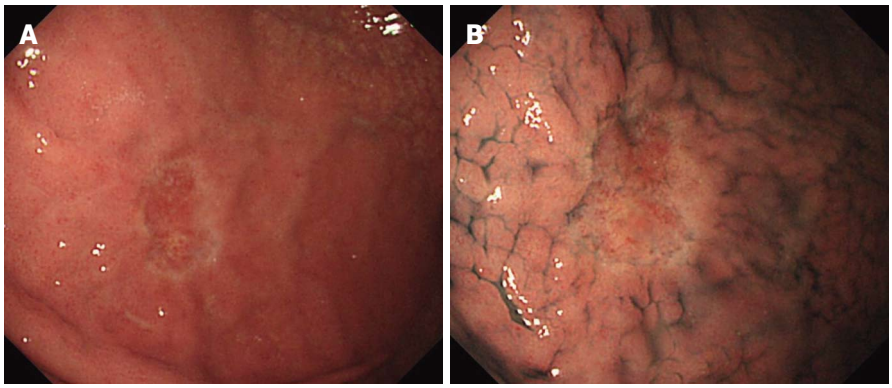

Figure 1 Pre-treatment endoscopic examination. Esophagogastroduodenoscopy revealed pale depressed mucosal lesion on anterior wall of middle gastric body approximately 15 mm in size with no ulcerative finding and non-atrophic background mucosa. A: Conventional white light endoscopy; B: With indigo-carmine dye staining.

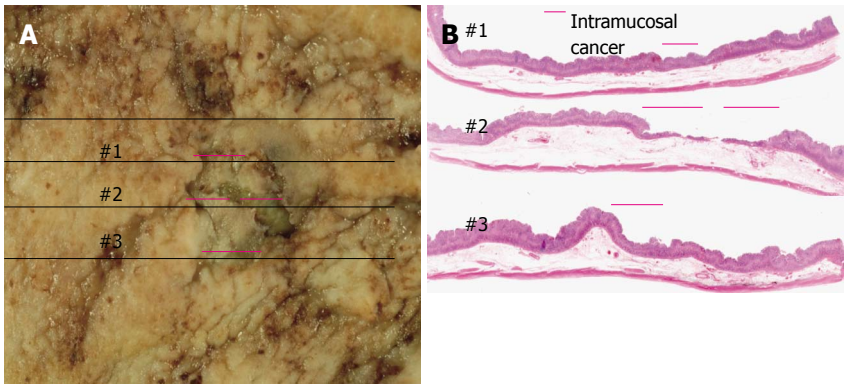

Figure 2 Surgically resected specimen. A: Formalin-fixed specimen from pylorus preserving gastrectomy; B: Panoramic view of lesion with hematoxylin and eosin staining. Mucosal lesion 15 mm × 12 mm in size with no ulcer finding and pink lines corresponding to lesion depression (hematoxylin and eosin staining, × 1).

in size without lymphovascular involvement or ulcerative findings and such lesions could satisfy the indications for ER. We encountered, however, a case of a small undifferentiated intramucosal EGC meeting all of the above-mentioned criteria that actually involved LN metastasis and decided to report this seemingly rare occurrence.

CASE REPORT

A 57-year-old female underwent an esophagogastroduodenoscopy (EGD) for a medical check-up in December 2008. The results indicated gastric cancer, and she was referred to our hospital for treatment. The EGD revealed a pale depressed mucosal lesion that was Type 0-Ⅱc in accordance with the Japanese Classification of Gastric Carcinoma (JCGC)[2] located on the anterior wall of the middle gastric body (Figure 1). The size of the lesion was approximately 15 mm, and there was no ulcerative finding. The biopsy specimen revealed a signet-ring cell carcinoma. Computed tomography (CT) indicated no distinct regional LN or distant metastasis. Laboratory results were within normal limits and the serum carcinoembryonic antigen level was normal. We diagnosed this lesion as clinical (c) T1(M), cN0, cM0, cStage I A according to the JCGC; therefore, pylorus preserving gastrectomy was performed as the standard treatment. The surgical specimen revealed a signet-ring cell carcinoma with a moderately to poorly differentiated adenocarcinoma limited to the mucosa without lymphovascular involvement or ulcerative findings (Figures 2 and 3A-E). The lesion was 15 mm × 12 mm in size, and the resection margin was free of tumor cells. In addition, tumor cells were positive for mucin (MUC)5AC and negative for MUC6 with an MUC5AC/MUC6 double layer and Ki-67 localization both absent (Figure 4). Some portions of the resected regional LNs (lesser curvature LNs; LN station 3), however, included metastatic cancer cells (3/32) that were also identified as a signet-ring cell carcinoma (Figure 3F). This lesion was then diagnosed as pathological (p)T1(M), ly0, v0, pN2, pM0, pStage ⅡA according to the JCGC. Four years after surgery, the patient was in good condition and disease free without any signs of tumor recurrence based on the latest follow-up EGD and CT examinations.

DISCUSSION

ER is widely accepted as a minimally invasive treatment for intramucosal EGC with virtually no risk of LN metastasis[2-6]. An earlier report, however, demonstrated undifferentiated intramucosal EGC had a higher probability of LN metastasis (4.2%)[3], and gastrectomy with regional lymphadenectomy has been considered essential treatment for such lesions. More recently, Hirasawa *et al*[1] reported small undifferentiated intramucosal EGCs < 20 mm in size without lymphovascular involvement or ulcerative findings had virtually no risk of LN metastasis (95%CI, up to 0.96%). Therefore, such small undifferentiated EGCs have become candidates for ER. It was within this context we experienced the present case involving a small undifferentiated intramucosal EGC < 20 mm in size without any lymphovascular involvement or ulcerative findings that evidenced LN metastasis.

Both Nasu *et al*[7] and Park *et al*[8] previously reported similar cases of a small undifferentiated intramucosal

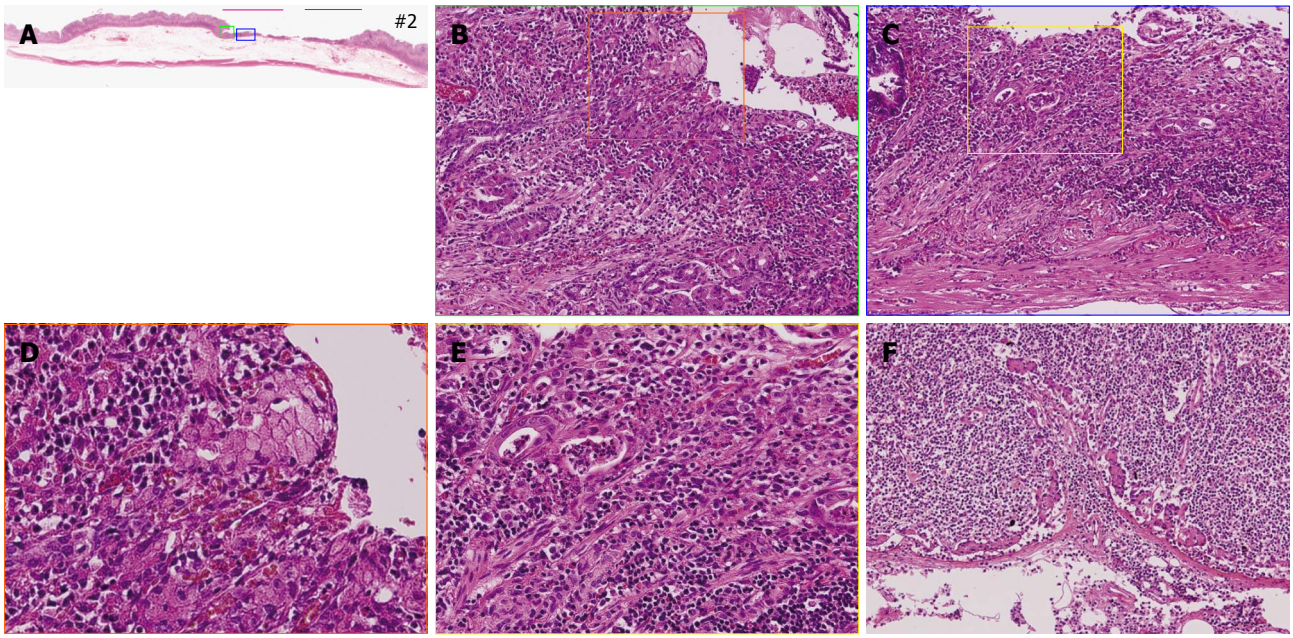

Figure 3 Histopathological findings. A: Panoramic view with hematoxylin and eosin staining (× 1); B: Signet-ring cell carcinoma identified at lesion edge (green frame in A, × 40); C: Moderately to poorly differentiated adenocarcinoma limited to mucosa visible in lesion center. Low magnification view (bule frame in A, × 40); D: High magnification view of red frame in B (× 200); E: High magnification view of yellow frame in C (× 200); F: Image showing signet-ring cell carcinoma invasion of station 3 lymph node (N2) (× 40).

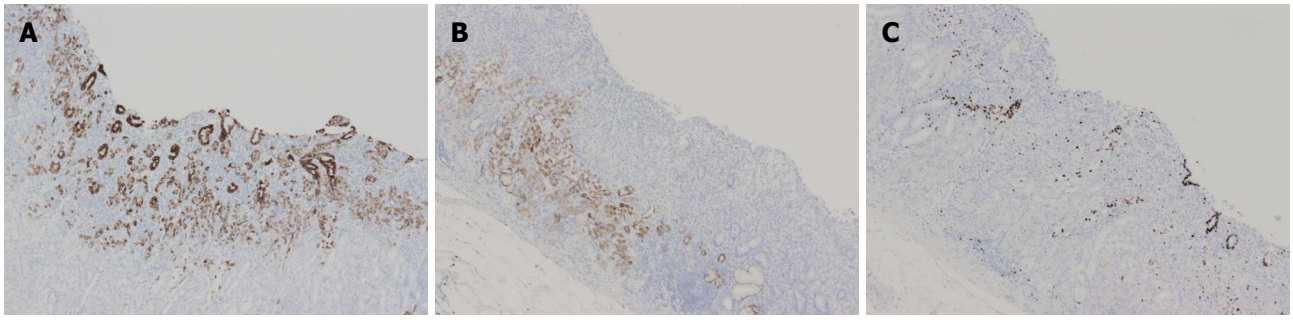

Figure 4 Immunochemical staining. Tumor cells positive for mucin (MUC)5AC (A, × 10) and negative for MUC6 with an MUC5AC/MUC6 double layer (B, × 4)and Ki-67 (C, × 4) localization both absent.

EGC < 20 mm in size (13 and 17mm, respectively) without lymphovascular involvement or findings of ulceration that involved LN metastasis. In addition, Abe *et al*[9] reported three cases involving LN metastasis of small undifferentiated intramucosal EGCs < 20 mm in size (10, 12 and 20 mm, respectively) without any ulcerative findings and concluded ER should not be indicated for undifferentiated EGCs > 10 mm in size. There was no reference in those three cases, however, regarding the existence of lymphovascular involvement. Hirasawa *et al*[10] also reported a case with LN metastasis of a small undifferentiated intramucosal EGC that was 13 mm in size and without an ulcerative finding. According to their case report, routine histological examination of the endoscopic submucosal dissection specimen sectioned at intervals of 2 mm indicated an intramucosal tumor without lymphovascular involvement or ulceration. Hematoxylin and eosin staining of one of the 60 additional deep-cut sections of the original resected specimen, however, revealed lymphatic involvement in the mucosa. The authors then suggested practical limitations in determining lymphovascular involvement through routine histological examinations may not always facilitate the detection of LN metastasis.

Based on the various published reports supportive of the findings in our particular case, the possibility of LN metastasis developing from a small undifferentiated intramucosal EGC < 20 mm in size without ulceration exists even if there is no apparent lymphovascular involvement[7,8]. It is highly desirable, therefore, to clarify the predictive factors for the development of LN metastasis with such small undifferentiated intramucosal EGCs. Takizawa *et al*[11] evaluated the conditions in which LN metastasis was unlikely and ER of undifferentiated EGCs had a greater probability of being effective. Such conditions included intramucosal cancers without lymphovascular involvement or ulcerative findings and: (1) lesions < 10 mm in size; (2) the presence of Ki-67 localization; (3) the presence of a double layer (MUC5AC/

MUC6); and/or (4) the presence of only a signet-ring cell carcinoma. The conditions in our case included a lesion > 10 mm in size, the absence of Ki-67 localization, the absence of a double layer (MUC5AC/MUC6), and the presence of not only a signet-ring cell carcinoma but also a moderately and poorly differentiated adenocarcinoma. In addition, more recently, Takizawa *et al*[12] reported that LN metastasis was significantly more common in mixed predominantly undifferentiated (MU)-type intramucosal cancer than in pure undifferentiated (PU)-type intramucosal cancer and concluded MU-type tumors might have greater malignant potential than PU-type tumors. Actually, the present case was MU-type intramucosal cancer with lymph-node metastasis.

If it were possible to evaluate the pathology of all resected specimens in detail using deep-cut sections and immunohistochemical staining as indicated in the above-mentioned reports[10-12], we might be able to predict those cases in which LN metastasis of small undifferentiated intramucosal EGCs would most likely occur, but it is not always feasible to perform such extensive pathological evaluations in clinical practice. We need to recognize the distinct possibility, therefore, of LN metastasis developing from small undifferentiated intramucosal EGCs < 20 mm in size without lymphovascular involvement or ulcerative findings because of the practical limitations related to such histological examinations[10]. It is, therefore, imperative that physicians thoroughly explain the possibility of LN metastasis to patients before performing ER for small undifferentiated intramucosal EGCs so as to obtain their informed consent and then conduct more careful follow-up surveillance examinations including CT scans, ultrasonography and/or EUS in addition to EGDs subsequent to ER treatment.

In order to satisfactorily demonstrate the validity of treating small undifferentiated intramucosal EGCs with ER, a large prospective study analyzing therapeutic outcomes will need to be conducted in the near future before the recently expanded indications for treating such lesions with ER should be accepted for general clinical use.

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CASE REPORT

Intraductal papillary neoplasm of the bile duct accompanying biliary mixed adenoneuroendocrine carcinoma

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Abstract

We present the first case of an intraductal papillary neoplasm of the bile duct (IPNB) accompanying a mixed adenoneuroendocrine carcinoma (MANEC). A 74-year-old woman presented with fever of unknown cause. Laboratory data revealed jaundice and liver injury. Contrast-enhanced computed tomography revealed a 20 mm polypoid tumor in the dilated distal bile duct, which exhibited early enhancement and papillary growth. Upper gastrointestinal endoscopy revealed mucus production from the papilla of Vater, characterized by its protruding and dilated orifice. Endoscopic ultrasonography visualized the polypoid tumor in the distal bile duct, but no invasive region was suggested by diagnostic imaging. Therefore, the initial diagnosis was IPNB. After endoscopic nasobiliary drainage, a pylorus-preserving pancreaticoduodenectomy was performed. Pathological examination of the resected bile duct revealed papillary proliferation of biliary-type cells with nuclear atypia, indicating pancreaticobiliary-type IPNB. In addition, solid portions comprised of tumor cells with characteristic salt-and-pepper nuclei were evident. Immunohistochemistry revealed expression of the neuroendocrine marker synaptophysin in this solid component, diagnosing it as a neuroendocrine tumor (NET). Furthermore, the MIB-1 proliferation index of NET was higher than that of IPNB, and microinvasion of the NET component was found, indicating neuroendocrine carcinoma (NET G3). This unique case of MANEC, comprising IPNB and NET, provides insight into the pathogenesis of biliary NET.

Key words: Neuroendocrine tumor; Intraductal papillary neoplasm of bile duct; Intraductal papillary neoplasm of the bile duct; Bile duct

Core tip: A 74-year-old woman presented with fever and jaundice. Computed tomography revealed a polypoid tumor in the dilated distal bile duct. Pylorus-preserving pancreaticoduodenectomy was performed. Pathological examination revealed the papillary proliferation of biliary-type cells with nuclear atypia in the dilated bile duct, indicating papillary neoplasm of the bile duct. A solid portion comprised of tumor cells with

characteristic salt-and-pepper nuclei was found. Immunohistochemistry revealed synaptophysin expression in the solid portion, diagnosing it as a neuroendocrine tumor (NET). This case provides insight into the pathogenesis of biliary NET.

Onishi I, Kitagawa H, Harada K, Maruzen S, Sakai S, Makino I, Hayashi H, Nakagawara H, Tajima H, Takamura H, Tani T, Kayahara M, Ikeda H, Ohta T, Nakanuma Y. Intraductal papillary neoplasm of the bile duct accompanying biliary mixed adenoneuroendocrine carcinoma. *World J Gastroenterol* 2013; 19(20): 3161-3164 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3161.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3161

INTRODUCTION

Intraductal papillary neoplasm of the bile duct (IPNB) is a new entity, defined as biliary neoplasms showing papillary or villous proliferation within the dilated lumens of intrahepatic and extrahepatic bile ducts by the 2010 World Health Organization (WHO) Classification of Tumors of the Digestive System[1]. IPNB encompasses several lesions, which were previously categorized as biliary papilloma, papillomatosis, papillary adenocarcinoma of the bile duct, and intraductal growth-type cholangiocarcinoma. The following three key pathologic features are characteristically evident in IPNB in varying combinations: (1) exophytic and papillary proliferation of neoplastic biliary epithelial cells, with delicate fibrovascular stalks within bile duct lumens; (2) mucin hypersecretion (macroscopic mucin is evident in some cases); and (3) variable dilatation or multilocular cystic changes of affected bile ducts.

Neuroendocrine tumors (NETs), including carcinoid tumors, are commonly found in several organs, including the pancreas and gastrointestinal tract. Most biliary NETs exist as a component of mixed adenoneuroendocrine carcinomas (MANECs)[2]. MANECs are found in hepatic hilar cholangiocarcinomas with hepatolithiasis, gallbladder cancers, and extrahepatic cholangiocarcinomas, and show a characteristic histology[3]. Moreover, since the NET components of biliary MANECs define prognosis, it is important to identify them and consider indications for adjunctive therapy, such as somatostatin analogues.

We encountered a patient with IPNB accompanying a NET in the extrahepatic bile ducts. This case is unique, has a different histology from most biliary MANECs, and provides insight into the histogenesis of NETs in biliary tumors.

CASE REPORT

Clinical course

A 74-year-old woman consulted a family physician for fever of unknown cause. Laboratory data revealed jaundice and liver injury. Computed tomography (CT) revealed an elevated lesion in the distal bile duct. She was admitted to our hospital for further examination and treatment. Enhanced CT revealed a 20 mm polypoid tumor, which exhibited early enhancement and papillary growth (Figure 1). Magnetic resonance image (MRI) and magnetic resonance cholangiopancreatography (MRCP) confirmed these findings. Mucus production was evident from the papilla of Vater, characterized by its protruding and dilated orifice. Endoscopic ultrasonography revealed a polypoid tumor in the distal bile duct. No invasive tumor regions were detected using these imaging techniques. Therefore, the initial diagnosis was IPNB. After endoscopic nasobiliary drainage (ENBD), a pylorus-preserving pancreaticoduodenectomy was performed. The postoperative course was uneventful, except for slight pneumonia.

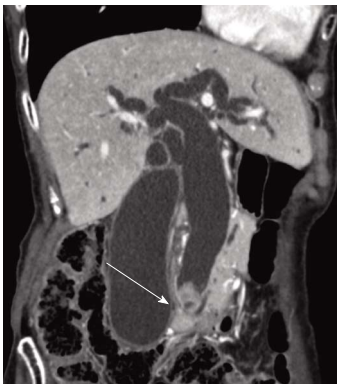

Figure 1 Computed tomography of the bile duct. Computed tomography revealed dilatation of the bile duct and an elevated lesion (arrow) at the bottom of the lower bile duct.

Pathology of the resected bile ducts

A whitish tumor occupying the dilated lumen of the distal bile duct was found, and the proximal portion of the bile duct was also dilated. As shown in Figure 2A, the tumor was comprised of two different areas: papillary and solid. The papillary proliferating area consisted of cholangiocyte-like columnar epithelial cells covering fine fibrovascular cores, and showed moderate-to high-grade intraepithelial neoplasia, indicating pancreaticobiliary-type IPNB (high-grade intraepithelial neoplasia) according to the WHO classification[1] (Figure 2B). Immunohistochemistry revealed expression of the biliary-type cytokeratin (CK): CK19 (Figure 3A) in these cells, but not CK20, confirming biliary-type IPNB. Conversely, the solid area lacking acinar/glandular structure (Figure 2A and B) was comprised of tumor cells with salt-and-pepper nuclei, a high nucleus-to-cytoplasm ratio, and increased nuclear chromatin (Figure 2C). Immunohistochemistry for neuroendocrine markers revealed synaptophysin expression (Figure 3B), but not chromogranin A and CD56 expression, indicating a NET component of MANEC. Furthermore, NET, as well as IPNB components, expressed CK19 (Figure 3A). The MIB-1 proliferation index of the NET component was significantly higher than that of the IPNB component (Figure 3C). Focal invasion of NET and IPNB components into the ductal wall was evident, but no pancreatic or lymph node metastasis was observed.

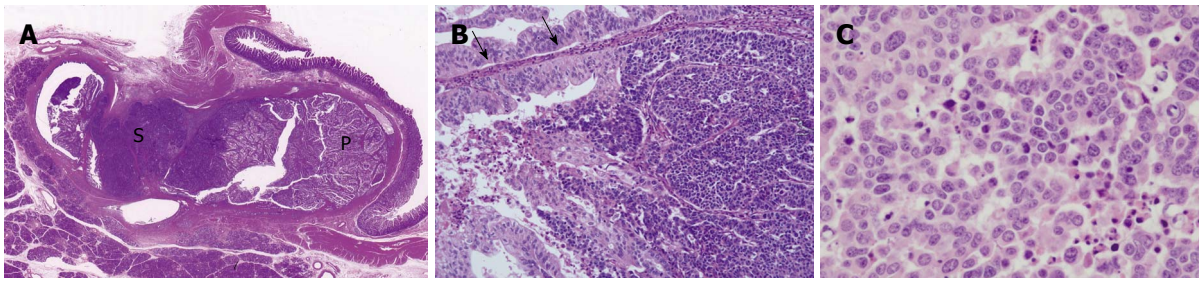

Figure 2 Pathological findings from the resected bile duct (hematoxylin eosin staining). A: A semi macro cross-sectional image of the resected extrahepatic bile duct. In the dilated bile duct, the tumor was comprised of two distinct parts: papillary (P) and solid (S); B: The boundary between the papillary (right) and solid (left) areas. The papillary area consisted of a papillary proliferation of cholangiocyte-like columnar epithelial cells covering fine fibrovascular cores (arrows). In the solid area, a lacunar tumor was evident, but lacked distinct acinar/glandular structures. Magnification: × 100; C: The solid tumor at higher magnification. Tumor cells exhibited characteristic salt-and-pepper nuclei, a high nucleus-to-cytoplasm ratio, and increased nuclear chromatin. Magnification: × 400.

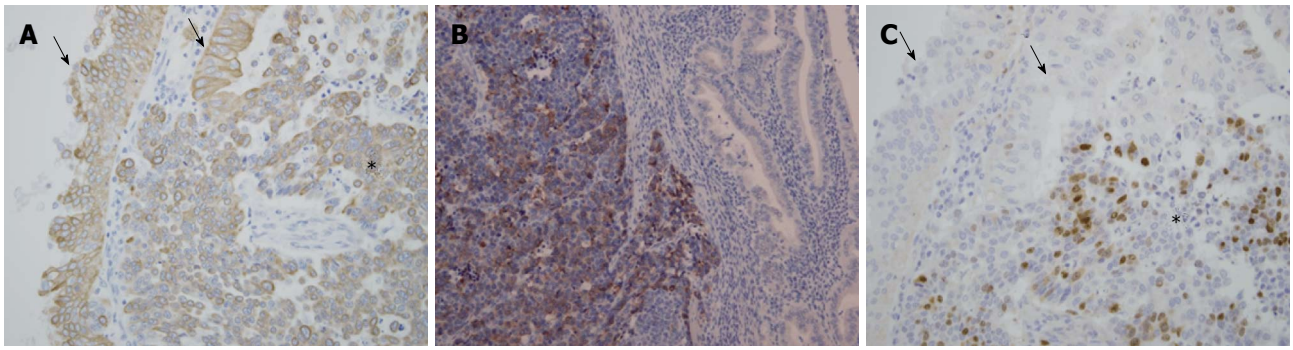

Figure 3 Immunohistochemistry for cytokeratin 19, synaptophysin, and Ki-67. A: Both the solid (asterisk) and papillary (arrows) components were positive for CK19. Magnification: × 200; B: Synaptophysin expression was evident in the solid component (left), indicating a neuroendocrine tumor, but not in the papillary component (right). Magnification: × 100; C: Although Ki-67-positive cells were scarce in the papillary component (arrows), many Ki-67-positive cells were identified in the solid component (asterisk). Magnification: × 200.

DISCUSSION

IPNB is characterized by a grossly visible, exophytic proliferation of neoplastic cholangiocytes with delicate fibrovascular cores. The WHO classification of digestive tumors[1] recognizes IPNB as a precancerous entity of cholangiocarcinoma. Surgical resection is the most optimal treatment for IPNB because mucin production by these tumors causes recurrent cholangitis and obstructive jaundice, even if these tumors are considered benign. Incomplete surgical resection often causes tumor recurrence; furthermore, recurrence in the remaining bile ducts can develop after apparently complete resection of even noninvasive tumors because of tumor multifocality[4,5].

Radiographic imaging modalities, including CT, MRI, and MRCP, are recommended noninvasive techniques for detecting masses and evaluating the tumor extent. Although cholangiography and cholangioscopy carry risks of complications, these diagnostic techniques are useful for evaluating tumor extent, operative planning, and biliary intervention[6,7]. In this case, it was not difficult to evaluate the degree of tumor invasion using noninvasive radiographic techniques. However, pathological confirmation could not be obtained via biopsies or cytology. Peroral cholangioscopy and intraductal ultrasonography may be necessary for pathological diagnosis. Unfortunately, we were unable to perform these invasive radiographies due to a lack of consent from the patient, who was suffering from pancreatitis after ENBD.

The degree of malignancy and histological atypia of IPNB ranges from borderline or low to moderate in the intraluminal papillary portions, to highly malignant and atypical in the invasive portions. IPNBs without invasive components are comprised of two subgroups: low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia. Moreover, the neoplastic cells of IPNB are cytologically classifiable into four phenotypes: pancreaticobiliary, intestinal, gastric (clear cell type), and oncocytic types[1]. This case was IPNB with associated pancreaticobiliary-type invasive carcinoma.

NETs in the digestive system, including in the gallbladder and extrahepatic bile ducts, are classified as: NET G1 [carcinoid; mitotic count: < 2 per 10 high-power fields (HPF); and/or ⩽ 2% Ki-67 index]; NET G2 (mitotic count: 2-20 per 10 HPF; and/or 3%-20% Ki-67 index); neuroendocrine carcinoma (large- or small-cell type); and MANEC, according to the 2010 WHO classification[2,8]. In biliary MANEC, the adenocarcinomatous component is located on the surface of the main tumor, and the majority of stromal invasion, including vascular invasion and lymph node metastasis, involves the NET

component[3]. Therefore, the NET component of biliary MANEC is considered to define prognosis[3]. In this case, contrary to typical biliary MANEC, the adenocarcinomatous component of MANEC was identified as IPNB. NET adjacent to the IPNB component resulted in both being intermingled in one mass (Figure 2B). Moreover, both NET and IPNB were immunophenotypically positive for CK19, suggesting that NET originated from the transformation of biliary tumor cells constituting IPNB. However, the NET component exhibited extensive proliferation and broad stromal invasion of the bile duct wall compared with IPNB, suggesting that the NET component defined the prognosis in this case.

In the pancreas, concomitant intraductal papillary mucinous neoplasm (IPMN; a pancreatic counterpart of IPNB) and pancreatic neuroendocrine tumor (pNET) are frequent, and the prevalence of pNET with IPMN was 2.8%-4.6% according to previous reports[9,10]. However, IPNB accompanying NET has not been previously reported. This case provides important insight into the histogenesis of biliary NET.

In summary, IPNB accompanying biliary MANEC in this patient provided important information for the elucidation of the histogenesis of NET in biliary tumors.

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CASE REPORT

Behçet's disease complicated by multiple aseptic abscesses of the liver and spleen

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Abstract

Aseptic abscesses are an emergent entity and have been described in inflammatory bowel disease, especially in Crohn's disease, and in other diseases. However, aseptic abscesses associated with Behçet's disease are extremely rare. We report a Japanese male diagnosed with an incomplete type of Behçet's disease who developed multiple aseptic abscesses of the spleen and liver. In 2002, the spleen abscesses were accompanied by paroxysmal oral aphthous ulcers and erythema nodosum. As the patient's response to antibiotic treatment was inadequate, a splenectomy was performed. Severe inflammatory cell infiltration, largely of polymorphonuclear neutrophils, was observed without evidence of bacterial or fungal growth. Although the patient had no history of ocular symptoms or genital ulcers, a diagnosis of incomplete Behçet's disease was made according to the Japanese diagnostic criteria because of the presence of paroxysmal arthritis and epididymitis since 2002. In 2005, multiple liver abscesses developed with right hypochondrial pain and seemed to be attributed to Behçet's disease because the abscesses yielded negative results during a microbiologic investigation and failed to go into remission under antibiotic therapy. Oral prednisone (15 mg/d) was started in May 2006, and the abscesses dramatically disappeared 4 wk after treatment. Although the patient had a relapse of the liver abscesses in association with the tapering of prednisone, the augmentation of prednisone dosage yielded a response. The abscesses of the liver and spleen were strongly suggested to be attributed to Behçet's disease. Clinician should be aware of the existence of aseptic abscesses as uncommon manifestations of Behçet's disease.

Key words: Behçet's disease; Aseptic abscess; Spleen; Liver; Prednisone

Core tip: We report a Japanese male diagnosed with an incomplete type of Behçet's disease who developed multiple aseptic abscesses of the spleen and liver. Spleen abscesses developed with paroxysmal oral aphthous ulcers and erythema nodosum. A splenectomy was performed, and severe neutrophil infiltration was observed without evidence of bacterial or fungal growth. Multiple liver abscesses also developed with right hypochondrial pain and seemed to be attributed to Behçet's disease because the abscesses yielded negative results during a microbiologic investigation. Oral prednisone (15 mg/d) was started, and the abscesses dramatically disappeared. Clinicians should be aware of the existence of aseptic abscesses as uncommon manifestations of Behçet's disease.

Maeshima K, Ishii K, Inoue M, Himeno K, Seike M. Behçet's disease complicated by multiple aseptic abscesses of the liver and spleen. *World J Gastroenterol* 2013; 19(20): 3165-3168 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3165.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3165

INTRODUCTION

Behçet's disease (BD) is a systemic inflammatory disease commonly characterized by oral and genital ulcerations, with involvement of the skin and eye. The manifestations of BD are protean, and all symptoms and signs tend to recur either alone or in combination. Aseptic abscesses (AAs) are characterized by deep, sterile, round lesions consisting of neutrophil infiltration that do not respond to antibiotic therapy but improve with corticosteroid and immunosuppressive drugs. Clinical reports and case series concerning AAs in patients with inflammatory bowel disease, neutrophilic dermatoses, and other diseases have been published[1-5]. Although BD belongs to a group of neutrophilic dermatoses, AAs associated with BD are extremely rare. Here, we describe a patient with BD complicated by AAs of the liver and spleen, which were successfully treated with corticosteroid therapy.

CASE REPORT

In February 2006, a 20-year-old Japanese male suffering from multiple liver abscesses associated with sporadic fever, right hypochondrial pain, and elevated inflammatory markers was admitted to our hospital. In 2002, at the age of 16, he developed oral aphthous ulcers, erythema nodosum, pericardial effusion, and multiple spleen abscesses (Figure 1A). As for the aphthous ulcers and erythema nodosum, resolution occurred spontaneously. Pericardiocentesis was performed. Effusive pericarditis was thought to be the cause of the pericardial fluid; however, it did not reaccumulate after pericardiocentesis. Because empirical antibiotic therapy had the least effect and symptoms such as fever and left hypochondrial pain persisted, an open splenectomy was performed in October 2002. Upon macroscopic examination, sections of the resected spleen showed multiple yellow nodular lesions (10-20 mm in diameter) (Figure 1B). Pathological examination confirmed the presence of severe inflammatory cell infiltration, largely of polymorphonuclear neutrophils, without evidence of bacterial or fungal growth. In addition to oral aphthosis and erythema nodosum, the primary symptoms of BD, paroxysmal arthritis and epididymitis have occurred since 2002. Although the patient had no history of ocular symptoms or genital ulcers, a diagnosis of incomplete BD was made according to the criteria of the BD Research Committee of Japan[6]. Spleen abscesses were suspected to be attributed to BD, but the association was difficult to prove. Paroxysmal oral aphthosis and arthritis persisted after splenectomy, and at the age of 19 in March 2005, multiple abscesses with an aggressive inflammatory response developed in the liver. Antibiotics were unable to demonstrate any benefit, and the liver abscesses seemed to be attributed to BD. Colchicine (with a 1.5 mg maximum daily dose) was started in July 2005. However, the patient had elevated inflammatory markers, fever, and right hypochondrial pain.

Upon physical examination, the patient did not present with fever, oral aphthosis, erythema nodosum, articular swelling, or abdominal tenderness at the time. The

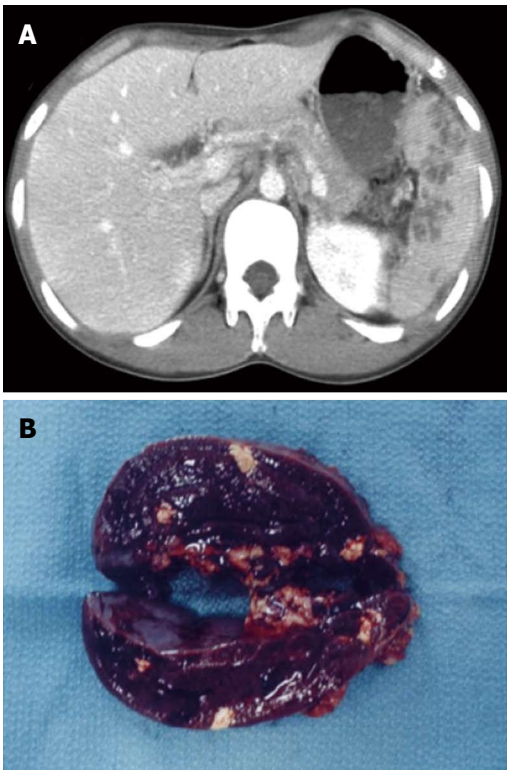

Figure 1 Contrast-enhanced abdominal computed tomography scan showing multiple spleen abscesses (A), and macroscopic findings of the cut surface of the resected spleen (B).

findings from the laboratory examinations are summarized as follows: white blood cell count, 13400/L (normal: 4000-9000/L) with 69% neutrophils; hemoglobin, 14.8 g/dL (normal: 12.0-16.0 g/dL); platelets, 57.1 × 10^4/L (normal: 14.0-40.0 × 10^4/L); aspartate aminotransferase, 21 U/L (normal: 10-40 U/L); alanine aminotransferase, 30 U/L (normal: 5-40 U/L); lactate dehydrogenase, 137 U/L (normal: 115-245 U/L); total bilirubin, 0.4 mg/dL (normal: 0.3-1.2 mg/dL); alkaline phosphatase, 583 U/L (normal: 115-359 U/L); gamma-glutamyl transferase, 123 U/L (normal: ≤ 70 U/L); and C-reactive protein (CRP), 11.0 mg/dL (normal: < 0.3 mg/dL). The results of the kidney function tests were within the normal limits. Antinuclear antibodies (speckled staining pattern) showed a 40-fold positive result (normal: < 40-fold). Rheumatoid factor, antineutrophil cytoplasmic antibodies, and HLA-B51 antigen were negative (HLA-A24, B52, and B60 antigens were positive). Colonoscopy showed only a small aphthous ulcer in the terminal ileum; pathological examination showed non-specific chronic inflammation without any evidence of Crohn's disease (CD), such as noncaseating granuloma. Blood and urine cultures were negative. An abdominal ultrasound scan was performed, which showed multiple round lesions in the liver. Contrast-enhanced abdominal computed tomography scan demonstrated multiple areas of low-attenuation with ring enhancement in the liver (Figure 2A). Despite empirical antibiotic therapy, no clinical improvement was achieved. Ultrasound-guided percutaneous aspiration of the abscess yielded pus containing numerous neutrophils; no

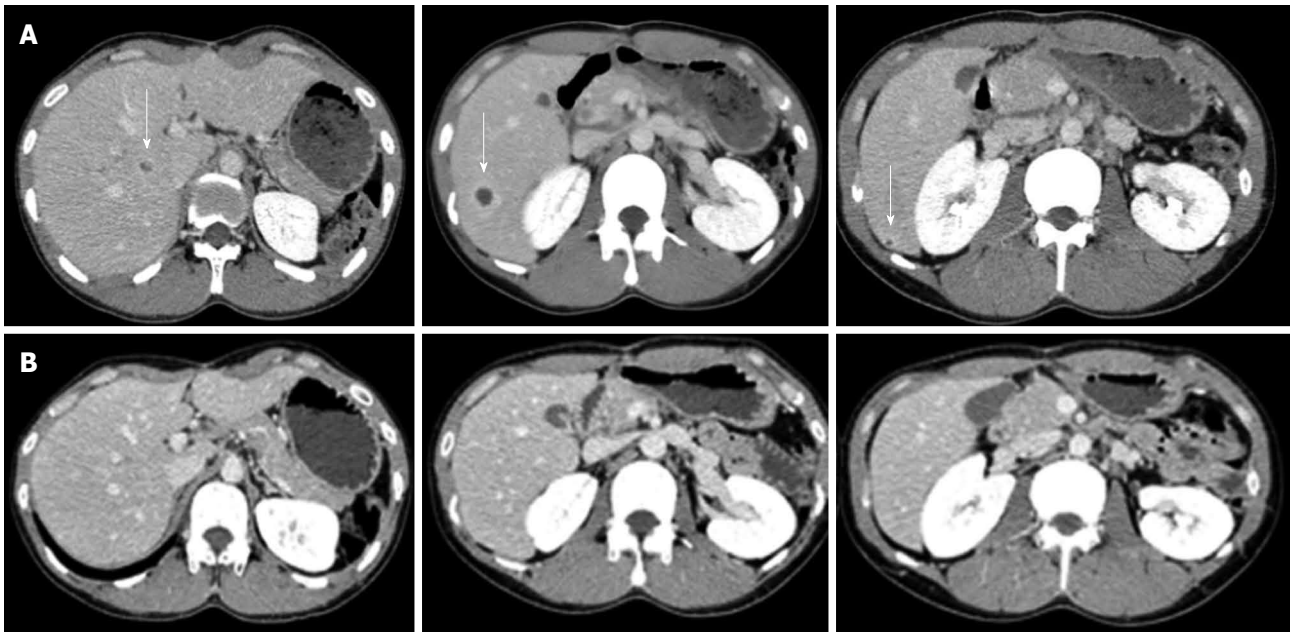

Figure 2 Contrast-enhanced abdominal computed tomography scan showing multiple liver abscesses (arrow) before (A) and 4 wk after corticosteroid therapy (B). Multiple areas of low-attenuation with ring enhancement were seen in the liver as indicated by the arrows (A). However, the areas vanished after treatment (B).

microbes were found in the culture. A fine needle biopsy of the liver lesions was performed, and a pathological examination revealed necrotic tissue containing inflammatory cells. Based on this evidence, these lesions were interpreted as aseptic liver abscesses associated with BD, and oral low-dose prednisone (15 mg/d), in addition to colchicine, was initiated in May 2006. A rapid improvement in the patient's symptoms occurred; the levels of CRP and liver enzymes reached normal range, and the liver abscesses disappeared within 4 wk of the initiation of corticosteroid therapy (Figure 2B). The dose of prednisone was gradually tapered; at 7.5 mg/d prednisone, however, the CRP level increased again, requiring a higher dose of prednisone. Therefore, the patient was maintained on low-dose prednisone therapy (10-12.5 mg/d) to restrain the inflammatory response. Elevated CRP was observed without obvious abnormality in August 2010, and the recurrence of AAs in the liver was detected by abdominal CT without abdominal symptoms in August 2011. After escalating the dose of prednisone to 20 mg/d, the liver abscesses vanished once again. The dose of prednisone was gradually tapered to 15 mg/d, and complete clearance of the liver lesions was achieved in February 2012.

DISCUSSION

We present a patient with BD who developed AAs in the spleen and liver. AAs are an emergent entity. This condition has been described in inflammatory bowel disease (IBD), especially in CD, as well as in other diseases such as Sweet's syndrome and pyoderma gangrenosum[1-5]. Most patients with AAs have some underlying disease, and it has been proposed that AAs belong to the spectrum of autoinflammatory multifactorial disorders[1]. The diagnosis of AAs is of exclusion on the basis of the following criteria suggested by André *et al*[1]: (1) deep abscess(es) upon radiologic examination, with neutrophilic features proven by surgical pathology or aspiration when performed; (2) negative blood cultures, negative serologic tests for bacteria, and, when surgical procedure or aspiration are performed, sterile standard, acid-fast bacillus and fungal cultures of pus; (3) failure of antibiotic therapy; and (4) rapid improvement by corticosteroids, sometimes in combination with immunosuppressive drugs. The main clinical manifestations of AAs are fever, abdominal pain, and weight loss. A rare case of BD complicated by multiple intrahepatic abscesses, which was dramatically resolved by antibiotic therapy, was reported[7]. There is also a case report of BD that was suspected to be complicated by sterile cerebral abscesses, although the clinical state of the patient gradually improved without immunosuppressive therapy[8]. In our patient, the liver and spleen abscesses were negative upon microbiologic investigation, and remission was not achieved under antibiotic therapy. Furthermore, the rapid resolution of the abscesses with corticosteroid therapy, even at the time of disease relapse in association with the tapering of prednisone, also favored a diagnosis of BD-associated AAs. Based on a PubMed search of the literature, our patient is the first unfailing case of sterile visceral abscesses associated with BD.

A differential diagnosis of CD *vs* BD can be difficult. Although ulcerative lesions at the ileocecal area are common in both diseases, we diagnosed the patient as having intestinal BD because of a lack of factors suggestive of CD: there was only one ulcerative lesion, its shape was not longitudinal, and histopathological examination showed no granulomatous lesions. It is also often difficult to dif-

ferentiate incomplete types of BD from Sweet's syndrome because some overlapping manifestations exist between BD and Sweet's disease. Unfortunately, a skin biopsy was not performed. However, we are convinced of the diagnosis of incomplete BD in this case because our patient had a chronic course with remissions and relapses, as well as a history of epididymitis and pericarditis, found in relatively rare manifestations of BD[9].

Neutrophilic dermatoses are well-recognized cutaneous manifestations of systemic diseases such as IBD, and the wide spectrum of the disease includes Sweet's syndrome and pyoderma gangrenosum. These diseases may share common features such as sterile infiltration of polymorphonuclear leukocytes; BD is analogous to this condition[10]. Extracutaneous neutrophilic infiltrates are observed in all forms of neutrophilic dermatoses, but they predominate in Sweet's syndrome[11]. Sweet's syndrome associated with BD has been reported, and there may be similarities in the pathogenesis of BD and Sweet's disease[12]. Therefore, it seems reasonable that BD was complicated by AAs, although the mechanism remains unclear. Classically, Th1 immune response polarization has been known to be the main characteristic of BD immunopathogenesis[13]. The interleukin 17 (IL-17)-mediated (Th17) immune response may also contribute to immunological aberrations of BD[14]. IL-8-producing T cells were suggested to orchestrate neutrophil-rich pathologies of BD[15]. It was proposed that liver disease occurring in IBD is mediated by an aberrant homing of gut-derived T cells to the liver, with subsequent extensive lymphocyte infiltration of the liver[16]. Therefore, the mechanism by which AAs of the liver and spleen occurred in our case might be explained by an aberrant T cell-mediated immune response.

A patient with CD and associated AAs who underwent successful splenectomy was reported[17]. However, in our study, the patient developed AAs in the liver after splenectomy and finally needed immunosuppressive therapy. The risks of postoperative infection and thrombosis after splenectomy are now widely accepted. Accordingly, splenectomy as a treatment option might not be advisable in cases of AAs associated with BD.

In conclusion, we present a case of aseptic liver and spleen abscesses as the presenting picture of incomplete BD, in which complete remission was only obtained by corticosteroid therapy. The current case illustrates the possibility of the existence of AAs as uncommon manifestations of BD. Thus, physicians should be aware of this possibility to avoid a delay in diagnosis and unnecessarily aggressive therapies such as splenectomy.

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CASE REPORT

Esophageal reconstruction with remnant stomach: A case report and review of literature

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Author contributions: Xie SP, Huang J and Cheng BC designed the report; Xie SP, Fan GH, Kang GJ and Geng Q managed the patients; Huang J performed surgical operation; Xie SP, Huang J and Cheng BC organized the report; Xie SP wrote paper.

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Abstract

The number of patients developing esophageal cancer after gastrectomy has increased. However, gastric remnant is very rarely used for reconstruction in esophageal cancer surgery because of the risk of anastomotic leakage resulting from insufficient blood flow. We present a case of esophageal cancer using gastric remnant for esophageal substitution after distal gastrectomy in a 57-year-old man who presented with a 1-month history of mild dysphagia and a background history of alcohol abuse. Gastroscopy showed a 1.2 cm × 1.0 cm bulge tumor of the lower third esophagus with the upper margin located 39 cm from the dental arcade. Computed tomography of the chest showed lower third esophageal wall thickening. The patient underwent en bloc radical esophagectomy with a two-field lymph node dissection of the upper abdomen and mediastinum *via* a left-sided posterolateral thoracotomy through the seventh intercostal space. The upper end of the esophagus was resected 5 cm above the tumor. The gastric remnant was used for reconstruction of the esophago-gastrostomy and placed in the left thoracic cavity. The patient started a liquid diet on postoperative day 8 and was discharged on the 10th postoperative day without complications. In this report, we demonstrate that the gastric remnant may be used for reconstruction in patients with esophageal cancer as a substitute organ after distal gastrectomy.

Key words: Gastric remnant; Distal; Gastrectomy; Esophageal cancer; Substitution

Core tip: Gastric remnant is very rarely used for reconstruction in esophageal cancer surgery because of the risk of anastomotic leakage resulting from insufficient blood flow. We present a case of esophageal cancer using gastric remnant for esophageal substitution after distal gastrectomy in a 57-year-old man, who was successfully treated with esophagectomy and remnant stomach reconstruction without micro-vascular anastomosis. The gastric remnant may be used for reconstruction in patients with esophageal cancer as a substitute organ after distal gastrectomy, with rapid recovery of bowel function and shorter hospital stay.

Xie SP, Fan GH, Kang GJ, Geng Q, Huang J, Cheng BC. Esophageal reconstruction with remnant stomach: A case report and review of literature. *World J Gastroenterol* 2013; 19(20): 3169-3172 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i20/3169.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i20.3169

INTRODUCTION

The number of patients developing esophageal cancer after gastrectomy has increased. Reconstruction is a challenge in esophageal cancer surgery when a previous subtotal gastric resection has been performed. In general, there has been a tendency to select the colon or jejunum

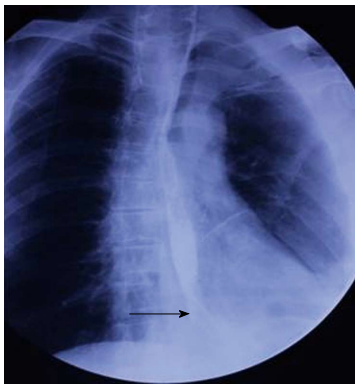

Figure 1 Barium esophagography. Esophagography shows a type 1 tumor, 1.5 cm in length, in the lower thoracic esophagus (arrow).

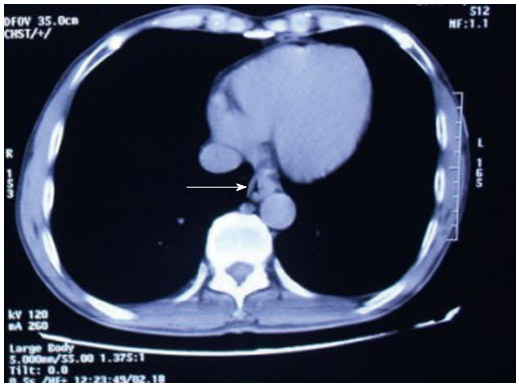

Figure 2 Thoracic computed tomography shows a tumor 1 cm in diameter (arrow).

for substitution of the gastric tube, in order to avoid anastomotic leakage resulting from insufficient blood flow, which requires complicated operative procedures and leads to higher operative morbidity and mortality[1,2]. Herein, we report a patient with esophageal cancer and previous distal gastrectomy, who was successfully treated with esophagectomy and remnant stomach reconstruction without micro-vascular anastomosis.

CASE REPORT

A 57-year-old man had undergone distal gastrectomy with lymph node dissection 7 years previously for gastric cancer with a Billroth Ⅱ anastomosis, pathologically diagnosed as highly differentiated adenocarcinoma, pT2N0M0; stage I b, according to the 7th edition of the UICC-TNM Classification of Malignant Tumors[3]. He was admitted to our hospital with a history of mild dysphagia with solids only for one month. His medical history was notable for alcohol abuse. Physical examination of the chest and abdomen revealed no abnormal findings. Barium esophagography revealed an approximately 1 cm arc filling defect in 1/3 of the lower esophagus with local mucosal damage and wall stiffness. The size of the gastric remnant was measured before surgery with contrast barium esophagography X-ray and was found to be 10 cm in length at the lesser curvature (Figure 1). Computed

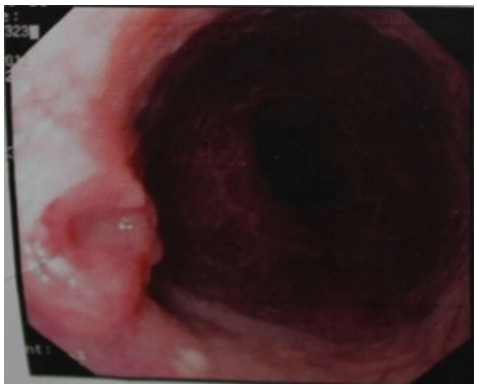

Figure 3 Upper gastrointestinal endoscopy shows a tumor 39 cm from the incisor, the anterior wall of the esophagus has a lip bulge about 1.2 cm × 1.0 cm.

tomography of the chest showed thickening of the lower third of the esophageal wall (Figure 2). Gastroscopy confirmed a 1.2 cm × 1.0 cm bulge tumor in the lower third of the esophagus with the upper margin located 39 cm from the dental arcade (Figure 3). The lesion did not stain with Lugol's solution. Pathological examination of the biopsy specimen revealed squamous cell carcinoma. Exclusion of cerebral, abdominal, skeletal, lymph node and other distal metastases (M0) was accomplished using [^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography, and distant metastases to other organs were not found.

Prior to surgery, the complete colon was examined by endoscopy, and the bowel was prepared by mechanical cleansing. The patient underwent en bloc radical esophagectomy *via* a left-sided posterolateral thoracotomy through the 7th intercostal space, with a two-field lymph node dissection in the upper abdomen and mediastinum. The upper end of the esophagus was resected 5 cm above the tumor, and the left gastric artery and short gastric artery were then divided. The remnant stomach was freed up to the gastro-esophageal junction, and appeared rosy with adequate bloody supply probably due to the efferent jejunal flap with its wide gastrojejunal anastomosis (Figure 4). We decided to preserve the remnant stomach for reconstruction. The jejunum was freed from surrounding tissues. A gastric tube was extended far enough to reach the proximal esophagus. An esophagogastric anastomosis was performed mechanically under the level of the left inferior pulmonary vein using a circular stapler by an anterior gastrotomy to the high point of the fundus of the gastric remnant. The gastric remnant was placed in the left thoracic cavity. A micro-vascular anastomosis was not performed. Enteral nutrition therapy was *via* a jejunal stoma. The operative time was 266 min and the estimated blood loss was 435 mL.

Histologically, the tumor was diagnosed as a highly differentiated squamous cell carcinoma with tumor-free margins (Figure 5), and metastases in the abdomen and mediastinum lymph node were not found. The pathological stage was pT2N0M0; stage I b, according to the 7th edition of the UICC-TNM Classification of Malignant Tumors[3].

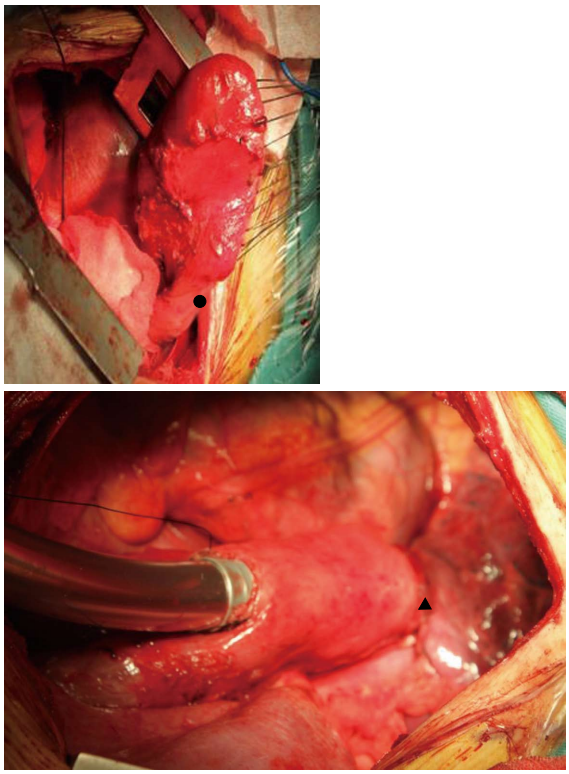

Figure 4 Esophagus is anastomosed to the remnant stomach. During surgery, after cutting the left, right and short gastric vessels, the remnant stomach relies on the blood supply of the anastomotic stoma alone and is still rosy. Circle: Jejunum-stomach anastomotic stoma; Triangle: Esophagus-remnant stomach anastomotic stoma.

The postoperative course was uneventful, with no complications resulting from insufficient blood supply. The integrity of the anastomosis of the esophago-gastrostomy was confirmed by water soluble contrast radiography on postoperative day 7, with no signs of leakage or stricture (Figure 6). The patient started a liquid diet the following day and was discharged on the 10th postoperative day without complications. On follow-up at 6 mo after surgery, the patient was alive with no evidence of recurrence. He had improved food intake; mainly managing a solid diet with an average intake per meal of approximately 450 mL. Barium esophagography confirmed the absence of stricture, with efficient emptying of the remnant stomach.

DISCUSSION

It was first reported in 1970 that a high incidence of gastrectomy (8.7%) was found in patients with esophageal cancer. Although surgical resection is the standard treatment for esophageal cancer after subtotal gastrectomy, the optimal management of this disease, including the surgical approach and the conduit for reconstruction, remains controversial. For patients with esophageal cancer and a history of gastrectomy, the esophagus is frequently reconstructed using colon interposition with a vascular pedicle[4]. The disadvantages of colon interposition include a long operative time for colon mobilization, highly

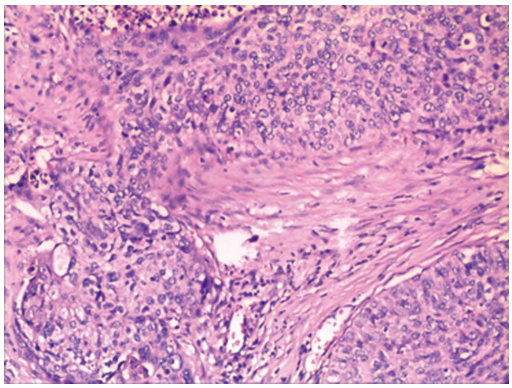

Figure 5 The tumor was diagnosed as highly differentiated squamous cell carcinoma.

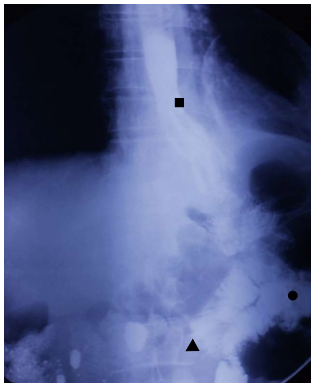

Figure 6 Barium esophagography on the 7th postoperative day. Esophagography shows that barium passes smoothly through the anastomotic stoma, without leakage. Square: Anastomotic stoma; Triangle: Afferent loop of jejunum; Circle: Efferent loop of jejunum.

invasive procedures and additional anastomosis, which increase the incidence of postoperative complications[5]. Increased blood loss was observed when resolving adhesions in the upper abdominal cavity caused by gastrectomy. When using the remnant stomach, the procedure is easier, and the aims are to minimize surgical insults and to maximize the patient's quality of life. A reduction in the number of surgical maneuvers below the transverse colon and fewer bowel anastomoses represent real advantages[6]. In addition, use of the gastric remnant for reconstruction may offer substantial advantages to the elderly population in terms of fewer cardiac and pulmonary complications, rapid recovery of bowel function, shorter hospital stay, and a faster return to physical activities.

A remnant stomach can last for more than five years before the diagnosis of esophageal cancer. Establishment of the collateral circulation is important for healing of the anastomotic stoma. The blood supply of the remnant stomach may be maintained by the reconstituted microvascular supply from the widely anastomosed jejunal loop or vascular adaptation of the stomach and anastomotic site[6], which may enhance perfusion of the gastric tube at the time of anastomosis and thus decrease anastomotic complications after esophagogastrectomy. Reavis demonstrated that gastrointestinal anastomoses were associated

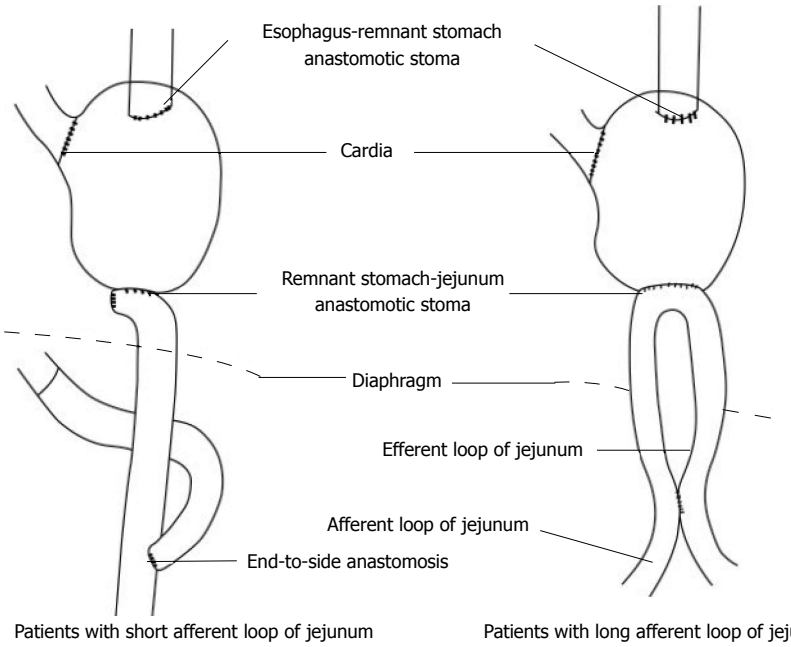

Figure 7 Scheme of the resection area of the gastric remnant and the esophagus (pre-operative), and reconstruction of the organs (post-operative).

with both vasodilation and angiogenesis and resulted in increased blood flow to the gastric fundus prior to esophagogastric anastomosis in animals, which translated into a decrease in anastomotic dehiscence[7]. In our case, wide gastrojejunal anastomosis was seen in this operation. During surgery, 30 min after the left, right and short gastric vessels were cut, the remnant stomach was still rosy (Figure 5). We think that this wide anastomosis maintained the blood supply in the remnant stomach by reconstituting the intramural network. No complications occurred during the postoperative period, and barium esophagography on the 7th postoperative day showed that barium passed smoothly through the anastomotic stoma, without leakage. Preoperative bowel preparation should be conducted in such patients. If a poor blood supply in the remnant stomach is seen during surgery, then colon interposition should be used to replace esophageal surgery.

If the remnant stomach can not be lifted to the thorax to perform tension-free anastomosis with the esophagus during esophagectomy, the length of the afferent loop of the jejunum should be observed, if the afferent loop is short, it should be cut at the anastomotic stoma and an end-to-side anastomosis with the efferent loop of the distal jejunum should be performed; if the afferent loop is long, a side-to-side anastomosis should be performed 10-15 cm from the afferent and efferent loops, thus increasing the lifting height of the remnant stomach and jejunal loop (Figure 7).

We report a patient with esophageal cancer and previous distal gastrectomy who was successfully treated with esophagectomy and remnant stomach reconstruction without micro-vascular anastomosis. It is important to select an appropriate operative method for patients with esophageal cancer after distal gastrectomy, and its location and stage must be determined. The remainder of the stomach can be used as an esophageal substitute depending on the curability of the esophageal cancer and the blood supply, which allows rapid recovery of bowel function and shorter hospital stay.

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