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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 33 September 7, 2010

EDITORIAL

4115 Delayed assessment and eager adoption of laparoscopic cholecystectomy: Implications for developing surgical technologies
Allori AC, Leitman IM, Heitman E

4123 Treatment of liver hydatidosis: How to treat an asymptomatic carrier?
Frider B, Larrieu E

REVIEW

4130 Epstein-Barr virus: Silent companion or causative agent of chronic liver disease?
Petrova M, Kamburov V

ORIGINAL ARTICLE

4135 Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome
Nossa CW, Oberdorf WE, Yang L, Aas JA, Paster BJ, DeSantis TZ, Brodie EL, Malamud D, Poles MA, Pei Z

4145 Effect of probiotics on pro-inflammatory cytokines and NF-κB activation in ulcerative colitis
Hegazy SK, El-Bedewy MM

BRIEF ARTICLE

4152 Chemoprotective effects of curcumin in esophageal epithelial cells exposed to bile acids
Bower MR, Aiyer HS, Li Y, Martin RCG

4159 ESWL for difficult bile duct stones: A 15-year single centre experience
Muratori R, Azzaroli F, Buonfiglioli F, Alessandrelli F, Cecinato P, Mazzella G, Roda E

4164 Outcome of surgical treatment of intestinal perforation in typhoid fever
Sümer A, Kemik Ö, Dülger AC, Olmez A, Hasirci I, Kişli E, Bayrak V, Bulut G, Kotan Ç

4169 Non-alcoholic steatohepatitis and influence of age and gender on histopathologic findings
Daryani NE, Daryani NE, Alavian SM, Zare A, Fereshtehnejad SM, Keramati MR, Pashaei MR, Habibollahi P

4176 Age-dependent eradication of *Helicobacter pylori* in Japanese patients
Mamori S, Higashida A, Kawara F, Ohnishi K, Takeda A, Senda E, Ashida C, Yamada H

4180 Efficient hemostatic method for endoscopic submucosal dissection of colorectal tumors
Yoshida N, Naito Y, Kugai M, Inoue K, Wakabayashi N, Yagi N, Yanagisawa A, Yoshikawa T

4187 Diagnostic sensitivity of imaging modalities for hepatocellular carcinoma smaller than 2 cm
Mita K, Kim SR, Kudo M, Imoto S, Nakajima T, Ando K, Fukuda K, Matsuoka T, Maekawa Y, Hayashi Y

4193 Effect of *Arctium lappa* L. in the dextran sulfate sodium colitis mouse model
Huang TC, Tsai SS, Liu LF, Liu YL, Liu HJ, Chuang KP

4200 Conservative management of chronic gastric volvulus: 44 cases over 5 years
Hsu YC, Perng CL, Chen CK, Tsai JJ, Lin HJ

4206 A newly designed big cup nitinol stent for gastric outlet obstruction
Shi D, Liao SH, Geng JP

4210 Relationship between genetic polymorphisms of ALDH2 and ADH1B and esophageal cancer risk: A meta-analysis
Yang SJ, Yokoyama A, Yokoyama T, Huang YC, Wu SY, Shao Y, Niu J, Wang J, Liu Y, Zhou XQ, Yang CX

4221 Prevalence of irritable bowel syndrome in Chinese college and university students assessed using Rome Ⅲ criteria
Dong YY, Zuo XL, Li CQ, Yu YB, Zhao QJ, Li YQ

4227 Efficacy evaluation of imatinib treatment in patients with gastrointestinal stromal tumors: A meta-analysis
Chen P, Zong L, Zhao W, Shi L

CASE REPORT

4233 Agressive inflammatory myofibroblastic tumor of the liver with underlying schistosomiasis: A case report
Pannain VL, Passos JV, Rocha Filho A, Villela-Nogueira C, Caroli-Bottino A

4237 Multi-site abdominal tuberculosis mimics malignancy on ^{18}F-FDG PET/CT: Report of three cases
Tian G, Xiao Y, Chen B, Guan H, Deng QY

World Journal of Gastroenterology
Volume 16 Number 33 September 7, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

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EDITORIAL

Delayed assessment and eager adoption of laparoscopic cholecystectomy: Implications for developing surgical technologies

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Abstract

Despite the prevailing emphasis in the medical literature on establishing evidence, many changes in the practice of surgery have not been achieved using proper evidence-based assessment. This paper examines the adoption of laparoscopic cholecystectomy (LC) into regular use for the treatment of cholecystitis and the process of its acceptance, focusing on the limited role of technology assessment in its appraisal. A review of the published medical literature concerning LC was performed. Approximately 3000 studies of LC have been conducted since 1985, and there have been nearly 8500 publications to date. As LC was adopted enthusiastically into practice, the results of outcome studies generally showed that it compared favorably with the traditional, open cholecystectomy with regard to mortality, complications, and length of hospital stay. However, despite the rapid general agreement on surgical technique, efficacy, and appropriateness, there remained lingering doubts about safety, outcomes, and cost of the procedure that suggested that essential research questions were ignored even as the procedure became standard. Using LC as a case study, there are important lessons to be learned about the need for important guidelines for surgical innovation and the adoption of minimally invasive surgical techniques into current clinical and surgical practice. We highlight one recent example, natural orifice transluminal endoscopic surgery and how necessary it is to properly evaluate this new technology before it is accepted as a safe and effective surgical option.

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Key words: Laparoscopy; Endoscopy; Minimally invasive surgery; Cholecystectomy; Natural orifice transluminal endoscopic surgery; Evidence-based medicine; Technology assessment; Comparative effectiveness research

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INTRODUCTION

With the introduction of laparoscopic cholecystectomy (LC) in the late 1980s, gastrointestinal surgery was forever changed. Arguably more than any other laparoscopic procedure, LC drove the endoscopic revolution. How-

ever, nothing has been more astounding than the speed with which the procedure was unanimously hailed as the new gold standard for surgical gallbladder removal.

Recently, the *Archives of Surgery* published the results of a randomized clinical trial comparing LC with open cholecystectomy (OC), which concluded that a small-incision variant of the open procedure is just as efficacious as LC with regard to primary and secondary clinical outcome measures, thus questioning the status of LC as the gold standard[1]. This article is notable not just for its unexpected conclusion but because it was published in 2008 - over 20 years since the first LC in France and 15 years since its global dissemination. Other studies have appeared in recent years, with similar conclusions[2-8]. These studies give rise to several questions: Why conduct randomized, controlled trials so many years after LC's incorporation into clinical practice? How did questions regarding LC's safety and efficacy come to linger unanswered, warranting review now? On what level of evidence was LC so widely practiced?

This article examines the evidence available to surgeons during the period of LC's rapid diffusion. We conclude that there was minimal evidence to support the use of LC over OC and that the early enthusiasm for LC was largely unfounded. In particular, the quality, methodologic design, scope, and timeliness of published studies were often poor, and they failed to adequately establish LC's safety, effectiveness, or cost savings over alternative therapies.

By critically appraising the development and diffusion of LC and identifying essential research questions that were ignored, we hope to illustrate the need for assessment that may be met in development of new surgical technologies. We conclude by considering the emergence of a more recent surgical innovation - natural orifice transluminal endoscopic surgery (NOTES) - and compare its current evaluation with the historical course of LC and the standards of technology assessment.

CASE HISTORY: LAPAROSCOPIC CHOLECYSTECTOMY IN THE LITERATURE

Originally described in 1882 by Langenbuch, open cholecystectomy remained the standard treatment for cholelithiasis and other diseases of the gallbladder for over 100 years[9]. The laparoscopic alternative to the open procedure was originally developed in France by Mühe[10-13] in 1985 in an attempt to reduce post-operative morbidity and related cost. It was popularized by Mouret[14-17] and Dubois[18-22] in 1987. LC was quickly adopted as the procedure of choice in many countries, and in the United States LC accounted for 90% of all cholecystectomies a mere four years following its introduction[23,24].

LC has proven its value over time, but from an evidence-based perspective, the enthusiastic preference for LC in the 1990s was largely unfounded. The problem was not with the procedure itself, but rather the lack of data supporting it: the literature that argued for the superiority of LC over OC did not adequately document LC's safety, effectiveness, or cost savings over alternative therapies.

In reviewing the LC literature, it is helpful to consider the publication timeline in light of McKinlay's heuristic stages of medical innovation[25]: LC went through an initial stage of the "promising report" (1985-1992), a middle stage of professional and organizational adoption (1993-1995), and a late stage of observational reports and standardization (1996-1999). A contemporaneous search of Medline *via* Ovid using keyword "cholecystectomy" revealed that in these 15 years (1985-1999), 2822 articles on LC were indexed in MEDLINE, predominantly in English-language journals[26].

Remarkably, there were no publications on LC before 1990 - a good 5 years after its introduction and 2 to 3 years after its world-wide popularization. Of the 228 articles published between 1990 and 1993, the majority were concerned with technique (58 articles, 25%), safety (39 articles, 17%), and instrumentation (14 articles, 6%). Despite the fact that LC was developed partly in response to economic concerns, only 4 articles (1.8%) evaluated its economic aspects or made any economic comparison with OC. Two articles (0.9%) described trends in practice, but only 8 (3.5%) discussed standardization of technique.

By the mid-1990s, LC had become widely adopted as the standard of care for the surgical treatment of gallbladder disease. Between 1993 and 1995, 1370 articles on LC were published. Three hundred and thirty-two (24%) of these covered safety issues, for which data were already available, although many of these articles considered special populations (e.g. the pregnant, pediatric, geriatric, or immunosuppressed patient). Another 228 (17%) discussed variations in methodology or instrumentation. Bridging these two categories were outcome-oriented studies, which consisted mainly of case reports. Despite the 10 years that had passed since its introduction, only 46 (3%) articles discussed trends in utilization or the development of standard practice. Forty-three articles (3%) focused on its economic aspects. Importantly, during this period, a number of commentaries questioned whether the overwhelming support for LC was warranted. Nonetheless, most objections were voiced only in editorials, and no studies sought to elucidate such issues systematically.

In the late phase of adoption (between 1996 and 1999), 1224 articles were published on LC. While the numbers of procedural, methodologic, and economic articles were comparable to those published in previous periods, a large percentage (294, or 24%) focused on observational accounts of safety, adverse reactions, and contraindications. This attention to safety is noticeably out of place for a technology that, after 15 years, should already have been properly evaluated for safety issues. Moreover, this ongoing concern is evidence of the rushed adoption of the LC in its earliest stage (Figure 1A). Another 29 articles (2%) in this late phase were dedicated to discussing trends in practice, standardization of methods, and utilization issues, representing increasing sentiment among surgeons and health-services researchers that there was a need for better assessment of LC.

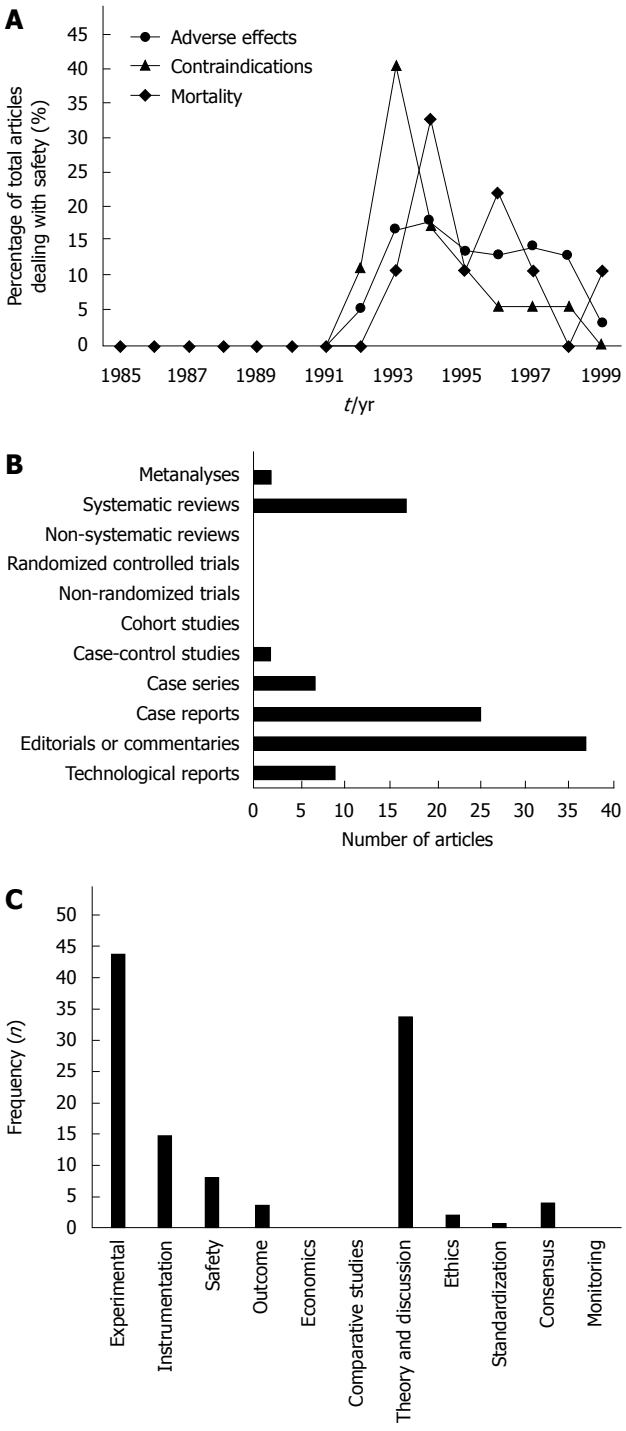

Figure 1 Evolution of technology, laparoscopic cholecystectomy and natural orifice transluminal endoscopic surgery. A: A timeline of the percentage of the total number of articles dealing with laparoscopic cholecystectomy's safety reveals the failure of the literature to satisfactorily answer essential safety issues early in the technology-assessment process; B: An evaluation of the study designs present in the natural orifice transluminal endoscopic surgery (NOTES) literature reveals that the vast majority of studies are technological reports, experimental studies in animal models or simulators, or editorials/commentaries. To date, there have been no clinical studies other than a handful of case reports/series; C: The present literature on NOTES is dedicated primarily to experimental procedures, instrumentation, and theoretical discussion such as advantages/disadvantages or indications/contraindications. Few articles have been devoted to safety or outcome studies, and no articles have compared NOTES procedures to their traditional open or laparoscopic counterparts.

Today, Medline has indexed over 8300 articles concerning various aspects of LC. The continued appearance of articles dealing with safety and effectiveness suggests lingering concerns.

CRITICAL APPRAISAL

In accordance with the principles of technology assessment[27,28], the first studies of LC evaluated the safety of the new procedure - with regard to complication rate, post-operative morbidity, and mortality - and the methods involved in the procedure itself. These studies were generally conclusive: LC was found to be a safe, viable procedure with no serious complications or post-operative morbidity, and it appeared to be as safe as the standard, open surgery. These studies also attempted to quantify such outcome variables as efficacy and effectiveness. Most outcome studies purported the main advantages of LC to be "decreased pain and disability and improved cosmesis without increased mortality or morbidity rates"[29]. Quantitative results supporting these conclusions differed from one study to another but were generally consistent: mean hospital stay for LC was reported to be one to two days, with many papers suggesting that LC could be done as an outpatient procedure; mean time to return to work after surgery was approximately 15 d[29]; post-operative pain was subjectively decreased[30]; and "quality of life" was improved[31]. However, despite the favorable results, the outcome data had serious limitations.

Study design and comparative controls

Most of the research published between 1985 and 1999 consisted of anecdotal evidence or retrospective case series. As the fervor for endoscopic surgery grew, OC was no longer being performed in adequate numbers to permit comparison studies. To complicate matters, as LC gained favor among surgeons and started to become the new standard, it became increasingly unlikely that prospective comparisons could be performed[32].

Consequently, one of the initial challenges in defining the appropriate role of LC in contemporary practice was to evaluate the outcomes and risks of OC, by which to compare outcomes from LC[32]. With great foresight, in 1992 Clavien *et al*[9] conducted a longitudinal evaluation of OC with the express purpose of serving as an historical control for future evaluations of LC. Remarkably, however, this study was rarely cited; by 1999, it had been referenced only 41 times, and fewer than half the citations were in work that addressed LC. While many LC studies alluded to vague OC statistics from historical controls, many studies failed to cite any specific sources of validated data. Without a real comparison group, the claims of LC's superiority were never truly validated.

Exclusion of confounding processes

Irrespective of methodologic inadequacies, the outcome literature also failed to account for the purported dif-

ferences between LC and OC. Specifically, some critics argued that the reported advantages of LC were not exclusive to LC, but could also have been achieved with OC, had the latter procedure and post-operative management been improved. For example, while LC was credited with shorter hospital stays, long-term post-operative observation even for standard elective OC was already viewed as unnecessary and was in the process of changing; the routine use of drains, which delayed discharge but were found to provide little benefit, was already less frequent; and routine post-operative administration of antibiotics was increasingly discouraged[33]. Therefore, some argue that the rapid adoption of LC, itself, did not deserve the credit for the dramatic changes in the care of patients, but rather served to catalyze rejection of unnecessary practices that increased costs or delayed discharge[33]. Whether or not such modifications would have yielded the same results in the absence of LC was a worthwhile question that was never answered.

Scope of assessment

Between 1985 and 1999, available studies in the LC literature assessed only a narrow scope of outcome variables (i.e. morbidity, mortality, and length of hospital stay) but ignored many other outcomes of interest (e.g. cost and important quality-of-life issues). Despite the fact that, from the very beginning, LC was purported to be more cost-effective than OC, few investigators performed economic analyses. The majority of economics-related investigations were rudimentary studies, conducted by clinicians, that discussed only the hospital costs of LC *vs* OC[34]; others considered costs indirectly *via* a cost-to-charge-ratio conversion of patient charges for both procedures[35,36]. Few of the early papers and none of the later studies gave detailed estimates of costs and no studies incorporated indirect costs or social impacts[37]. Only two robust economic analyses were performed during the period of LC's adoption: a cost-effectiveness analysis by Kesteloot *et al*[37], and a cost-utility analysis by Cook *et al*[38]. While both of these were favorable to LC, they were published in the economics literature, where they remained unknown to, or underappreciated by, those in clinical practice.

Patient-reported outcomes

Many studies advocated LC based upon "patient preference". One investigator found that patients became convinced of the benefits of LC by the surgeons' enthusiasm rather than their own understanding of the procedure, and that a thorough informed consent process eliminated this preference[39]. Most studies on LC limited patient-reported outcomes to consideration of such simple Yes/No questions as, "Are you glad you got this procedure?" or, "Would you recommend this procedure to others who suffer from gallbladder disease?"[26]. Detailed investigations of the impact of LC on patient satisfaction, quality of life, and general well-being were never performed. Lacking such analysis, these statistics cannot be used to make convincing claims regarding quality of life[28].

Reaching consensus

The first attempt to perform the necessary critical review of LC was conducted by the National Institutes of Health (NIH) in a Consensus Conference held in 1992[40]. The NIH Consensus Panel's assessment reviewed the numerous studies to date and concluded that LC was a safe and effective procedure that "leads to increased quality of life over other methods of treating gallbladder disease" (extracorporeal shock wave therapy, bile acid therapy, and open cholecystectomy). It acknowledged that the frequency of common bile duct injury was higher with LC than with OC, but suggested that this was in large part due to the experience, skill, and judgment of the surgeon[30,40]. The Panel also concluded that all patients with gallbladder disease should undergo LC in preference to OC except for the following contraindications: cardiopulmonary contraindications to a general anesthetic, hepatic cirrhosis with portal hypertension or coagulopathy, acute pancreatitis, acute gangrenous cholecystitis, septic shock, the third trimester of pregnancy, and previous upper abdominal surgery[31].

While the NIH Consensus Conference attempted to come to terms with the flood of new information regarding LC, it did not critically appraise the clinical studies that it consulted. The poor quality of evidence for LC at that time was noted by one Australian surgeon: "It is of particular note that the (NIH) review has 99 references and yet few are of real scientific substance. The published papers in this rapidly developing area still consist essentially of anecdotes. We have no adequately constructed clinical trials. We have very little good comparative study. We have essentially no long-term follow up and we have little in the way of objective measures of outcomes prepared by independent observers"[41].

Because it was based on sparse and incomplete data, the NIH consensus could be no more than "a rapid agreement for the most appropriate procedure" and amounted to little more than informed opinion[31]. Critics later concluded that, despite the "consensus," the issue was far from decided, especially with regard to such concerns as management of acute cholecystitis and choledocholithiasis and treatment of the pregnant patient[31].

Accessiblity of data

Whereas the NIH consensus was widely publicized, other better-constructed studies appear to have been less accessible to surgeons, who may not have looked at work conducted outside their discipline. As noted earlier, the Kesteloot *et al*[37] and Cook *et al*[38] studies - both far superior to any other economic study published in the surgical literature, and both favorable toward LC - were not recognized by the surgical community. By the late phase of LC's adoption in 1999, Kesteloot *et al*[37] had been cited a total of only ten times, only five of which were in surgical journals. Cook *et al*[38] was cited 15 times, but only twice in surgical journals. Although publication in peer-reviewed economics journals attested to the rigor of their research methods, an unfortunate consequence of their location

was that the studies' audience did not include the surgeons who performed LC, the hospital administrators who invested in equipment, or the third-party payors who were interested in how well LC compared to OC.

The accessibility problem is best exemplified by the excellent multi-stage prospective assessment - arguably the best study ever done on LC - published in a journal dedicated to technology assessment. In 1994 the *International Journal of Technology Assessment in Health Care* published a cautionary article on the increased use of LC following a multi-stage study commissioned to evaluate LC's impact on patients, the hospital, and staff after its introduction to the Greater Victoria Hospital Society (GVHS) in Canada in 1991[42]. In prospective, case-control fashion, the study demonstrated that cost of the LC was approximately 47% of that of OC, with the difference being attributed to reduction in length of stay. As had been observed elsewhere, operative time was found to increase by more than 20 min for LC. Post-operative hospital stay was significantly less for LC than for OC (3.2 d *vs* 13.1 d, respectively). The GVHS assessment even included qualitative patient-reported outcomes regarding need for pain medication and time to resumed normal daily activities as part of the evaluation process. Data collection was repeated annually for three years, and the integration of these multiple assessments was used incrementally to form a maturing consensus.

The study resulted in a favorable view of LC but concluded that the cost savings promised by LC "could only be realized by capitalizing on the reduced length of stay by removing the surgical beds from service" (i.e. earlier discharge)[42], not from reduced costs of the procedure itself. In the 4 years covered by the assessment, the inpatient bed complement at the GVHS decreased by 155 beds, while the number of total surgical procedures was unaffected.

However, despite the strengths of the GVHS study, it, too, appears to have gone largely unnoticed by surgeons. Similar to the two economic evaluations published in the social science literature, by the end of the late phase of adoption in 1999, the GVHS study had never been cited in any medical or surgical literature. These important and fundamental studies might have influenced the diffusion of LC, yet their very existence seems to have been unknown to the medical profession.

ANALYSIS

The conversion from open to laparoscopic cholecystectomy has been called the most "precipitous and rapid" of all changes to modern surgical medicine[32]. With its first demonstration in 1985, LC spearheaded a revolution in general surgery toward minimally invasive procedures that forever changed the nature of surgery[41,43]. A rigorous positive and normative evaluation of LC relative to OC would have indicated whether the tremendous physician preference for the laparoscopic procedure was warranted; but in the absence of meaningful outcome-based and economic research, the evaluation of LC fell short of standards of evidence-based medicine, and many important questions remained unanswered.

Why there was so little critical evaluation of LC is cause for debate. Indeed, concern for evidence-based practice and systematic evaluation of innovation was already widespread during the mid- to late-1980s when LC was popularized. First developed in the early 1970s, technology assessment had already matured to become a robust discipline essential to both public and private policy-making in many contexts[28,44,45]. Proponents of technology assessment, including the United Kingdom's National Health Service and the United States' Institute of Medicine, recognized its ability not only to examine safety and efficacy, but also to describe, both qualitatively and quantitatively, the indirect and delayed social, environmental, economic, legal, and ethical implications of a given medical technology - concepts that also bear strong relation to such clinically important concepts as quality of care, quality of life, and patient well-being in general[27,46,47].

Certainly, the case of LC was unique in many aspects. LC was conceived and popularized not at academic centers but by private clinics[32]. Its explosive rate of adoption was led by market forces[31,43]. Experimental studies were not published, and the first articles appeared several years after LC's introduction to clinical practice - quite late according to the standards of academic research, even considering the necessary time lag for publication.

Some academic surgeons did voice early concerns about LC and called for better evidence. For example, only a few years after the introduction of LC, O'Brien published his critical observations on laparoscopic surgery and the surgeons who embraced it: "There has been little shyness and little reticence by surgeons in testing the limits of (the) possibilities (of laparoscopic surgery). Any and every part of the gut from the oesophagus to the rectum can be removed endosurgically. … Trivial procedures … can be made difficult, and difficult procedures … have been made even more difficult. Common sense at times is overridden by the surgeons' tenacity and blind commitment"[41].

In a survey of British surgeons regarding the necessity of a randomized trial comparing LC and OC, McMahon noted that only 58% of responders considered such a trial to be necessary[48]. Few researchers questioned surgeons' preference for LC, despite the paucity of scientific evidence. It is likely that the necessary conceptual questions were never seriously considered because they appeared to be self-explanatory[49]. Clearly, the incentive for surgeons was to improve care for their patients, and minimally invasive surgery appeared intuitively superior to alternative treatments: it just "made sense" that a less invasive procedure would necessarily decrease morbidity and would be associated with faster recovery.

It seemed to make sense to patients also: LC quickly became, in popular opinion, the ideal for surgical treatment of gallbladder disease. Because neither surgeons nor patients could claim equipoise any longer, a prospective, randomized controlled trial comparing LC to OC quickly became ethically impossible[50]. Despite long-standing

criticism over the details of its assumed superiority, the claimed advantages of LC over OC were never elucidated, and the laparoscopic revolution continued unabated.

IMPLICATIONS FOR DEVELOPING SURGICAL TECHNOLOGIES

With the emergence of further advances in minimally invasive technology, approaches such as single-port surgery and NOTES were inevitable. In the case of NOTES, there is already excitement over its potential applications[51], and much experimental work has already been started[52-62]. The first report of NOTES in an animal model appeared in 2002[63], and the first report of NOTES in clinical patient care was published in 2004[64]. Ninety-nine articles were published in the next five years. A search was conducted on March 20, 2009, in MEDLINE *via* Ovid using the intersection of medical subject headings (laparoscopy, endoscopy, or minimally invasive) with keywords (endoluminal, transluminal, translumenal, natural orifice, peroral, transgastric, transanal, transrectal, transvaginal, transcolonic, or transvesical) with results through January 2008. Standard endoscopic procedures such as percutaneous endoscopic gastrostomy, endoscopic polypectomy, biopsy, needle aspiration, and bilio-pancreatic procedures such as endoscopic retrograde cholangiopancreatography were then manually excluded from search results. The vast majority were descriptions of animal models (37%), non-systematic reviews (17%), or editorials/commentaries (25%). Only 7 case reports and 2 case series dealt with human patients, and only two systematic reviews were indexed (Figure 1B).

These articles discussed novel, experimental procedures or instrumentation, followed by theoretical discussion such as advantages/disadvantages and indications/contraindications. However, of the 37 experimental studies, only 8 (21%) attempted to evaluate safety, and only 3 (8%) evaluated outcome. There are no definitive safety or outcome data yet available for human patients (Figure 1C). Nevertheless, the first case report of successful transvaginal cholecystectomy in a human being was published in 2007[52].

Recognizing the paucity of safety and outcome data, and perhaps learning from the experience with LC in the 1990s, clinicians have sought to set early responsible guidelines for research and development of NOTES. To their credit, in 2005, surgeons and gastroenterologists formed the Natural Orifice Surgery Consortium for Assessment and Research (NOSCAR)[65,66]. Their SAGES/ASGE white paper[66] outlined the state of NOTES procedures and specified research that must still be done prior to the stepwise introduction of NOTES into clinical practice.

But is NOTES really ready for prime time? Private and academic medical centers are already hosting NOTES training seminars for surgeons, gastroenterologists, and even residents in these specialties to train them for this next frontier in minimally invasive surgery[67]. There is tremendous appeal in "surgery without scars", but proceeding directly from promising reports and the short-term evaluation of NOTES' basic safety to its incorporation into clinical practice once again skips the crucial aspects of an evidence-based technology assessment that would justify its adoption.

WHERE DO WE GO FROM HERE?

McKinlay has argued that "there is a double standard in the acceptance of reports of surgical *vs* medical treatments, and that this arises from professional and lay attitudes"[25]. However, the tenets of evidence-based medicine and the process of technology assessment are just as pertinent to the field of surgery as to other medical disciplines - and their warning resounds just as clearly. There should be a strict evidence-based progression from early safety studies to subsequent comparative outcome studies and economic analyses. Systematic critical appraisal of the evidence should be conducted periodically and favor large, contemporaneous, prospective, blinded, randomized and controlled studies over studies using other methodological approaches[44]. Assessments may be performed at various stages of maturity and diffusion over the lifetime of a given medical technology. Even when a technology is assessed in its earliest stages of development, it is possible and important to articulate the goals for basic outcome assessment set during these conceptual and experimental stages[44,49,68,69].

As a testament to the importance of formal technology assessment in health policy and planning, many countries have established centers for comparative effectiveness research - such as the National Institute for Health and Clinical Excellence in the United Kingdom, the Canadian Coordinating Office for Health Technology Assessment in Canada, and the Institute for Quality and Efficiency in Germany[70-72]. In the United States, the U.S. Congress Office of Technology Assessment evaluated multiple technologies before being disbanded in 1995[73]. The Agency for Healthcare Research and Quality, now the primary federal agency charged with improving the quality, safety, efficiency, and effectiveness of health care, was recently allocated $50 million in FY2009 to conduct comparative effectiveness research, and the Comparative Effectiveness Research Act recently introduced in Congress proposed the establishment of an institute dedicated to organizing and conducting such research[74].

With such a framework in place, surgeons worldwide are well-poised - and ethically bound - to ensure that scientific evidence for a surgical procedure supports its advantages over other surgical options. Proponents of developing surgical technologies must carefully follow the established principles of clinical research, technology assessment, and evidence-based medicine to safeguard the integrity of surgical practice and meet the professional responsibilities that monumental technological changes create.

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EDITORIAL

Treatment of liver hydatidosis: How to treat an asymptomatic carrier?

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Abstract

Liver hydatidosis is the most common clinical presentation of cystic echinococcosis (CE). Ultrasonographic mass surveys have demonstrated the true prevalence, including the asymptomatic characteristic of the majority of cases, providing new insight into the natural history of the disease. This raises the question of whether to treat or not to treat these patients, due to the high and unsuspected prevalence of CE. The high rate of liver/lung frequencies of cyst localization, the autopsy findings, and the involution of cysts demonstrated in long time follow-up of asymptomatic carriers contribute to this discussion. The decision to treat an asymptomatic patient by surgery, albendazole, or puncture aspiration injection and reaspiration or to wait and watch, is based on conflicting reports in the literature, the lack of complications in untreated patients over time, and the spontaneous disappearance and involution of cysts. All these points contribute to difficulties of individual clinical decisions. The patients should be informed of the reasons and the risks of watchful/waiting without treatment, the possibility of complications, and the risks of the other options. As more information on the natural history of liver hydatidosis is acquired, selection of the best treatment will be come easier. Without this knowledge it would be very difficult to establish definitive rules of treatment. At present, it is possible to manage these patients over time and to wait for the best moment for treatment. Follow-up studies must be conducted to achieve this objective.

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Key words: Hydatid cyst; Liver; Hepatic cystic echinococcosis; Albendazole; Liver ultrasonography; Puncture aspiration injection and reaspiration; Ultrasonography screening; Asymptomatic liver hydatidosis

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INTRODUCTION

The liver is the most frequently affected organ by hydatid cysts, and asymptomatic liver hydatidosis is the most common clinical presentation of cystic echinococcosis (CE). To date, its treatment is subject of controversy. Echinococcus granulosus is the agent of hydatid disease, affecting humans, as well as domestic and wild animals. CE has spread to all continents becoming a major health problem, particularly amongst populations that practice sheep husbandry. Humans become infected by the ingestion of

eggs contaminated with the feces of dogs. Most transmission occurs in rural areas, but numerous dogs live in urban areas and often have access to viscera disposed off carelessly from slaughterhouses or from animals slaughtered in private homes[1,2].

NATURAL HISTORY OF CE

Background

New imaging techniques such as ultrasonography (US), computed tomography scan (CT), magnetic resonance (MR), and advances in immunology and molecular biology have had a major impact on the epidemiology, diagnosis, prognosis, management, and understanding of the natural history of diseases. The "imaging finding" in asymptomatic cases is a new clinical feature that appeared as a consequence of applying these technologies in asymptomatic or symptomatic individuals[3]. Some examples of this "new asymptomatic pathology" are gallbladder stones, cysts and other benign liver tumors, and other asymptomatic or presymptomatic pathologies[4,5]. These "imaging findings" replaced the "autopsy finding" and give a more accurate picture of the prevalence of asymptomatic diseases; they also avoid the known bias of necropsy. The application of US in CE allowed the detection of hydatid cysts in the liver in early asymptomatic stages and gives a better understanding of the natural history[6-9].

Asymptomatic liver hydatidosis is defined as the presence of CE detected incidentally in patients who do not have symptoms or have symptoms that are not thought to be due to CE. The diagnosis is made during routine US or imaging techniques, such as CT or MR, for other abdominal conditions, or in specific situations for detecting CE, for example, examination of cohabitants of CE patients or mass screening in endemic areas.

Ultrasonographic mass screening

In the early 1970s, it was though that "with the use of new diagnostic techniques, it is likely that the prevalence rates in humans in endemic areas will increase because of the low sensitivity of serology in detecting CE in lungs". "An example of this, being the tripling of the prevalence rate in Rio Negro, Argentina, when mass miniature radiography [miniature chest X-ray (MXR)] was used as a screening method"[10]. This was also observed when "Control Programs" in Argentina introduced such detection with dd5 (double diffusion of arc five)[11]. The use of US in mass screening of the abdomen demonstrated a similar phenomena as the use of MXR and dd5, increasing the prevalence rates in the liver by detecting 2-3 times more hydatid cysts than serology[5,6,8]. Surveys developed in the early 1980s using portable US devices showed an unsuspected prevalence of hydatid cysts in asymptomatic populations[1,6,8,11,12]. Thereafter, several surveys confirmed those findings and demonstrated the high sensitivity and specificity (near 100%) of US in the detection of CE. US is much more precise in detecting abdominal cysts than immunodiagnostic tests, as the latter exhibit relatively high rates of false-negative and false-positive results[2,6,8,13-19]. Mass surveys using portable US scanners have been carried out in rural areas of Argentina[6,8,20], Tunisia[21,22], Libya[23,24], Uruguay[25,26], China[27,28], Perú[17,29,30], Turkey[31], and Morocco[32] amongst others and demonstrated asymptomatic characteristic in the majority of these cases[6-9,16,33]. The unsuspected high prevalence of the disease in asymptomatic patients has a major influence on the decision to treat or not to treat such patients.

Liver/lung frequencies of cysts localization and autopsy findings

Frequencies of localization in liver and in lungs differ whether based on hospital communications, surgery, autopsies, or screening in asymptomatic cases with simultaneous examination of abdomen by US and lungs by chest X-ray[6,7,9,12,33]. Cases of CE who underwent surgery represent only a small proportion of infected persons in any area. The Argentine National Register of Hydatidosis reported a liver/lung ratio of localization of 1.8/1 in 11 589 cases (1935 to 1963), based on hospital notifications (symptomatic cases)[34]. In a Chilean investigation, of 63 436 hydatid cases from hospital registries, the ratio was 1.73/1. In 732 CE autopsies, the ratio was 4.27/1. In a review of 21 573 autopsies from the University of Buenos Aires Hospital, (Argentina, 1879-1985) the prevalence of CE was 1.6% (349 cases), 80% in the liver and 20% in lung with a 4/1 liver-to-lung ratio. The ratio in this study was 2.6/1 in complicated cases. The ratio in uncomplicated cases was 7/1[35]. These results were similar to those found in a US and chest X-ray simultaneous screening performed in 1126 asymptomatic individuals: 71 (90.1%) in liver and seven (9.9%) in lung (ratio 9/1)[34]. This has allowed the determination of the true frequency of liver/lung cyst localization in asymptomatic carriers (prevalence), based till then mostly on data from hospital notifications of surgical cases[6,9,34,35]. Some reports showed predominantly pulmonary localization, with slower growth and less frequent complications with the G8 strain of CE compared to other genotypes[36]; however, in Argentina, the G8 strain was not detected[37]. The liver/lung ratio determination revealed the true prevalence of CE infestation and raised questions about its natural history, mainly in the liver. The growth of cysts depends on the evolutionary potential of the hexacanth embryo, the host tissue in which it is harbored, and its resistance. The lung, due to the elasticity of its tissue, offers limited resistance, resulting in fast cyst growth with early appearance of clinical symptoms. In the liver, the resistance of the surrounding tissue is strong and in many cases the growth of the cyst is slow or even null for several years, producing a high percentage of asymptomatic carriers[32,33]. The great tolerance of the liver to CE is another point to consider when therapeutic modalities are proposed.

Long-term follow-up of asymptomatic cases

Follow-up of asymptomatic liver cyst carriers represents a useful contribution to the better understanding of the disease[33]. The demonstration by US of the evolution of cysts over time, their spontaneous disappearance, and the

absence or slow growth of liver cysts through long-term longitudinal studies changed some concepts of the natural history of CE infection. Long-term follow-up showed that most asymptomatic liver hydatid patients remain symptom-free for years, regardless of the cyst size or type, with a low risk of developing complications; thus it is difficult to establish specific rules for their therapy, if any[33]. A retrospective study was done in 42 asymptomatic liver CE cases that emigrated to Buenos Aires City (non endemic) from endemic provinces between 25 to 45 years ago, derived as "liver imaging findings", at our Hepatology Unit (tertiary university hospital). In this population, cysts detected according to World Health Organization (WHO) classification were: type CE 4, 45.2% (19/42), CE 5 21.4% (9/42) CE 2 or CE 3, 33.3% (14/42) (unpublished data: Frider *et al*). The fact that these asymptomatic carriers lived years outside endemic areas, the unsuspected high prevalence of CE detected by mass US and the rate of cyst localization in liver /lung poses questions about the natural history. These results also raised doubts about what treatment, if any, should be administered to those asymptomatic patients. Waiting and watching (wait & watch) is an option in the management of some types of cysts and could be the rule in the majority of them[6,8,33]. There are few studies based on follow-up of asymptomatic individuals; thus, the decision to treat is difficult and is based on some cyst features that are an indirect method of evaluating the viability of cysts[38,39].

Classifications based on different stages of the natural evolution of hydatid cysts exist. Gharbi's and the WHO classification are the most commonly used[40,41]. A longitudinal study in asymptomatic CE children without treatment showed that 11.4% (8 /70) of cysts disappear over a 44 mo of follow up and 25% (4/16) at 10 years[42-44]. Hyaline cysts, CE1, are common in children and young men, and CE4 or CE5 are common in older people; nevertheless, different types of cysts could be observed in the same liver. A long-term follow-up of CE liver carriers showed that all CE1 cysts disappeared at 12 years and most cysts had evolutionary changes; however, the remarkable phenomenon was that three quarters of them persisted asymptomatically[33]. The evolution of cysts is a slow spontaneous involution that can be accelerated by local or systemic treatment[33,38,39,43]. Calcification of the cyst wall or in the membranes, is better detected by CT than by US[45]. Pancreatic calcifications in chronic pancreatitis are better demonstrated by CT than by US[46]. This is a physical phenomenon of X-rays and the diagnosis of calcification of the cyst wall or its membranes is not an exception to this rule. A significant amount of calcium is needed to see calcifications with US, whereas small amounts of calcium can be detected in a high proportion by CT, not just in the inactive WHO types CE4 and CE5, but also in CE1, CE2, and CE3 cysts. The role of calcification for staging CE is controversial. Some authors affirm that cyst content solidification over time and the disappearing of inner cysts or septa predicts cyst inactivity more reliably than calcification[33]. Although US is of an enormous benefit in CE, it is still a crude method for observing parasite tissues, and minimal alterations of hydatid membranes are not seen by this method or by other imaging techniques. Degeneration of the germinal layer has been detected by electron microscopy, but is not picked up by US[38]. CT or MR has the same difficulty, perhaps Positron Emission Tomography could detect more early lesions, but this methodology is costly for the majority of the endemic areas. Despite minimal alterations of the hydatid membrane not being detected by imaging methods, calcifications are an indirect sign that reflect disturbance of membranes or of the cyst wall[38]. Some classifications like Gharbi's were done with older (1981) US devices, with which calcifications were more difficult to detect. There are two well-known pathological types of calcification, dystrophic and metastatic. Dystrophic calcification occurs in degenerated or necrotic tissue as a reaction to tissue damage. The different types of calcifications seen in CE, from sprinkled, eggshell-like to circular content[45] are also signs of involution and aging of cysts. Nevertheless calcifications, except for totally calcified cysts ("rocky cysts"), do not always guarantee the absence of complications. Abscesses can develop in calcified type IV as in other cysts with partial calcifications. This emphasizes the need for follow-up of all types of cysts, calcifies or not. In spite of the caution needed in the definition of cyst viability by imaging alone (immunology and possibly molecular biology could aid in the diagnosis of CE), US invaluable in terms of diagnosis, prognosis and spontaneous or induced changes of CE in the liver[16,33,39,42,43]. More studies with more cases and more years of follow-up are needed to gain a better understanding of the natural history CE in the liver.

TREATMENT OF CE

The widespread use of US and the detection of unsuspected cysts have given rise to a great deal of controversy regarding the optimal management of asymptomatic liver CE. The incomplete knowledge of the natural history also contributes to this controversy and to the choice of a definitive therapeutic strategy. The scarcity of trials with long-term follow-up of treated or untreated asymptomatic carriers has contributed to the absence of evidence-based clinical guidelines[33,47]. Reviews and meta-analysis have tried to form conclusions as a palliative to this situation[48-52]. The most significant challenge in the evaluation of patients with upper digestive symptoms who are found to have hydatid cysts, is whether the cyst is the cause of the symptoms or is an incidental finding. This difference is important, because upper digestive symptoms are common in the general population, and the presence of CE is not always related to these symptoms. This is a crucial issue in the definition of symptomatic or asymptomatic CE and in the subsequently choices for the management of the disease. For many years, surgery was the only treatment option. The possibility of detecting CE in the early stages before complications appear, and the use of precocious surgery led to mass screening with immunological tests and US in endemic areas[8,12,24,53-56].

Apart from surgery, the current treatment options for CE include chemotherapy with benzilimidazole

(BMZ) compounds, albendazole (ALZ) and Mebendazole (MBL)[57-59], PAIR with injection of scolicides (alcohol, hypertonic sodium solution, *etc*.)[59,60-62] laparoscopy, radiofrequency ablation (still very restricted), and also the watch/wait modality (no treatment at all, only observation)[8,33,43,49]. Each of these therapeutic tools has limitations depending on the individual case. The evidence supporting any of these modalities from carefully designed clinical studies is insufficient and the choice of treatment options remains controversial[33,49-52]. Surgery is still the mainstay of radical treatment in symptomatic or complicated cases, but chemotherapy and the PAIR method with concomitant chemotherapy offer new options. According to the WHO recommendations, surgery is indicated for large hepatic cysts with multiple daughter cysts, for single hepatic cysts situated superficially (risk of spontaneous or trauma rupture), for infected cysts, and for cysts communicating with the biliary tree and/or exerting pressure on adjacent vital organs. Curative surgery is not always possible, the risk of relapse and morbidity is considerable, particularly when the surgery is repeated. Surgery has progressed, with shorter treatment duration, decreased post-operative complication rates, and increased curative rates[57,58]. Overall recurrence is seen in about 6% of cases and mortality in about 0.5% and 4%, depending on the type of surgery and medical facilities[52,63-65]. PAIR or a laparoscopic surgical approach[61,65] are emerging, but despite being minimally invasive techniques, morbidity, recurrence, and low mortality rates are reported. Surgery is contraindicated in patients in whom general contraindications for surgery apply, for example, pregnant women, inactive asymptomatic cysts, very small cysts, multiple cysts or cyst that are difficult to access[52]. Cyst rupture can occur spontaneously, while surgical damage with spillage and widespread dissemination in the peritoneal cavity is possible[62]. In addition to contraindications for surgery, in some highly endemic regions, long hospital waiting lists and a lack of adequate medical facilities and/or experienced staff exist[48].

Over the past 30 years, ALZ and MBL have increasingly been used to treat CE[52,66]. Chemotherapy is important because completely curative surgery is not always possible, and PAIR also involves a 2% to 15% risk of relapse. The outcome of benzimidazole (BZM) therapy is related to the size and age of the parasite, calcification and fibrosis. "Recent" cysts and/or those with thin walls are more accessible to drugs than "old" cysts with thick or calcified walls[48]. ALZ can be used on patients of any age, and is less limited by the patient's status than surgery or PAIR. ALZ showed better absorption and tissue distribution than MBZ[58,59]. Close surveillance of signs of hepatotoxicity is mandatory in all patients receiving ALZ, and it is contraindicated in advanced chronic liver diseases. Chemotherapy cure can be expected in about 30% of patients and improvement in 30%-50%, after 12 mo of follow-up[52,67]. Chemotherapy is indicated in inoperable liver or lung cysts, or cysts in more than two organs, in peritoneal localizations, in multiple small cysts deeply localized in the liver parenchyma, in patients with incomplete surgery or relapse, and to prevent spillage associated to surgery or PAIR. Chemotherapy is contraindicated in large cysts that have a risk of rupture, especially in superficial or infected cysts, because of the possibility of hydatid abscess (two unpublished own cases) and in early pregnancy[66,67]. Studies have shown the cysts' response to chemotherapy can be clearly demonstrated by US[16,43,52,57,58,68]. Decreasing size, margins of the cyst wall, detachment of the inner membrane, the appearance of echogenic material (matrix) in the cyst cavity or the disappearing of cysts are sensitive and specific signs of a good response[39,50]. In some series, a certain degree of response was observed in more than 75% of patients[58,69]. Evaluations at up to 12 mo, showed about 30% cyst disappearance, 30%-50% cyst degeneration and/or a significant size reduction, but 20%-40% exhibited no changes. There is only one prospective, controlled, randomized, open study of ALZ in patients with liver CE in which parasite viability after treatment was assessed in all patients[69]. In 65 asymptomatic children there were no statistical differences between ALZ-treated with and untreated at 29 mo of follow-up. However, four years after treatment, the differences were significant, 76% (treated) *vs* 38.5% (untreated)[43]. Interestingly, in the same population, the non treated group (watch/wait) comprised 14 (87.6%) who were CE 1 or CE2 and two (12.6%) who were CE4 and CE5, but at 10 years follow-up three (18.8%) were CE1 or CE2 and eight (50.1%) were CE4 or CE 5, reflecting a degree of involution. In the treated ALZ group, two (8%) were CE1 or CE2 and 17 (68%) were CE4 or CE5, with no significant differences between the two groups[44].

PAIR was introduced in 1986, is widely used and indications and contraindications are well-described elsewhere. PAIR seems to have greater clinical efficacy, and lower rates of complications and disease recurrence compared to surgery[52,60]. A randomized study comparing drainage with ALZ to ALZ alone or to drainage alone, showed that a maximum size reduction was observed in cysts treated with combination of PAIR and ALZ[70]. A subsequent study demonstrated that PAIR combined with ALZ is an effective and safe alternative to surgery for the treatment of uncomplicated liver cysts and requires a shorter hospital stay[71]. Surgery should be reserved for patients with secondary bacterial infection or for those with difficult-to-manage cyst-biliary communication or obstruction[72-74]. Another unresolved point is for how long is it necessary to treat patients with ALZ? Treatment for three months is generally used and some cysts show a rapid response, others show non response during treatment or months after stopping the drug. In these cases long-term use of ALZ can be useful and should be investigated. In some disseminated CE cases, the use of ALZ for years resulted in a cure of the disease with a disappearance of the majority of cysts[75]. The adherence of patients, the strict control of possible toxicity (liver enzymes and granulocytes), and imaging are essential for this extended treatment. The continuous and long-term use of ALZ in the treatment of alveolar echinococcosis (AE) is safe and well-tolerated, with good results and without significant adverse events[76]. The results of long-term use in AE allowed the longer use

of ALZ in CE. Some other presentations of ALZ, such as an emulsion can offer better results[77]. Future advances in chemotherapy might be achieved by identifying drugs with higher efficiency. Today, ALZ should be considered as the primary choice of treatment for patients who are not candidates for surgery (inoperable), recurrent cases, those with peritoneal involvement or with multiple cysts in several organs, those who refuse surgery or PAIR, and, perhaps, for asymptomatic carriers[72].

CONCLUSION

The great tolerance of the liver and the asymptomatic character of CE infestation is an established fact. This tolerance raises the questions of "to treat or not to treat" these asymptomatic patients[6,8,33,43,44]. The debate to whether a particular asymptomatic patient should undergo treatment or only enter in a watch/wait option is based on conflicting reports. These are: the lack of complications in untreated patients over years, the beneficial effect of existing therapies, the difficulties of accurately predicting prognosis and the possibility of developing severe complications, even mortality, because of the growth of cysts[6,8,9,33,43,52,65,73]. Large cysts are more likely to develop such complications when they are located superficially[52,74]. Surgery and PAIR are not innocuous and mortality, morbidity and relapse are possible[78]. All these points demonstrate the difficulties of individual clinical decisions. The patients should be informed of the reasons and the risks of no treatment (watch/wait) and also about the risks of the other options. The long-term response and the good tolerance of ALZ in children have raised expectations about this treatment in the early stage of infestation. The current conditions surrounding asymptomatic liver hydatidosis are still confusing and we can not draw definite conclusions about the treatment of these carriers. Due to the scarcity of controlled and follow up studies of this neglected illness, treatment decisions are difficult to take. We are sure that further investigations are essential to determine the proper treatment in these cases. We emphasize the urgent need for more comparative studies between treatment or watch and wait, with close follow up. We feel that the treatment of liver hydatidosis has been performed without a clear discrimination between symptomatic or asymptomatic patients, especially in some surgery papers. An increase of follow-up studies is also the way to continue the path opened by the use of US initiated in the early 1980s, with the access to medical control of an enormous number of asymptomatic CE carriers. The knowledge of the natural history of hydatidosis is ongoing and a better understanding of this history will allow us to select the best therapeutic option to be administered at the right moment.

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REVIEW

Epstein-Barr virus: Silent companion or causative agent of chronic liver disease?

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Abstract

The Epstein-Barr virus (EBV) has an important and multifaceted role in liver pathology. As a member of the herpes virus family, EBV establishes a persistent infection in more than 90% of adults. Besides acute hepatitis during primary infection, many clinical syndromes of interest for the hepatologist are associated with EBV infection. The role of EBV in the evolution of chronic hepatitis from hepatotropic viruses is considered. Chronic EBV-associated hepatitis is suspected in immunocompetent adults with compatible serology, suggestive histology and detection of the viral genome in the liver and/or increase of specific circulating cytotoxic T-lymphocytes. EBV is the main cause of post-transplant lymphoproliferative disorders which occur in up to 30% of cases. EBV-driven lymphoproliferative diseases are also recognized in non-immunocompromised patients and liver is involved in up to a third of the cases. Directly implicated in the pathogenesis of different tumors, EBV has a disputable role in hepatocellular carcinoma carcinogenesis. Further research is required in order to establish or reject the role of EBV in human liver cancer. This paper attempts to discuss the range of EBV-associated chronic liver diseases in immunocompetent patients, from mild, self-limiting mononuclear hepatitis to liver cancer.

PRIMARY INFECTION, LATENCY AND REACTIVATION

The Epstein-Barr virus (EBV) is a member of the *Gammaherpesvirinae* subfamily of herpes viruses. It infects more than 90% of the adult population all over the world. EBV shares the tendency of establishing latency in the host with other herpes viruses[1]. Primary infection leads to transitional viremia, followed by a strong T-cell adaptive immune response, which actually holds the infection latent in immunocompetent individuals[2]. Short episodes of spontaneous reactivation and consequent viral replication normally occur in healthy individuals[3]. In the immunocompetent individual the occurrence of EBV reactivation leading to immortalization of B-lymphocytes is strongly regulated by cytotoxic T lymphocytes (CTLs) specific for lytic and latent antigens[4]. It is believed that almost no specific symptoms indicate these events. Viral replication may cause significant clinical entities and even severe complications in patients with diminished cell-mediated immunity[5,6]. The latter refers to immunosuppression, physical or psychological stress, other infections, *etc*[7].

The EBV DNA encodes more than one hundred pro-

teins expressed during the lytic infection. Only ten of these proteins are expressed in latently infected B-cells *in vitro*[8]. Limited gene expression during latency ensures successful escape from CTL recognition. However, this has led to sophistication of the diagnostic tools for detection of EBV gene products in different tissues. Different types of latency regarding expression of EBV latent genes are established in different diseases and states. EBV-encoded nuclear antigen 1 (EBNA-1) and small RNAs (EBER) are found in Burkitt's lymphoma, whereas latency type of Hodgkin's disease is represented by additional expression of the latent membrane proteins (LMP-1 and LMP-2)[9]. These differences further hamper the diagnosis of EBV-associated disease and challenge both the clinicians and molecular biologists to reveal the causal links.

The classical form of primary EBV infection in the immunocompetent individual is infectious mononucleosis (IM, or first-kiss-disease). Albeit not a challenge for general physicians, in some cases it could be of interest for hepatologists. Almost half of patients with IM have hepatitis, while jaundice is present in 5%-10% of cases[10,11]. Besides transient liver enzyme elevations, cholestatic pattern of severe hepatitis[12-14] and even fatal liver failure might complicate the IM[15-17].

CHRONIC ACTIVE EBV INFECTION

Chronic active EBV infection (CAEBV) is thought to be a rare disorder, defined by chronic severe illness, which begins as a primary EBV infection associated with abnormal EBV serology, histological evidence for organ disease (including hepatitis) and demonstration of EBV gene products in tissue. The CAEBV infection might progress to a chronic or recurrent infectious mononucleosis-like disease[18]. This is a result of a disturbance in the host-virus balance and Th1/Th2 disbalance. It is reported that CAEBV is associated with an aggressive clinical course[19] but the pathogenesis and pathophysiology of CAEBV are still not well characterized. Patients with iatrogenic, congenital, or acquired immunodeficiency are at increased risk for EBV-associated lymphomas and CAEBV. It seems that CAEBV in Western countries is milder than in Asian countries[20]. The mild forms from the Western world are characterized by intact immune control of B-cells, relatively low viremia and EBV-specific CTL expansion comparable to seropositive donors. Under the heading of the popular clinical term CAEBV, novel EBV lymphoproliferative diseases (LPDs) have been identified recently in non-immunocompromised hosts, both in Asian and Western countries. Immune senescence in the elderly is also associated with both reactive and neoplastic EBV-driven LPDs[21]. Almost a third of the patients with some EBV-driven LPDs have liver involvement, and liver failure is an important cause of death in this group[22].

EBV-ASSOCIATED CHRONIC HEPATITIS

Some reports have suggested EBV to be a trigger agent for an autoimmune hepatitis[23]. It should be kept in mind that liver involvement in CAEBV might mislead even experienced physicians to misdiagnose an autoimmune hepatitis (AIH)[24]. Nobili *et al*[25] identified EBV and hepatitis A virus infections as possible initiating agents in a cohort of AIH type I patients. In similar cases a suppressor T-cell dysfunction and consequent loss of control and raising of ASGRP antibodies have been seen to be involved in the evolution of the viral disease[26-28]. EBV has been suspected as a probable cause of particular granulomas in the liver[29] and even in a rare vanishing bile duct syndrome[30].

In some patients with chronic liver disease caused by a major hepatotropic virus, an infection with other viral agents may be discovered. We previously evaluated patients with chronic hepatitis B and C regarding their EBV serology. Our patients with reactivated EBV infection had lower levels of HBV DNA and higher mean values of serum hepatitis C virus (HCV) RNA respectively, compared to EBV-seropositive patients without reactivation[31]. Several hypotheses were put forward to explain those results. Reactivation of the EBV-specific T cells leads to significant production of several cytokines, especially interferon-γ (IFN-γ), interleukin (IL)-1, IL-2 and IL-10[32]. Additionally, the EBV BCRF1 shares high sequence homology with human IL-10 and exerts the same activity. Exogenous IL-10 enhances HCV replication[33]. IFN-γ inhibits HBV replication in the absence of cell necrosis[34]. It is unclear why the effect on viral replication was opposite in HBV and HCV cases. Most probably, T cell cross-activation could explain the hepatitis virus reactivation. The latter conclusion has been drawn from a small group of patients whose EBV reactivations preceded HBV flares[35]. Sugawara *et al*[36] have shown *in vitro* that EBNA1 promotes HCV replication. Speculative viral or immune interference in cases of chronic hepatitis B or C and EBV infection needs further research.

Bertolini *et al*[37] investigated an experimental model (nu/nu mice) of chronic EBV hepatitis with typical intrasinusoidal lesions after inoculation of EBV-infected B-cells. The study provided evidence for EBV replication in a very rare proportion of hepatocytes. Despite lack of evidence, we might speculate that infection of non-lymphoid cells is possible *via* a specific integrin-dependant mechanism[38]. Hesitant reports have linked EBV to chronic hepatitis in non-immunocompromised humans. The existence of EBV-associated liver disease is still accepted with caution as EBV has not been detected in human hepatocytes[13]. Specific latent antigens as well as EBER transcripts are detected in infiltrating lymphocytes. It has been proposed that liver cells suffer from a form of collateral damage[13]. Chronic hepatitis might also be induced by a soluble Fas-ligand, tumor necrosis factor and IFN-γ[39-41]. In most cases the infiltrating lymphocytes are CD8+ CTLs. It has also been found that activated CD8+ cells might be selectively trapped in the liver as they are bound to specific adhesive molecules expressed by Kupffer cells[42]. In the last year, several studies have tried to lift the curtain regarding chronic hepatitis associated with EBV infection. Drebber *et al*[43] proposed a list

of criteria to establish the diagnosis: presence of suggestive histopathological features, serological profile and detection of viral genome in the liver tissue. In spite of some methodological limitations, this study addressed the necessity of new scoring systems regarding the possible EBV-associated chronic liver disease. Their work revived the interest in this issue both for pathologists and molecular hepatologists[43]. The critics of a hypothesis for chronic EBV liver disease in non-immunocompromised patients discuss so called "incidental virus" or amplification of the genome in circulating B cells which turn up in the liver. The existence of acute mononuclear hepatitis during primary EBV infection has been already accepted. Hence, it is most likely that the reactivation leading to liver damage occurs whether the infected lymphocytes are incidentally or intentionally in the liver. We previously discussed cases of EBV-associated chronic hepatitis in patients with reactivated EBV infection and increased specific CTL-response to a lytic EBV-epitope compared to healthy controls[44]. We presumed frequent reactivations in these patients because we had found an increased percentage of terminally differentiated CD28(-) CD27(-) CD8(+) T cells, suggestive of chronic antigen stimulation and replicative senescence. Diminished expression of co-stimulatory molecules CD28 and CD27 could further compromise CD8+ reactivation and characterize these terminally differentiated cells as more resistant to apoptosis[45,46]. Focused T-cell pull with low expression of CD28 and CD27 has low ability to control reactivations and is a typical finding in an elderly group. Surprisingly, very similar changes were found in younger patients under chronic cytomegalovirus and EBV antigen stimulation[47].

We surmise that a chronic liver disease might be a manifestation of a chronic EBV infection with frequent reactivations and persistent, moderate or low level of viral load. Based on recent studies and cases we can propose the expansion of Drebber's criteria by adding recurrent EBV reactivations, increased circulating EBV-specific CTLs, and increased CD38 B-cell expression. We are also inclined to accept as additional clinical signs the increase of LDH levels, mild spleen enlargement and tendency for thrombocytopenia.

LIVER TRANSPLANTATION AND EBV INFECTION

EBV infection is the main cause of post-transplant lymphoproliferative disease (PTLD). The incidence of PTLD ranges from 0.5% to 30% depending on the organ being transplanted, the EBV status of the transplant recipient and donor, and the therapies used to achieve immunosuppression[48,49]. EBV seronegativity at the time of transplant and pediatric age are predisposing factors. The disorder occurs commonly in combined liver-kidney transplants, followed by cardiac, liver, lung, and then kidney transplants. In addition, constitutional factors such as cytokine gene polymorphism may play a predisposing role. Little is known about the chronic carrier state and its relation to late PTLD. Most pediatric liver transplant candidates are EBV-naïve when listed for orthotopic liver transplantation (OLT). Those immunosuppressed, EBV-naïve children, who receive an adult EBV-positive liver, are unable to adequately control EBV primary infection, hamper viral replication and avoid lymphoproliferation[50]. For this reason, PTLD has become one of the major threats in transplantation, complicating the course of up to 10% of pediatric liver graft recipients, with a mortality reported up to 50%[51]. All data support the recommendation of strict EBV monitoring in the first months after the transplantation. However, little is known about late and chronically persistent infection. Helpful insights into how often and how long we should monitor viremia during follow-up are insufficient. D'Antiga *et al*[52] revealed that none of their pre-OLT seropositive patients had sustained positivity of EBV serology or polymerase chain reaction (PCR) for 6 mo or longer, versus as many as 65% of those who were EBV-negative pre-OLT. A prolonged EBV viral load was strictly related to pre-OLT immunity against the virus and appeared to be associated with the occurrence of PTLD[52]. In fact, it is well established that persistence of EBV is a risk factor for development of lymphomas[53]. Patients who demonstrate a sustained viral detection are at risk for the development of late PTLD which implies necessity for preliminary testing as well as for long-term EBV infection follow-up. The development of molecular genetic diagnosis for EBV (PCR) means that progressive disease, or PTLD, may be prevented by preemptive reduction of immunosuppression in response to rising EBV titers.

EBV AND LIVER CANCER

EBV was the first human virus directly implicated in carcinogenesis. Since its discovery it has been considered as a major player in the development of a wide range of cancers both in immunocompetent and immunocompromised individuals. Recent studies have concluded EBV itself or infected cell clones might promote replication of the HCV[31,36]. Presumably, EBNA1 is responsible for supporting HCV replication, suggesting that EBV may be involved in the development of hepatocellular carcinoma (HCC)[54]. The detection of EBV gene products in HCC additionally supports this assumption. Sugawara *et al*[55] reported a higher amount of EBV DNA in HCV-positive HCC compared to HBV-associated HCC. EBV-infected cells support HCV replication better than uninfected cells, suggesting that EBV may act as a helper virus to promote HCV replication in the HCV-positive HCCs, especially in Japanese patients. Li *et al*[56] found that almost 30% of liver cancers harbored EBV DNA (*vs* 8% in the control group, $P < 0.05$) suggesting that the herpes virus might be involved in hepatocellular carcinogenesis in China. However, studies from Western countries do not confirm the hypothesis that EBV may promote the development of HCC. Akhter *et al*[57] examined cancer tissues of 31 non-cirrhotic, HCC patients. None of the tumor samples were found to be positive for EBV DNA. Being unable to con-

firm an association between EBV and infection with hepatitis viruses in HCC, the authors suggested environmental or genetic factors which could predispose Chinese and Japanese patients to develop EBV-infected HCC. On the other hand, a possible source of detected EBV DNA might be the infiltrating lymphocytes. Furthermore, a study from The Netherlands concluded that the absence of epithelial-specific *BARF1* transcripts and other EBV transcripts and proteins in EBV DNA PCR-positive cases argues strongly against a role for EBV in HCC[58]. However, weak signals for EBV DNA in 5 of 16 HCCs (31.25%) and in two of four HCV-related hepatitis cases were detected and actually no EBV DNA was found in non-neoplastic and normal liver tissue, using DNA PCR-Enzyme Immunoassay for the detection of the *Bam*HI W repeat fragment of EBV. The weak positivity of EBV DNA in some liver tissues was explained by amplification of EBV DNA in the lymphoid infiltrate or blood, reflecting a high EBV DNA load in these patients. Chen *et al*[59] showed that circulating EBV-positive peripheral B cells express *BARF0* transcripts which could also explain the negative *Bam*HI A rightward transcripts RNA *in situ* hybridization results. Moreover, we cannot exclude the presence of EBV-positive lymphocytes or EBER-positive hepatocytes in the larger amounts of tissue, as most studies usually test routine histology sections (5 μm thick paraffin-embedded or cryosections). This fact raises the question whether usual techniques such as immunohistochemistry and in situ hybridization lack enough power to detect the virus in the liver regardless of its place of persistence - infiltrating lymphocytes, hepatocytes or Kupffer and other liver cells. We would welcome better evidence for or against the causative (and/or supportive) role of EBV in human HCC carcinogenesis.

Undeservedly neglected within hepatological society, EBV-associated chronic liver disease demonstrates the challenges in discovering the intimate mechanism of viral-host interactions leading to different clinical syndromes. Since proven to induce acute hepatitis during IM, to affect the liver in a PTLD setting, possibly to contribute to liver carcinogenesis, it would not be surprising if EBV causes chronic hepatitis. Undoubtedly, follow-up of patients is required to reveal the long-term complications of EBV-associated liver disease before we decide that this viral infection belongs in a group of meaningless companions of cryptogenic disease.

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ORIGINAL ARTICLE

Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome

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Abstract

AIM: To design and validate broad-range 16S rRNA primers for use in high throughput sequencing to classify bacteria isolated from the human foregut microbiome.

METHODS: A foregut microbiome dataset was constructed using 16S rRNA gene sequences obtained from oral, esophageal, and gastric microbiomes produced by Sanger sequencing in previous studies represented by 219 bacterial species. Candidate primers evaluated were from the European rRNA database. To assess the effect of sequence length on accuracy of classification, 16S rRNA genes of various lengths were created by trimming the full length sequences. Sequences spanning various hypervariable regions were selected to simulate the amplicons that would be obtained using possible primer pairs. The sequences were compared with full length 16S rRNA genes for accuracy in taxonomic classification using online software at the Ribosomal Database Project (RDP). The universality of the primer set was evaluated using the RDP 16S rRNA database which is comprised of 433 306 16S rRNA genes, represented by 36 phyla.

RESULTS: Truncation to 100 nucleotides (nt) downstream from the position corresponding to base 28 in the *Escherichia coli* 16S rRNA gene caused misclassification of 87 (39.7%) of the 219 sequences, compared with misclassification of only 29 (13.2%) sequences with truncation to 350 nt. Among 350-nt sequence reads within various regions of the 16S rRNA gene, the reverse read of an amplicon generated using the 343F/798R primers had the least (8.2%) effect on classification. In comparison, truncation to 900 nt mimicking single pass Sanger reads misclassified 5.0% of the 219 sequences. The 343F/798R amplicon accurately assigned 91.8% of the 219 sequences at the species level. Weighted by abundance of the species in the esophageal dataset, the 343F/798R amplicon yielded similar classification accuracy without a significant loss in species coverage (92%). Modification of the 343F/798R primers to 347F/803R increased their universality among foregut species. Assuming that a typical

polymerase chain reaction can tolerate 2 mismatches between a primer and a template, the modified 347F and 803R primers should be able to anneal 98% and 99.6% of all 16S rRNA genes in the RDP database.

CONCLUSION: 347F/803R is the most suitable pair of primers for classification of foregut 16S rRNA genes but also possess universality suitable for analyses of other complex microbiomes.

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Key words: Foregut; Microbiome; 16S; 454 sequencing; Primer

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INTRODUCTION

Currently there is a broad international collaboration underway aimed at defining the microbiome (http://nihroadmap.nih.gov/hmp/) on the internal and external surfaces of the human body [Human Microbiome Project (HMP)]. The goals of the HMP collaborative project are to determine a core microbiome, assess how it changes during disease, and establish whether a change in the microbiome is associated with disease. It is anticipated that knowledge gained from the HMP will shed light on the etiology and pathogenesis of idiopathic chronic diseases that have a high impact on human health.

The human body is a highly complex conglomeration of human cells, bacteria, Archaea, fungi, and viruses, in which the number of microbes outnumbers the human cells by a factor of 10 to 1. Despite their intimate association, the microbial influence upon human development, physiology, immunity, and nutrition remains largely unstudied. This can be partly attributed to the lack of robust techniques needed for exploring a complex microbial community.

Previous attempts to fully characterize the human microbiome have had certain limitations. The classical method of culturing bacteria from human subjects excludes a large number of unculturable or not-yet-cultivated bacteria, and also misrepresents the abundance of some species due to selection by culture conditions. To overcome these drawbacks, culture-independent methods have been developed. The most commonly used culture-independent method relies on amplification and analysis of the 16S rRNA genes in a microbiome[1]. 16S rRNA genes are widely used for documentation of the evolutionary history and taxonomic assignment of individual organisms[2-6] because they have highly conserved regions for construction of universal primers and highly variable regions for identification of individual species[7]. Analysis of a microbiome is classically performed by amplifying all 16S rRNA genes in a sample, cloning the 16S amplicons into a vector transformed into *Escherichia coli* (*E. coli*), randomly selecting transformed colonies, producing high copies of the amplicon containing plasmid, purifying the plasmids, and sequencing the 16S rRNA inserts by the Sanger method[8].

Advantages of this protocol are potential identification of both cultivatable and uncultivable organisms and acceptable adequacy of single pass Sanger sequencing of 900-1000 bases for classifying bacteria. Disadvantages include annealing bias[9], cloning bias[10], as well as time and expenses. The high cost associated with this approach has been prohibitive for in-depth sampling of a complex microbiome. Inadequate sampling overlooks low abundance species. As a result, a low abundance species that could be a microbiome core species often cannot be consistently observed amongst different individuals. For example, a recent 16S rRNA gene survey of the human distal esophageal microbiome yielded 6800 sequences but revealed only one of the 166 species, *Streptococcus mitis*, shared by all 34 subjects sampled[11].

High throughput sequencing technology introduces a new way to perform microbiome surveys without limitations of cloning/Sanger sequencing. One 454 sequencing run can produce 1.2 million sequences in 8 h[12], which would require months or years of work with older methods. Because many sequences are acquired in one run, the unit cost per sequence read is a very small fraction of that for Sanger sequencing. This improvement allows sufficient sampling of a complex microbiome at affordable cost. The new technology also eliminates the cloning bias by directly sequencing the 16S rRNA genes generated by polymerase chain reaction (PCR). Therefore, high throughput sequencing is ideal if adaptable to meet the requirements needed for microbiome work.

The main limitation of high throughput sequencing is read length. Reads from next generation sequencing technologies are considerably shorter than those from Sanger sequencing. Illumina's Solexa and Applied Biosystem's SOLiD platforms generate reads of about 25-100 bases[13,14], while 454 sequencing technology reads up to 400-500 bases per sequence. The general approach to analyzing microbiomes in health and disease focuses at 2 levels. Population level analyses, such as the Fst test, *P* test, Unifrac analysis, clustering analysis, and normal reference range, relate samples of microbiomes by combined genetic distance between the samples. These types of analyses are relatively insensitive to variation in read length[15]. In Unifrac clustering analysis, reads of 100-200 nucleotides can yield the same clustering as full-length sequences if

the correct regions are chosen for sequencing. Detailed analyses at the level of the operational taxonomic unit (OTU) depend on read length - the longer the more accurate[16]. Compared with full length sequences, single pass reads of approximate 900 bases (of full length 1500-1600) from Sanger sequencing is associated with a slight loss of classification accuracy, which has been acceptable considering the significant reduction in sequencing cost from sequencing the entire 16S rRNA genes.

In the recent studies utilizing 454 sequencing technology to perform 16S rRNA gene surveys of microbiomes[17-20], a major concern has been reduction of classification accuracy with short sequence reads. Several strategies have been tried to maximize the information obtained from short sequences. One is to utilize a paired-end sequencing strategy to increase sequence length[21]. Another is to target certain hypervariable regions (HVR) that are most informative for a specific microbiome of interest. Currently, various HVRs, individually[17,19] or in combination[18,22], have been used in analysis of a microbiome but their efficacies often are not validated.

Early studies using Sanger sequencing revealed interesting associations between human microbiomes and disease, such as that seen between the human foregut microbiome, dental/periodontal diseases and gastroesophageal reflux diseases (GERD)[11,23,24]. To further study such associations, the Human Microbiome Project[25] (part of the NIH Roadmap Initiative) currently supports in-depth analysis of the association between the human microbiome of various anatomical sites and related diseases. Our area of focus is the foregut microbiome during disease progression from GERD to esophageal adenocarcinoma (the fastest increasing cancer in the Western world). As the first step of the foregut microbiome project, we identified the most informative HVRs, and designed and validated a broad range primer set most suitable for the foregut microbiome. These will be used to facilitate our in-depth analysis of the human foregut microbiome and its role in GERD-related disease progression.

MATERIALS AND METHODS

Sequence collection

Sequences collected for our *in silico* analysis were obtained from separate, previously conducted 16S rRNA gene surveys of 3 foregut sites. Esophageal 16S sequences (6800) were obtained from research by Yang *et al*[11], oral 16S rRNA gene sequences (2458) were from research by Aas *et al*[26], and gastric 16S rRNA gene sequences (839) were from research by Bik *et al*[27], totaling 10097 sequences. In addition, we removed sequences that were derived from patient gastric samples with *Helicobacter pylori* (300), chimera sequences (127), as well as sequences with more than 8 bases missing after *E. coli* position 27 (186). The final foregut dataset contained 9484 sequences (2373 oral, 6626 esophageal, 485 gastric). These sequences were represented by 220 species. Because 16S rRNA-based operational classification criterion does not have sufficient discriminatory power to differentiate between *Streptococcus pseudopneumoniae* and *Streptococcus pneumoniae*[28], as the two species differ by only 5 base pairs (bp) between their 16S rRNA genes corresponding to a 0.03% difference (16S-based operational threshold for separation between two species is 3% diversity), they were considered as one species in this study. As a result, 219 species were represented in the dataset.

Sequence alignment

The 219 16S rRNA gene sequences were aligned using the NAST alignment program[29]. The program was set to recognize sequences at least 1250 bases long with at least 75% identity. Because NAST may remove bases not found to be sufficiently homologous, and thus unalignable, several sequences were truncated after alignment, particularly at the 5' end. The missing portion of these sequences was manually replaced after alignment with the corresponding region from the GenBank sequence of the same species so that all 16S sequences would be complete, beginning from position 28 of the *E. coli* 16S rRNA gene.

Amplicon design

To simulate the sequence data that would be obtained using specific primer pairs, representative amplicons were constructed from full length 16S rRNA gene sequences. The criteria for design of the representative amplicons was based on 454 requirements. Amplicons could be no more than 600 bases in total (including primers and nucleotide barcode) for optimal emulsion PCR. Because on average 454 read lengths are approximately 400 bases, only the portion of the amplicon that would be sequenced was considered. For example, for a forward read amplicon of a total 500 base pairs with 50 bases of forward primer and nucleotide barcode, only the first 350 bases after the primer would be read, with the final 50 bases ignored. These amplicons were designed using 6 universal primer sets from the European Ribosome Database[30] as shown in Table 1.

Sequence trimming

The simulated amplicons were generated by trimming full length sequences of the 219 foregut species using the MEGA version 4 program (MEGA4)[31]. Aligned FASTA sequences were uploaded onto MEGA4, with the sequence file including an aligned *E. coli* 16S rRNA gene sequence as a positional template.

To simulate data that would be obtained by cloning/Sanger sequencing methods, amplicons of approximately 900 bases downstream of the starting position were first generated for the 219 sequences. These amplicons were used to theoretically compare how reads obtained by 454 sequencing technologies would compare to Sanger sequence reads. The 8F primer was located in the 219 aligned sequences (bases 8-27) and all bases upstream of 28 were deleted. The gaps from the aligned sequences were then removed, and all bases downstream of 928 were deleted leaving the sequences between positions 28 and 928 in *E. coli* (900 bases total) as a reference for the

Table 1 Primers used in the study

Primer	# bases	Sequence (5′ to 3′)	Species with identical match (%)
Forward primers			
8F	20	AGAGTTTGATCCTGGCTCAG	n/a[1]
343F	15	TACGGRAGGCAGCAG	99.1
517F	17	GCCAGCAGCCGCGGTAA	93.2
784F	15	AGGATTAGATACCCT	90.0
917F	16	GAATTGACGGGGRCCC	84.0
1099F	16	GYAACGAGCGCAACCC	73.1
Reverse primers			
534R	18	ATTACCGCGGCTGCTGGC	91.8
798R	15	AGGGTATCTAATCCT	90.0
926R	20	CCGTCAATTYYTTTRAGTTT	83.0
1114R	16	GGGTTGCGCTCGTTRC	74.9
1407R	16	GACGGGCGGTGTGTRC	91.3
1541R	20	AAGGAGGTGATCCAGCCGCA	n/a[1]

[1]Sequences near the ends of the 16S rRNA genes are often designed for primer binding and are not included in sequences deposited in GenBank. n/a: Not available. R = A, G; Y = C, T.

Sanger sequencing data that would be obtained for the 219 species.

For length-based amplicon comparison, aligned sequences were again trimmed upstream of *E. coli* position 28, representing the first base after the 8F forward primer. This *E. coli* position 28 was set as base #1. All gaps were then deleted from the aligned sequences and downstream bases were trimmed to leave amplicons with 900, 800, 700, 600, 500, 400, 300, 200, 100 and 50 bases. These were compared against full length 16S rRNA gene sequences for classification accuracy.

For amplicons based on different primer sets (within varying regions of the 16S rRNA gene), sequences of reverse and forward reads from both ends of the amplicons were analyzed. To trim full length sequences to the amplicons of interest, the position of the forward or reverse primer was first located by searching for the primer sequence. For theoretical forward reads of the amplicons, the sequence upstream and including the forward primer was deleted. Gaps were then deleted in the aligned sequences leaving all sequences with the base after the forward primer as base #1. From this position, the site of 350 bases downstream of position #1 was determined, and all bases downstream of #350 were deleted. This represented 454 sequencing's ability to read the 350 bases downstream of the primer (after the first 50 or so bases of the primers and barcodes were read). For theoretical reverse reads of amplicons, the position of the reverse primer was located as before. From the 3' end of the reverse primer complement (sense strand) the first base of the sequence of interest was designated position #1'. Using the *E. coli* 16S rRNA gene as a reference, the site of 360 bases upstream of base #1' was determined and set as #360'. All bases upstream of #360' were deleted. The gaps in the aligned sequences were then deleted and the number of bases in each sequence was determined. For any sequences with more than 350 bases, extra bases were removed manually from the 5' end so that all sequences were 350 bases long. All sequence trims were then saved as FASTA files for analysis.

Sequence classification and comparison of amplicon accuracy

Sequence trims for size and designed amplicons were classified at the phylum to genus level using RDP Ⅱ Classifier[32] and at the species level using RDP Ⅱ Seqmatch[33].

For classification at the phylum to genus level, FASTA files were uploaded onto the RDP Ⅱ Classifier at 80% confidence threshold and results were viewed at a display depth of 7 to see assignment data down to the genus level. The resultant classification dataset for the trimmed sequences was individually compared to the dataset obtained using full length sequences and/or 900 bp Sanger length sequences. The number of discrepant assignments was recorded.

For classification at the species level, FASTA files were uploaded onto RDP Ⅱ Seqmatch. The following parameters were selected: typed and non typed strains, uncultured and isolates, ⩾ 1200 bases, good quality, nomenclature taxonomy. The species classification of every sequence was individually analyzed and compared to the species that the full length version of the sequence was assigned to, as described previously[11].

Homology between universal primers and foregut bacterial species

Universal 16S rRNA primers were obtained from the European ribosomal RNA database[30]. The primer sequences are shown in Table 1. Determination of the percentage of the 219 foregut species with the exact primer sequence was done using MEGA4 software[31].

Evaluation of the universality of the foregut primers in the domain Bacteria

The optimized primers were searched using Probe Match against the bacterial 16S rRNA gene database at Ribosomal Database Project (http://rdp.cme.msu.edu).

RESULTS

Accuracy of taxonomical classification is dependent on amplicon length

To compare shorter reads that would be obtained with high throughput sequencing against longer reads that would be obtained using Sanger sequencing, we performed *in silico* analysis; looking at the capability of different length 16S rRNA gene sequences to accurately classify foregut bacteria. Using full length sequences from 219 representative foregut species, we created sequence truncations that were 50-900 bases long beginning from base 28, the first base after the 8F primer. A length of 900 bases was chosen as the longest sequence to analyze because it is the length of a single pass Sanger sequence and generally accepted as accurate taxonomically. Each of the truncated versions of the 16S rRNA gene sequences was analyzed using RDP Ⅱ classifier down to the genus level. The results showed that loss of classification accuracy was length dependent (Figure 1). At the genus level, sequences as small as 200 bases showed relatively good classification accuracies (94.1% accuracy for 900 base se-

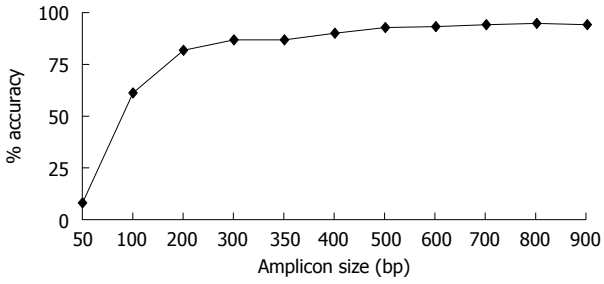

Figure 1 Classification accuracy is dependent on amplicon size. Full length sequences were trimmed to 900, 800, 700, 600, 500, 400, 350, 300, 200,100, and 50 bases with each amplicon starting at *Escherichia coli* base 28. Each sequence trim was uploaded onto Ribosomal Database Project Ⅱ classifier and the results at each taxonomical level were compared to results obtained using the full length sequence. Percent classification accuracies at the genus level for each amplicon size trim are shown.

quence, 90.0% for 400 bases, and 81.7% for 200 bases). Once the sequences became smaller than 200 bases, the classification accuracies decreased considerably, with the 100 base sequence having only 61.2% accuracy and the 50 base sequence having only 8.2% accuracy at the genus level. Overall, for identification at the genus level, the data showed that sequence sizes generated by Solexa or SOLiD platforms would have drastically lower classification accuracies (less than 50 bases, 8%-61% accuracy) than those sequence sizes generated by 454 technology (over 400 bases, over 90% accuracy).

Accuracy of taxonomical classification varies with the region of the 16S rRNA gene sequenced

The 16S rRNA gene contains 9 defined HVR[7]. These HVRs are of varying usefulness in classification depending on which species is being classified[22]. Therefore it would be necessary to choose the most informative region to sequence if less than 400 bases can be covered. To determine which region provided the most accurate classification data for the 219 foregut representative species, we undertook an analysis of different 350-bp regions of the 16S rRNA gene.

In the above size-based analysis, the 350-bp amplicon (determined to have 86.8% classification accuracy) (Figure 1) was located at the beginning of the 16S rRNA gene, starting immediately after the 8F primer, spanning bases 28-428 and including HVRs 1 and 2. To identify other potential 350-bp regions within the 16S rRNA gene that may have better classification accuracy, we analyzed 6 350-bp regions that simulated amplicons generated using various combinations of universal primers and covered the 9 HVRs (Table 2). Sequence reads from the designed amplicons were simulated by trimming the full length 16S rRNA gene sequences of the 219 foregut species as described in Materials and Methods. Because all amplicons were longer than the maximum read length of 454 technology, 2 sequence reads were simulated from the amplicons. A forward read, analyzing the first 350 bases from the 5' end of the sense strand, and a reverse read analyzing the first 350 bases from the 3' end. In total, 6 amplicons were created (A-F), and 12 reads

Table 2 Amplicons designed for analysis

Amplicon	Primers		Total length	HVR(s) included
	F	R		
A	8F	534R	527	1,2,3
B	343F	798R	456	3,4
C	517F	926R	410	3,4,5
D	784F	1114R	331	5,6
E	917F	1407R	491	6,7
F	1099F	1541R	443	7,8,9

HVR: Hypervariable region; F: Forward primers; R: Reverse primers.

were analyzed (forward reads A-F and reverse reads A'-F'). The location and direction of the reads from these amplicons is illustrated in Figure 2.

Using the RDP Ⅱ classifier, we evaluated the classification accuracy of the 12 reads at the phylum, class, order, family, and genus levels (Table 3). At the phylum level, classification accuracies for the 12 proposed reads ranged between 97.7% and 100% and at the genus level from 84.5% to 91.8%. Read B' was the most accurate at the genus level, covering bases 433-783 including the HVR 3 and 4. Its accuracy *vs* the 900-bp Sanger mimics was 93.6%.

To determine the classification accuracy of sequence read B' at the species level, we used the B' region from the full length 219 foregut species sequences and assigned each B' sequence to a species level operational taxonomic unit (SLOTU) using RDP Ⅱ Seqmatch. The assigned SLOTUs using sequence read B' were compared to the SLOTUs assigned with full length or 900 bp 16S rRNA gene sequences. At the species level 14 of the 219 foregut species were misclassified *vs* full length sequences (93.6% accuracy) (Table 4), compared to 10 of the 219 foregut species misclassified *vs* 900 bp sequences (95.4% accuracy) (data not shown).

It is important to note that our species level analyses of amplicon B' was an unweighted analysis where each species was represented equally. Because not all of the 219 species are equally represented in the foregut, we used the results of the species level classification along with the experimentally determined relative abundance of each species in the foregut to give a weighted accuracy value. This provided a relative value of how many total sequences might be misclassified instead of how many species are misclassified. The relative abundance of each species in the foregut was based on the number of sequences of each species found in the foregut.

Of the 9484 sequences from the 3 studies, 671 would have been found to be classified inaccurately with amplicon B', or approximately 7% giving a weighted species level classification accuracy of 92.9% for amplicon B' (unweighted was 93.6%). Of those 671 sequences misclassified using sequence read B', about half (369, or 55.0%) belonged to *Prevotella melaninogenica* and a significant number (193, or 28.8%) belonged to *Streptococcus infantis*, which was incorrectly identified as *Streptococcus mitis*.

Next, we directly examined the efficacy of amplicon B' with every sequence from the esophageal study (n =

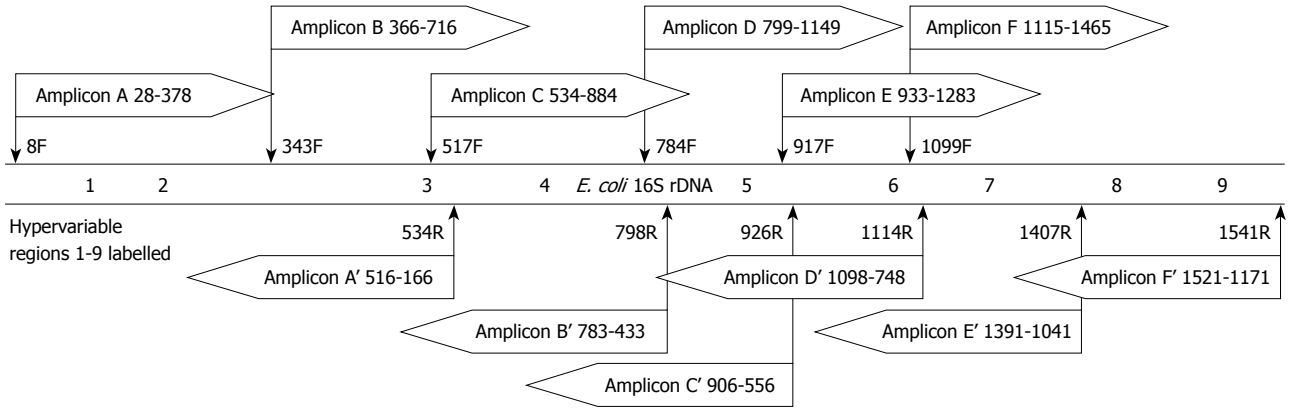

Figure 2 Design of amplicons for *in silico* evaluation. Amplicons designed using the 6 universal primer sets as described in Table 3 were evaluated for theoretical forward and reverse reads. The schematic shows the relative position of each amplicon read and read direction, as well as which bases would be included in the sequence. The positional template is the *Escherichia coli (E. coli)* 16S rDNA gene with the approximate locations of the hypervariable regions labeled.

Table 3 Accuracy of taxonomic classification of 219 foregut species using 350-bp sequences

Amplicon	% accuracy compared to 900-bp amplicon/full length				
	Phylum	Class	Order	Family	Genus
Forward reads					
A	97.7/97.7	96.3/95.9	95.9/95.4	93.2/94.5	87.7/86.8
B	99.1/99.1	98.2/96.8	97.7/96.3	97.3/95.9	91.8/89.0
C	99.5/99.5	98.2/96.8	97.7/96.8	97.3/95.9	90.4/88.1
D	98.6/99.1	98.6/98.2	97.3/97.3	95.9/96.3	88.1/86.8
E	98.2/98.6	97.7/98.2	95.4/95.9	92.2/93.6	85.4/84.9
F	97.7/98.2	96.3/97.3	95.0/95.4	91.8/93.6	83.6/84.5
Reverse reads					
A′	98.6/98.6	97.3/97.7	97.3/97.7	95.0/96.3	90.0/90.9
B′	99.5/99.5	98.2/96.8	97.7/96.8	97.7/96.3	93.6/91.8
C′	99.5/99.5	98.2/96.8	97.7/96.8	97.3/95.9	91.3/90.0
D′	99.5/100	98.6/99.5	96.8/98.2	94.5/96.3	87.2/89.5
E′	98.2/98.6	96.8/98.2	94.5/96.3	91.3/94.1	83.6/87.2
F′	98.2/98.6	96.8/98.2	95.0/96.4	92.2/95.0	82.6/85.8

6800). The oral and gastric sequences were not analyzed because some were too short to span the full amplicon B' region. We compared the taxonomical classifications obtained using the amplicon B' truncations to those obtained using the original sequences (approximate 900 bp in length). Amplicon B' accurately classified 6332 of the 6800 sequences (93.1% accuracy) at the species level. The 468 misclassified sequences belong to 14 species (Table 5).

Optimization of primers used to generate amplicon B'

To maximize the probability of amplifying all bacterial species in the foregut, the 343F (15mer between positions 343 and 357) and 798R (15mer between positions 784 and 798) primer set were examined against 16S rRNA genes from these species to determine their universality. Of the 219 species, 217 (99.1%) had 100% homology to 343F and 197 (90.0%) had 100% homology to 798R. While the 343F primer had favorable homology with all sequences, it was extended 8 bases to position 365 and deleted 4 bases between positions 343 and 346 because the original primer sequence was too short, having a low melting temperature that would not have worked with PCR conditions when used in tandem with the 798R primer. This new primer, designated as 347F is a 19mer but this modification resulted in only 113 sequences (51.6%) with 100% homology. The 798R primer was also lengthened to base 803, but shortened from 784 to 785 to ensure CG at 5' end of replication and to bring the melting temperature closer to the forward primer's. This modified primer, designated as 803R is a 19mer and the modification resulted in only 180 sequences (81.4%) with 100% homology. To improve the universality of the modified 343F and 798R primers, we analyzed both at each individual base (Figure 3). For both the modified 343F and 798R there are positions of relatively low consensus. In the modified 343F, the consensus bases at positions 359 and 360 match with only 77.2% and 74.4% of the 219 species, respectively. In 798R, the consensus bases at positions 798 and 784 have 90.0% matches, respectively. To improve on these mismatches degenerative primers were constructed. Thus bases 359 and 360 in the modified 343F were changed from G (guanine) to R (guanine or adenosine). For 798R, base 798 was changed from A (adenosine) to R (guanine or adenosine) resulting in improved base matching as shown in Figure 3. The optimized 347F was 100% homologous with 205

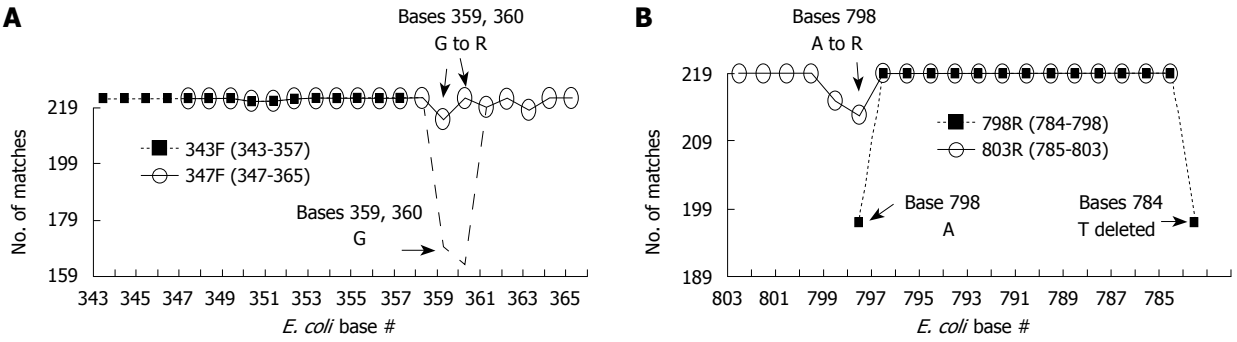

Figure 3 Optimization of primers used to generate amplicon B. European Ribosome Database primers 343F (A) and 798R (B) were optimized to generate maximal % match with corresponding region in the 16S sequences of foregut species on a base by base manner. Each primer base was analyzed for the number of matches with the corresponding base of all 219 foregut species studied [base # assigned by position within *Escherichia coli (E. coli)* 16S sequence]. Most bases for both 343F and 798R showed 100% match (219/219), however some bases had slight mismatch % and a few bases had significant mismatch % (base 798, 784, and 359, 360). To have a better homology between the primers and the foregut 16S sequences, the bases with significant mismatch were adjusted to result in lower % mismatch. This was accomplished by changing 798A to R (increasing match from 197 to 213/219) and by changing 359G and 360G to R (increasing match from 169 and 163 to 212 and 219/219, respectively). Further modifications of primers were made to make them more suitable for polymerase chain reaction (PCR) reactions. R primer 5' end was shifted from 798 to 803, and 3' end from 784 to 785. F primer 5' end was shifted from 343 to 347 and 3' end from 357 to 365. These changes provided suitable melting and annealing temperatures for the designed primer pairs in our PCR reactions. Resulting primers were designated 347F and 803R.

Table 4 Foregut species misclassified using amplicon B' compared with full length sequences

Species	Weight (of 9484)	Species identified using amplicon B
Atopobium AY959044	8	*Atopobium parvulum*
Bacteroides vulgatus	1	Uncultured bacterium
Bradyrhizobium japonicum	0	*Blastobacter denitrificans*
Bradyrhizobium liaoningense	1	*Blastobacter denitrificans*
Escherichia fergusonii	1	*Shigella sonnei*
Escherichia flexneri	4	*Shigella sonnei*
Haemophilus aegyptius	17	*Haemophilus influenzae*
Haemophilus haemolyticus	34	*Haemophilus*
Lactobacillus gasseri	2	*Lactobacillus johnsonii*
Leptotrichia wadeii	10	*Leptotrichia shahii*
Neisseria macaca	28	Uncultured bacterium
Prevotella melaninogenica	369	Uncultured bacterium
Pseudoramibacter	3	Uncultured bacterium
Streptococcus infantis	193	*Streptococcus mitis*
Total	671	

Table 5 Esophageal species misclassified using amplicon B' compared with Sanger sequences

Species	Weight (of 6800)	Species identified by Amplicon B'
Actinomyces naeslundii	2	*Actinomyces viscosus*
Atopobium AY959044	8	*Atopobium parvulum*
Bacteroides vulgatus	1	Uncultured bacterium
Bradyrhizobium japonicum	1	*Blastobacter denitrificans*
Campylobacter showae	4	*Campylobacter*
Escherichia flexneri	2	*Shigella sonnei*
Haemophilus aegyptius	17	*Haemophilus influenzae*
Haemophilus haemolyticus	33	*Haemophilus*
Lactobacillus gasseri	2	*Lactobacillus johnsonii*
Leptotrichia wadeii	7	*Leptotrichia shahii turn*
Neisseria macaca	25	Uncultured bacterium
Prevotella melaninogenica	288	Uncultured bacterium
Pseudoramibacter	1	Uncultured bacterium
Streptococcus infantis	77	*Streptococcus mitis*
Total	468	

(93.6%) species and 803R had 100% homology with 209 (95.4%) species. The sequences of the optimized primers are 5'-GGAGGCAGCAGTRRGGAAT (347F) and 5'-CTACCRGGGTATCTAATCC (803R).

347F/803R primer set has a broad taxonomic coverage of domain bacteria

The 347F/803R primer set has a broad taxonomic coverage of common foregut bacterial species identified from the limited number of sequences generated by Sanger sequencing, but deep sampling of the foregut microbiome by 454 sequencing will uncover numerous species that were below the detectable level by Sanger sequencing. To evaluate their potential to detect species not included in the foregut dataset, 347F/803R primer sequences were searched against the 16S rRNA gene database at RDP (http://rdp.cme.msu.edu/). The database has a collection of 16S rRNA genes from 5165 type strains and 433 306 high quality, near full length 16S rRNA genes from both cultured and uncultured bacterial species, representing 36 phyla. For optimized primer 347F, the introduction of ambiguity codons improved the coverage to 91.1% from 65.7% for the type strain sequences and to 90.4% from 63.7% for all 16S rRNA genes (Table 6). In comparison, the optimized primer 803R had an identical match with 91.8% of the type strain sequences and 84.9% of all 16S rRNA genes. Assuming a typical PCR can tolerate 2 mismatches between a primer and a template, the modified 374F will be able to anneal 99% of the type strain sequences and 98% of all 16S rRNA genes, compared with 99.9% and 99.6% for 803R primer, respectively.

DISCUSSION

The introduction of next generation sequencing has been a boon to many fields of research, but has not yet been fully applied to microbial ecology. One reason for

Table 6 Taxonomic coverage of domain bacteria by primers 347F and 803R

Primer	Optimization	Total species	Coverage at mismatches *n* (%)			Total sequence	Coverage at mismatches *n* (%)		
			0	1	2		0	1	2
374F	Before	5165	3392 (65.7)	4835 (93.6)	5042 (97.6)	433306	275801 (63.7)	406626 (93.8)	418613 (96.6)
	After	5165	4703 (91.1)	4996 (96.7)	5114 (99.0)	433306	391695 (90.4)	418832 (96.7)	424756 (98.0)
803R	Before	5165	4584 (88.8)	5091 (98.6)	5159 (99.9)	433306	352827 (81.4)	417612 (96.4)	430967 (99.5)
	After	5165	4741 (91.8)	5131 (99.3)	5159 (99.9)	433306	367771 (84.9)	427791 (98.7)	431725 (99.6)

this is that previous read lengths of approximately 200 bases for 454 sequencing were not sufficient to accurately classify bacteria based on their 16S rRNA genes[33]. However, with the recent improvements to 454 sequencing which allows for longer (approximately 400 base) read lengths, this is now changing.

This increase in read length greatly improves 454's applicability to 16S rRNA gene studies. We have shown that read lengths comparable to current 454 output (300-400 bases) give satisfactory 16S rRNA gene classification of bacteria at the genus and species level while shorter read lengths (such as those from Solexa and SOLiD technology) do not. However, with a change in sequencing read lengths of 16S rRNA from about 900 bp (as is commonly used with Sanger sequencing) to read lengths of 400, it is important to revisit which portion of the 16S rRNA gene to focus on to gain the most information from shorter reads. With the selection of the most informative stretch of the 16S rRNA gene, it is also important to ensure that the designed primers achieve a level of universality to be able to detect a wide array of microbial species. Although the primer pairs we chose to analyze were already available from the European Ribosomal Database, Wang has recently reported coverage rates of existing and predicted primers which span various regions of the 16S rRNA gene[34]. Some of those predicted primers with good coverage overlapped those that we had chosen.

Using the designed primers and 454 sequencing data, a complex microbiome can also be characterized by the relative abundance of 16S rRNA genes representing bacterial species in the microbiome, by assigning the 16S genes to specific species and calculating their relative weights. This is a much more accurate method of determining species relative abundance within the microbiome than the traditional methods such as colony forming unit counting (which is biased against fastidious bacteria) or cloning (which may be skewed due to cloning bias). As a comparison, the absolute amount of 16S rRNA genes from each organism can be determined by quantitative PCR (qPCR) but the number of species that can be tested in qPCR is limited.

The primer pairs we analyzed could theoretically be used for a wide range of bacteria-containing sources, such as environmental and clinical samples. By concentrating on species known to reside in the foregut (mouth, esophagus, and stomach), we have confirmed that 454 sequencing is an appropriate method for 16S rRNA gene analysis of the foregut microbiome. Careful choice of our primer set allowed us to find a region of the 16S rRNA gene that gives maximum classification accuracy within the current size limitations of 454 sequencing. This region was between the universal primers 343F and 798R, encompassing bases 361-784 and HVR 3 and 4. Focusing on just the 219 species of the foregut also allowed us to tailor our primers for better match, resulting in optimization of primers 343F and 798R (which we have modified to 347F and 803R).

We are using these primers in several foregut microbiome projects including one supported by the Human Microbiome Project. Without the time and cost restraints of cloning, 454 sequencing will allow us to analyze many more sequences than in previous foregut microbiome research (thousands *vs* 50-200 sequences per sample as was done previously). We expect that this more in-depth analysis of the foregut microbiome made possible by 454 sequencing will allow us to more clearly characterize the association between commensal bacteria of the esophagus and GERD-related esophageal disease progression to esophageal adenocarcinoma. In addition to identifying bacteria already shown to occupy the foregut, 454 sequencing will broaden the range of bacteria found within the human foregut with the increased coverage. This may lead to discovery of additional species, as well as previously undiscovered species, residing within the human foregut and a more accurate estimation of the species diversity of the foregut in normal and disease conditions.

Any species that was not included in our computational analyses will have a good chance of being identified by the foregut primers, based on our data showing the broad taxonomic coverage of these primers within domain bacteria. Thus, even though these primers were optimized for foregut species, they could potentially be used for other 16S rRNA based applications such as microbiome analysis on other anatomic sites or environmental samples. These carefully selected and designed primers will be essential tools in our efforts to harness the maximum potential that 454 sequencing offers to the field of microbial ecology of the human body.

COMMENTS

Background

The study of the human foregut microbiome has generated interest recently because of its association with gastroesophageal reflux disease-related complications. New technologies, such as 454 pyrosequencing, have increased the capability and scope of the study of complex microbiomes but have not yet been adapted for use with foregut microbiome samples.

Research frontiers

16S rRNA gene analysis has previously been used to characterize the foregut microbiome. However, this was done with older techniques, such as Sanger sequencing, which did not allow for truly in-depth sequencing. In this study, the authors outline the size and location requirements for primer sets suitable to adapt 16S rRNA gene surveys to 454 pyrosequencing.

Innovations and breakthroughs

Previous 16S rRNA gene surveys used an amplicon of near full length, reading the first 900 or so bases. However, the restrictions of 454 sequencing limit amplicon size to 600 bases and read sizes to 400 bases. This is the first effort to systematically design a primer set to study the foregut microbiome using 454 pyrosequencing by testing different, suitably sized regions within the 16S gene for maximum accuracy of classification.

Applications

By designing a primer pair allowing accurate classification using 454 sequencing, this study will allow for in-depth characterization of the foregut microbiome, which is known to be associated with disease. Characterization of the disease-associated foregut microbiome may eventually lead to novel cures or diagnostics.

Terminology

454 pyrosequencing is a next generation sequencing technology that allows for sequencing of DNA in a high throughput fashion (1 200 000 reads per run). 16S rRNA genes are ubiquitous, highly conserved markers that code for a ribosomal RNA unit. This gene marker is commonly used to identify bacteria at the species level.

Peer review

The paper is well written and clearly illustrates its findings. The findings of this study are significant, as it provides a comparatively cheap and rapid way of identifying foregut bacteria. The study design and approach are sound. These results may replace the labor-intensive, time-consuming Sanger sequencing method. More importantly, this may lead to a detection method for foregut disease conditions.

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ORIGINAL ARTICLE

Effect of probiotics on pro-inflammatory cytokines and NF-κB activation in ulcerative colitis

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Abstract

AIM: To demonstrate the therapeutic effect of probiotics in patients with ulcerative colitis (UC), and their effect on inflammatory mediators and nuclear factor (NF)-κB activation in these patients.

METHODS: Thirty patients with mild to moderate UC were randomly classified into two groups: sulfasalazine group, who received sulfasalazine 2400 mg/d; and probiotic group, who received sulfasalazine 2400 mg/d with probiotic. The patients were investigated before and after 8 wk of treatment with probiotic (*Lactobacillus delbruekii* and *Lactobacillus fermentum*). Colonic activity of myeloperoxidase (MPO) was assayed with UV spectrophotometry, the colonic content of interleukin (IL)-6 was determined by enzyme-linked immunosorbent assay (ELISA), fecal calprotectin was determined by ELISA, and expression of NF-κB p65 and tumor necrosis factor (TNF)-α proteins in colonic tissue was identified by immunohistochemistry and reverse transcription polymerase chain reaction, respectively.

RESULTS: At the start of the study, colonic mucosal injury and inflammation were demonstrated in UC patients by hematoxylin and eosin staining, and an increase in colonic MPO activity, fecal calprotectin, and expression of colonic TNF-α and NF-κB p65 proteins. The use of probiotic for 8 wk significantly ameliorated the inflammation by decreasing the colonic concentration of IL-6, expression of TNF-α and NF-κB p65, leukocyte recruitment, as demonstrated by a decrease in colonic MPO activity, and the level of fecal calprotectin compared to sulfasalazine group and the control group ($P < 0.05$).

CONCLUSION: Oral supplementation with probiotics could be helpful in maintaining remission and preventing relapse of UC.

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Key words: Ulcerative colitis; Probiotics; Interleukin-6; Nuclear factor-κB

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Hegazy SK, El-Bedewy MM. Effect of probiotics on pro-inflammatory cytokines and NF-κB activation in ulcerative colitis. *World J Gastroenterol* 2010; 16(33): 4145-4151 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i33/4145.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i33.4145

INTRODUCTION

Ulcerative colitis (UC) is one of the two major forms of inflammatory bowel disease (IBD), and is a chronic and relapsing inflammatory condition that is characterized by colonic tissue edema, increased colonic epithelial permeability, and extensive infiltration of leukocytes in the colon[1]. The pathogenic mechanism of UC involves dysregulation of the intestinal immune response to intestinal

environmental antigens, such as intestinal microflora[2-4]. UC occurs in the colon where many intestinal microbes reside[5,6]; it does not significantly develop or progress in germ-free animals, which indicates that intestinal microflora play an important role in initiating and perpetuating colonic inflammation. Normal intestinal microflora comprises an estimated 400 different bacterial species that reach their highest concentrations in the terminal ileum and colon[7,8]. Intestinal microflora produces toxic compounds, such as Gram-negative bacterial endotoxin, and harmful enzymes, such as β-glucuronidase and tryptophanase, which produce cytotoxic or carcinogenic agents[9-11]. Cytotoxins and endotoxins might interact at the apical intestinal surface and induce responses in intestinal epithelial cells, which produce pro-inflammatory cytokines and other mediators that induce inflammatory activation of the mucosal immune system.

At present, medical treatment of UC relies mainly on traditional drugs: aminosalicylates, corticosteroids, and immunosuppressants. These drugs reduce inflammatory injury and attenuate the expression of some pro-inflammatory molecules, but their side effects and systemic activity severely disturb the quality of life of patients, particularly during long-term treatment[12]. Manipulation of the mucosal microbiota to reduce the inflammatory potential of colonizing bacteria is therefore an attractive therapy for UC. One option is to use antibiotics to remove species involved in inducing the inflammatory response[13]. However, antibiotic therapy has had limited success in UC, possibly due to the fact that treatment needs to be customized for individual patients.

An alternative is to use probiotic bacteria that interact with the host epithelium to resolve inflammation. Probiotics have been defined as live microbial food supplements that beneficially affect the host by improving its intestinal microbial balance[14]. The most widely used probiotics in humans are bifidobacteria and lactobacilli. Lactic acid bacteria (LAB) are safe microorganisms that improve disturbances in the indigenous microflora, ameliorate the development of microflora[15], have anti-diabetic and anti-hyperlipidemic effects[16,17], inhibit carcinogenesis[18], have anticolitic effects[18], and induce nonspecific activation of the host's immune system[19]. Nevertheless, the anticolitic mechanism of LABs has not been thoroughly examined. Therefore, this study was conducted to demonstrate the probable therapeutic effect of probiotics in patients with UC, and to evaluate their effect on the inflammatory mediators and nuclear factor (NF)-κB activation in these patients.

MATERIALS AND METHODS

Patients

The subjects of this study were selected from UC patients with chronic diarrhea who were seen at the outpatient clinic of Tanta University Hospitals, and some inpatients at the Internal Medicine Department. Patients were newly diagnosed by colonoscopy and biopsy. They were classified as follows: 10 healthy volunteers (six male and four female) as the control group, and 30 patients (23 male and seven female) with mild to moderate UC assessed by Mayo score[20]. Patients were excluded if they had been on corticosteroids or any other immunosuppressant, had colorectal carcinoma, bilharzia, pregnancy, or hepatic or renal dysfunction.

Table 1 Demographic characteristics

Parameter	Control (n = 10)	Sulfasalazine group (n = 15)	Probiotic group (n = 15)
Age (yr)	46 ± 1.89	48 ± 1.90	47 ± 1.59
Sex (M:F)	6:4	11:4	12:3
Smoking (yes/no)	3/7	2/13	1/14
Mayo score	0	4	4
Extent of the disease			
Left side colitis	0	4	5
Proctitis	0	5	4
Pancolitis	0	6	6
ESR (mm/h)	27 ± 2.1	66.3 ± 1.3	69.2 ± 1.2
Albumin/globulin ratio	1.9 ± 0.2	1.1 ± 1.3	1.2 ± 1.1
Leukocyte count (cells/mm)	4.3 ± 1.2	8.9 ± 1.3	8.5 ± 1.1
Hemoglobin (%)	14 ± 1.5	10.5 ± 1.1	10.7 ± 1.6
Stool frequency (/d)	1	4	4
Blood in stool	-	+	+
Fever	-	-	-
Pulse rate (/min)	80 ± 2	93 ± 3	94 ± 2

ESR: Erythrocyte sedimentation rate; +: Minimal.

All the patients were subjected to full history taking, thorough clinical examination, endoscopic examination, biopsy and histological examination, and some laboratory investigations (Table 1).

Informed consent was obtained from all the participants. The protocol of the study was approved by the Ethical Committee of the University.

The patients were randomly sub-divided into two equal groups: the sulfasalazine group, who were treated with oral sulfasalazine 2400 mg/d with placebo (starch) for eight consecutive weeks[21]; and the probiotic group, who were treated with sulfasalazine 2400 mg/d with a probiotic preparation (Lacteol Fort; Rameda, Egypt) for eight consecutive weeks. The probiotic preparation was provided in sachets, which contained powder with 10 billion CFU of *Lactobacillus delbruekii* and *Lactobacillus fermentum*, to be dissolved in 50 mL fresh water.

Assessment of colon macroscopic and histological damage

Biopsy samples were obtained from inflamed colonic mucosa. Colon tissues were fixed in 4% paraformaldehyde, dehydrated, paraffin embedded, and stained with hematoxylin and eosin. At the same time, colon samples from the same sites were obtained and snap-frozen at -80℃ for subsequent determinations.

Measurement of stool fecal calprotectin by enzyme-linked immunosorbent assay

Stool samples were collected and suspended in extraction buffer, and homogenized for 25 min. One milliliter

of the homogenate was transferred to a tube and centrifuged for 20 min, and the supernatant was collected and frozen at -20℃. Calprotectin was analyzed by enzyme-linked immunosorbent assay (ELISA)[22].

Measurement of colonic myeloperoxidase and interleukin-6 levels

Colon tissues were weighed and homogenized in a solution that contained 0.5% hexadecyl trimethyl ammonium bromide dissolved in 10 mmol/L phosphate buffer (pH 7), and centrifuged for 30 min (20000 *g*) at 4℃. An aliquot (50 μL) of the supernatant was added to a reaction mixture of 1.6 mmol/L tetramethylbenzidine and 0.1 mmol/L H_2O_2, and incubated at 37℃. The absorbance was obtained at 460 nm. Myeloperoxidase (MPO) activity was defined as the quantity of enzyme that degraded 1 μmol/mL of peroxide at 37℃, expressed in units per gram wet tissue[23].

Colon tissues were minced, suspended in 2 mL 10 mmol/L phosphate buffer (pH 7.4) and incubated in a shaking water bath (37℃) for 20 min. The samples were then centrifuged (9000 *g* for 30 s), and the supernatant was kept at -70℃ until interleukin (IL)-6 ELISA[24].

Detection of colonic of NF-κB p65

Expression of NF-κB p65 in colonic tissue was assessed by immunohistochemistry. Tissue sections were deparaffinized in xylene and rehydrated in a descending ethanol series. After dewaxing and rehydration, antigen retrieval was done by microwave for 15 min. Endogenous peroxidase activity was blocked by 20 min incubation in 3% H_2O_2 in methanol at room temperature. The sections were incubated with 1:100 diluted specific polyclonal rabbit anti-rat NF-κB p65 serum (NeoMarkers, Fremont, CA, USA) for 12 h at 4℃, or incubated with 1:100 diluted normal rabbit serum under the same conditions as the negative control. After phosphate buffered saline (PBS) washing, the slides were incubated with a biotinylated horseradish-peroxidase-conjugated secondary antibody and 0.1% diaminobenzidine substrate. Sections prepared by substituting PBS for the primary antibody served as the negative control. Positive expression of NF-κB p65 was shown by brown deposited granules in the cytoplasm and/or nucleus. The results were evaluated semi-quantitatively according to the percentage of positive cells in 10 randomly selected fields under high power microscopy (700 × magnification).

Detection of mRNA expression of tumor necrosis factor-α by reverse transcription polymerase chain reaction

mRNA expression for tumor necrosis factor (TNF)-α was assessed using reverse transcription polymerase chain reaction (RT-PCR) standardization by co-amplification of the housekeeping gene β-actin, which served as an internal control. Total RNA from colonic tissues was isolated using Trizol reagent (Sigma, St. Louis, MO, USA) by the single step method, and was reverse transcribed into cDNA. The resultant cDNA was used as template for subsequent PCR. The rat-specific primers (sense and antisense primers) for TNF-α and β-actin were 5'-CATGATCCGAGATGTGGAACTGGC-3' and 5'-CTGGCTCAGCCACTCCAGC-3' (TNF-α, 315 bp) and 5'-ATGGATGACGATATCGCTG-3' and 5'-ATGAGGTAGTCTGTCAGGT-3' (β-actin, 568 bp), respectively. Amplification was performed in 30 cycles, with initial incubation at 95℃ for 3 min and final extension at 72℃ for 7 min; each cycle consisted of denaturation for 30 s at 95℃, annealing for 45 s at 55℃, and extension for 1 min at 72℃. PCR products were separated in 2% agarose gel and stained with ethidium bromide.

Statistical analysis

Data were statistically analyzed by paired Student's *t* test to compare between the results before (baseline) and after treatment within the same group, and unpaired Student's *t* test to compare between means of the different groups using the computer program SPSS for Windows version 10 (Chicago, IL, USA). All results were expressed as mean ± SD. The level of significance was set at $P < 0.05$.

RESULTS

Macroscopic presentation and histological evaluation

The histological findings of colonic tissues are presented in Figure 1. There was erosion in the mucosa and submucosa, mucosal edema, vascular congestion, focal hemorrhage, and infiltration of polymorphonuclear cells, plasma cells and neutrophils. No changes were observed in the control group. The sulfasalazine group showed attenuation of the extent and severity of the histological signs. Administration of probiotic plus oral sulfasalazine inhibited the extent of inflammation, prevented mucosal injury, and alleviated colitis.

Determination of colonic MPO and IL-6 levels

Evaluation of leukocyte recruitment was assessed by measurement of MPO activity. Compared to the control group, MPO activity was significantly increased in the colonic mucosa of UC patients before treatment. Administration of oral sulfasalazine with probiotic decreased MPO activity. The decrease was most significant in the probiotic group ($P < 0.05$, Figure 2).

As shown in Figure 2, a significant increase in the level of IL-6 was observed in the colon of UC patients. Both the sulfasalazine and probiotic groups showed a significant decrease in the level of IL-6 compared to that before treatment ($P < 0.05$).

Determination of fecal calprotectin level

Fecal calprotectin level was significantly higher in UC patients at the beginning of the study. Both the sulfasalazine and probiotic groups showed a significant decrease in calprotectin level compared to the UC patients, and the decrease in the probiotic group was significantly greater than that in the sulfasalazine group ($P < 0.05$, Figure 2).

Calprotectin showed a significant positive correlation

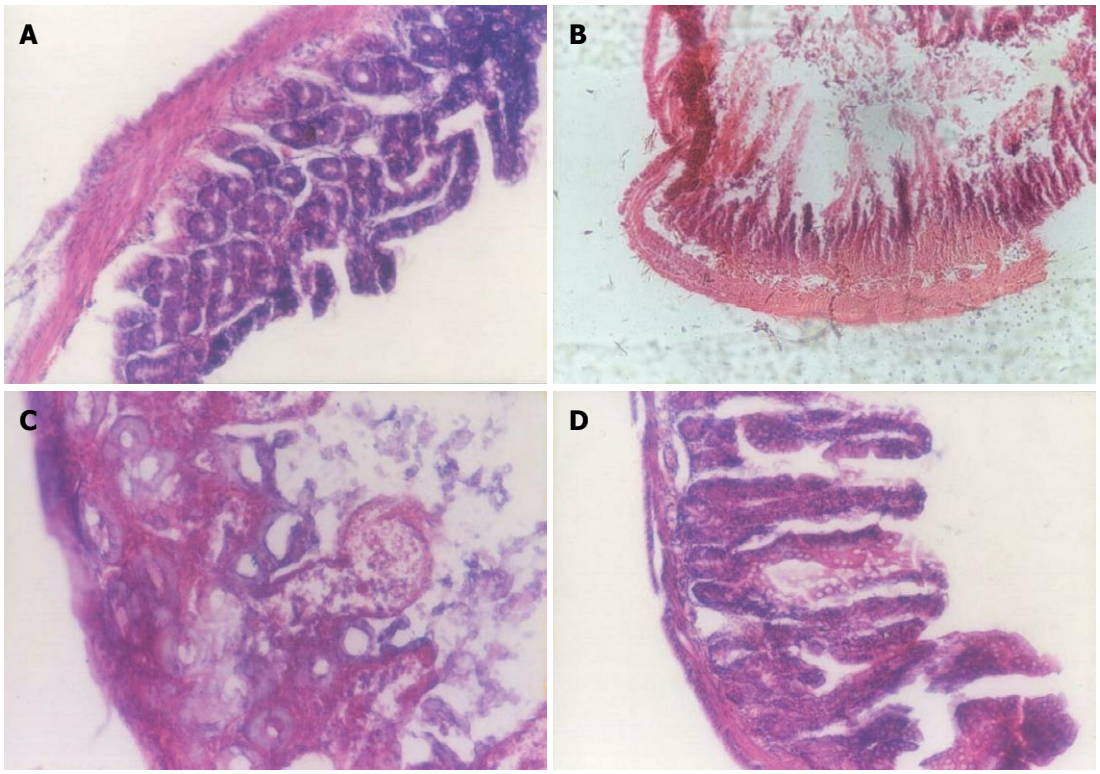

Figure 1 Hematoxylin and eosin staining of colonic tissue (HE, × 250). A: Control group showed no damage; B: Patients before treatment showed necrotic destruction of the epithelium, inflammatory cellular infiltration, and ulceration of the mucosa and submucosa; C: Sulfasalazine group showed attenuation of the extent and severity of the histological signs; D: Probiotic group showed inhibition of the extent of inflammation, and prevention of mucosal injury.

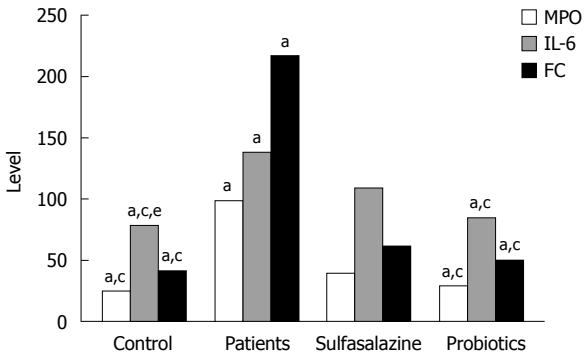

Figure 2 Myeloperoxidase activity (g/U), interleukin-6 level (pg/mL), and fecal calprotectin (g/kg) in colon of ulcerative colitis patients and controls. Data are presented as mean ± SD. $^{a}P < 0.05$ *vs* sulfasalazine; $^{c}P < 0.05$ *vs* patients; $^{e}P < 0.05$ *vs* interleukin-6 level of the probiotics. MPO: Myeloperoxidase; IL-6: Interleukin-6; FC: Fecal calprotectin.

with both MPO ($r = 0.93$, $P < 0.05$) and IL-6 ($r = 0.91$, $P < 0.05$).

Immunohistochemistry

Expression of NF-κB p65 in UC patients before treatment was significantly higher than that in the control group. NF-κB-p65-positive cells were predominantly located within the mucosa and had a brown-yellow cytoplasm. Administration of sulfasalazine resulted in a significant reduction in colonic NF-κB p65 levels. Compared with the patients before treatment, expression of NF-κB p65 in the placebo group weakened significantly, and the expression in the probiotic group was the lowest (Figure 3 and Table 2).

Table 2 Semi-quantitative index of nuclear factor-κB p65 expression in controls and ulcerative colitis patients

Reaction	Controls	Patients	Sulfasalazine	Probiotics
NF-κB	+ (6)	++++ (6)	+++ (4)	++ (2)
Immunostaining			++ (2)	+ (4)

Brackets indicate number of samples; $n = 6$ for each group. The intensity of staining for nuclear factor-κB (NF-κB) is represented in a semi-quantitative evaluation: +: Weak; ++: Moderate; +++: Strong; ++++: Intense reaction.

RT-PCR

The m-RNA level of TNF-α in UC patients at the beginning of the study was significantly higher than in the control group. TNF-α m-RNA expression was inhibited after treatment with oral sulfasalazine and probiotic. Maximum inhibitory effect was observed with the probiotic preparation (Figure 4).

DISCUSSION

The pathogenesis of UC remains unknown[25]. Genetic and environmental factors are obviously contributory. Luminal bacteria could play a major role in the initiation and perpetuation of chronic UC[26]. Thousands of endogenous bacteria live in the large intestine and might be an essential factor in certain pathological disorders. In human UC,

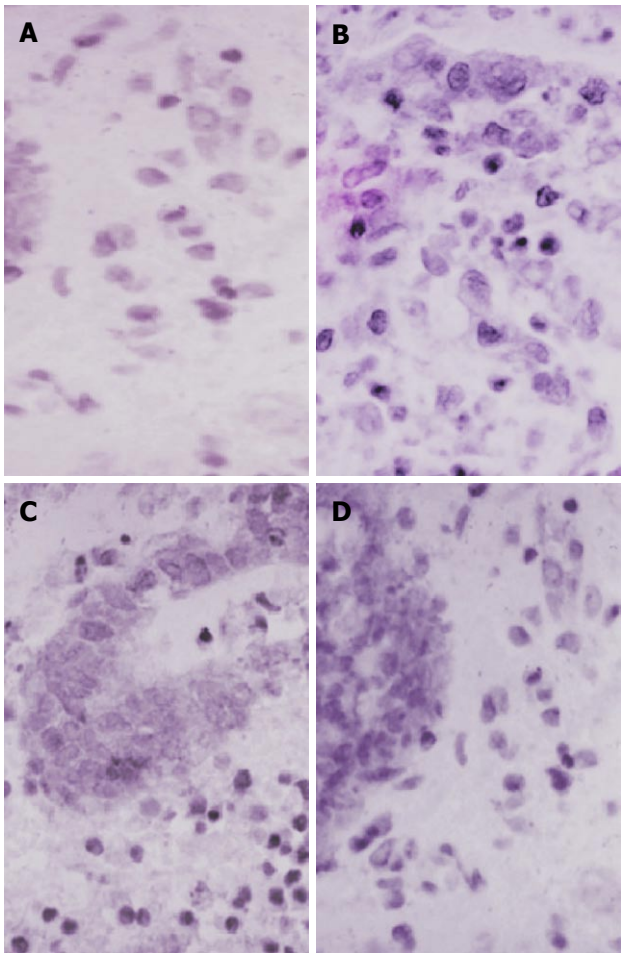

Figure 3 Immunohistochemical staining for nuclear factor-κB p65 (Brown staining, SP × 700). A: Section of colon from controls showing normal structure and architecture; B: Section of colon from ulcerative colitis patients before treatment showing extensive nuclear factor (NF)-κB (brown) expression; C: Section of colon from sulfasalazine group showing limited NF-κB (brown) expression; D: Section of colon from probiotic group showing minimal NF-κB (brown) expression.

inflammation is present in parts of the gut that house the highest concentration of bacteria. Enhanced mucosal permeability could play a pivotal role in maintaining a chronic inflammatory state due to genetic predisposition or direct contact with bacteria or their products. Manipulation of the colonic bacteria with antibiotics and probiotics has been shown to be more effective and tolerable than immunosuppressants[27].

It is well known that there is an inflammatory cascade within the gut tissues of UC that is characterized by the recruitment of circulating leukocytes into the gut tissues and the release of pro-inflammatory mediators[1]. In the present study, we observed this cascade of inflammatory cells in colitis. First, our results showed that MPO activity, an index of leukocyte infiltration, was increased significantly in UC patients, which suggested recruitment of leukocytes. Second, the increase in the level of fecal calprotectin represented mucosal neutrophil infiltration. Third, the expression of IL-6 and TNF-α protein was upregulated significantly, as demonstrated by ELISA and PCR. These results are supported by some investigators who have shown the development of such a cascade of

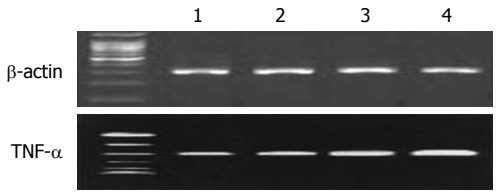

Figure 4 Expression of tumor necrosis factor-α mRNA. Lane 1: Control group; Lane 2: Probiotic group; Lane 3: Sulfasalazine group; Lane 4: Ulcerative colitis patients before treatment. TNF-α: Tumor necrosis factor-α.

inflammatory events in dextran-sulfate-sodium-induced colitis in mice[28]. Probiotic administration for 8 wk reduced MPO activity significantly, as well as the expression of IL-6 and TNF-α. These results are in agreement with those of Federico *et al*[29], who have found that a symbiotic preparation that contained *Lactobacillus paracasei* B 20160 restored the serum level and mRNA expression of IL-6, IL-8, and TNF-α in UC patients. Other investigators have found that *Lactobacillus HY* 7801 blocks tri-nitrobenzene-sulfonic-acid-stimulated MPO activity in the intestine, as well as the expression of IL-1β, and TNF-α[30].

A prominent feature of mucosal histology in patients with active IBD is infiltration by neutrophilic granulocytes[31]. Calprotectin is a major protein of neutrophils and macrophages, and accounts for about 60% of the cytosol of these cells. In the present study, there was a good correlation between fecal calprotectin and MPO and IL-6. This result agrees with that of Wagner *et al*[32], who have found that fecal calprotectin has the potential to be used as a surrogate marker for successful treatment outcome in IBD patients. Also, they have demonstrated that fecal calprotectin and MPO provide superior discrimination to eosinophil protein X in determining treatment outcome in UC patients.

It is well known that NF-κB plays a pivotal role in expression of inflammatory mediators. NF-κB is usually found in the cytoplasm conjugated to an inhibitory protein IκB. Phosphorylation of IκB by IκB kinase after inflammatory signal transduction leads to degradation of IκB *via* proteasomes, which results in transfer of NF-κB to the nucleus and its activation. NF-κB regulates transcriptional activity by binding to specific DNA sequences in inflammatory genes that are involved in inflammatory and immune processes[33]. In the present study, the expression of NF-κB p65 was detected by immunohistochemistry. Much more NF-κB p65 protein was activated in the UC patients, and the lowest level was observed in the probiotic group. These data were consistent with the finding that *Bifidobacterium longum* downregulates TNF-α and IL-8 production and inhibits NF-κB activation of lamina propria mononuclear cells in inflamed mucosa of active UC, without any adverse effect on the viability of colonic cells[34].

Recent data have demonstrated that the mucosal immune response is involved in patients with IBD. NF-κB is a key regulator of inducible expression of many genes that are involved in immune and inflammatory responses in the gut. Stimuli like cytokines (IL-6, TNF-α), bacteria, and viruses can release NF-κB from their cytoplasmic

form to the nuclei. NF-κB can activate anti-apoptic genes, including TNF-receptor-related genes, Bcl-2 homologenes, and repress the apoptosis of some inflammatory cells such as neutrophils and activated macrophages, thereby elongating and worsening tissue inflammatory injury[35]. More potent and selective treatment strategies with anti-sense p65 and proteasome inhibitors have been aimed at preventing NF-κB activation in mucosal macrophages and T lymphocytes. However, NF-κB-regulated genes are also involved in survival responses of epithelial cells. Selective inhibition of NF-κB activation in inflammatory cells could be an option for management of IBD[36].

In the present study, administration of probiotics not only decreased the NF-κB DNA binding activity, but also reduced the accumulation of leukocytes, and downregulated IL-6 and TNF-α production, and thereby ameliorated the severity of the colitis. Therefore, supplementation with probiotics could be helpful in maintaining remission and preventing relapse of UC. These results are in agreement with those of Nikfar *et al*[37] who demonstrated that probiotics can improve the symptoms of irritable bowel syndrome, and can be used as a supplement to standard therapy. Also, other investigators[38,39] have demonstrated the efficacy of probiotics in maintaining remission of human UC and prevention of disease relapse.

COMMENTS

Background

Medical treatment of ulcerative colitis (UC) relies mainly on traditional drugs: aminosalicylates, corticosteroids, and immunosuppressants. These drugs reduce inflammatory injury but their side effects and systemic activity severely disturb the quality of life of patients severely, particularly during long-term treatment. An alternative is to use probiotic bacteria that interact with the host epithelium to resolve inflammation. The aim of the present study was to demonstrate the therapeutic effect of probiotics in patients with UC, and to evaluate their effect on the inflammatory mediators and nuclear factor (NF)-κB activation in these patients.

Research frontiers

Various *in vitro* studies have been performed in attempts to suppress the inflammation in experimentally-induced UC. They have suggested that suppression of the activity of NF-κB could control the production of several inflammatory mediators. The present study was undertaken to evaluate the therapeutic role of probiotics in UC patients and their effect on the inflammatory mediators and NF-κB activation in these patients.

Innovations and breakthroughs

The present study suggested that administration of probiotics not only decreased NF-κB DNA binding activity, but also reduced the accumulation of leukocytes, downregulated interleukin-6 and tumor necrosis factor-α production, and thereby ameliorated the severity of the colitis.

Applications

These findings suggest that supplementation with probiotics could be helpful in maintaining remission and preventing the relapse of UC. Probiotics are safe microorganisms that protect patients from the side effects of medical treatment that disturbs quality of life.

Peer review

It is an important paper and can be published after making some revisions.

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BRIEF ARTICLE

Chemoprotective effects of curcumin in esophageal epithelial cells exposed to bile acids

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Abstract

AIM: To investigate the ability of curcumin to counteract the impact of bile acids on gene expression of esophageal epithelial cells.

METHODS: An esophageal epithelial cell line (HET-1A) was treated with curcumin in the presence of deoxycholic acid. Cell proliferation and viability assays were used to establish an appropriate dose range for curcumin. The combined and individual effects of curcumin and bile acid on cyclooxygenase-2 (*COX-2*) and superoxide dismutase (*SOD-1* and *SOD-2*) gene expression were also assessed.

RESULTS: Curcumin in a dose range of 10-100 μmol/L displayed minimal inhibition of HET-1A cell viability. Deoxycholic acid at a concentration of 200 μmol/L caused a 2.4-fold increase in *COX-2* gene expression compared to vehicle control. The increased expression of *COX-2* induced by deoxycholic acid was partially reversed by the addition of curcumin, and curcumin reduced *COX-2* expression 3.3- to 1.3-fold. HET-1A cells exposed to bile acid yielded reduced expression of *SOD-1* and *SOD-2* genes with the exception that high dose deoxycholic acid at 200 μmol/L led to a 3-fold increase in *SOD-2* expression. The addition of curcumin treatment partially reversed the bile acid-induced reduction in *SOD-1* expression at all concentrations of curcumin tested.

CONCLUSION: Curcumin reverses bile acid suppression of gene expression of *SOD-1*. Curcumin is also able to inhibit bile acid induction of *COX-2* gene expression.

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Key words: Esophageal cancer; Curcumin; Cyclooxygenase-2; Superoxide dismutase; Chemoprevention

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Bower MR, Aiyer HS, Li Y, Martin RCG. Chemoprotective effects of curcumin in esophageal epithelial cells exposed to bile acids. *World J Gastroenterol* 2010; 16(33): 4152-4158 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i33/4152.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i33.4152

INTRODUCTION

Over the past three decades the incidence of esophageal adenocarcinoma has increased over four-fold, and the current five year survival remains only 10%-20%[1]. Esophageal adenocarcinoma is known to develop in the distal esophagus in the setting of exposure to both bile acids and low pH[2-4]. The exact cellular mechanisms underlying this

process are largely unknown. Oxidative stress has been theorized to play a significant role in the staged progression from reflux esophagitis to Barrett's esophagus and eventually to esophageal adenocarcinoma. Increased levels of reactive oxygen species (ROS) and oxidative injury have been detected in esophageal cells exposed to low pH and bile acids[5-7], and the level of free radicals in esophageal tissue has also been associated with the degree of esophagitis[8]. In addition, we have previously shown an association between the development of esophageal adenocarcinoma and increased levels of 8-hydroxy-deoxyguanosine, an indicator of oxidative damage, using an esophageal duodenal anastomosis rat model of reflux esophagitis[9].

Based on these findings, compounds with antioxidant properties may hold promise as chemopreventative agents in the Barrett's metaplasia-dyplasia-adenocarcinoma sequence. Curcumin is a phenolic compound derived from the plant *Curcuma longa*. Curcumin is known to have both anti-inflammatory and antioxidant properties. A number of animal and *in vitro* models have also shown curcumin to be a potent chemopreventative agent[10,11]. Previous work by Li *et al*[12] demonstrated in a rat model of esophageal reflux that intraperitoneal injections of curcuma aromatic oil, a volatile oil extract of *Curcuma aromatica*, helped prevent the development of esophageal adenocarcinoma. However, the molecular mechanisms by which curcumin may inhibit the development of esophageal adenocarcinoma have not been fully defined.

In the current study, HET-1A cells were used as an *in vitro* model to study the potential impact of curcumin on the initial changes induced by bile acids in the esophagus. HET-1A cells are a well characterized, non-cancerous, SV-40 T-antigen immortalized human esophageal epithelial cell line[13]. HET-1A cells have been shown to produce ROS after brief exposures to low pH and to bile acids[6,14]. HET-1A cells develop gene expression changes consistent with the development of Barrett's esophagus upon exposure to deoxycholic acid[15], and carcinogens have been found to induce tumorigenic characteristics within these cells[16,17]. Furthermore, previous work by Rafiee *et al*[18] has shown that curcumin has an anti-inflammatory effect on HET-1A cells by inhibition of acidic pH-induced secretion of cytokines interleukin (IL)-8 and IL-9.

We tested the hypothesis that curcumin prevents the bile acid-mediated impairment of HET-1A cellular mechanisms for managing oxidative stress. Specifically, we investigated the impact of bile acid and curcumin on expression of the human gene forms of superoxide dismutase (*SOD-1* and *SOD-2*). The ideal curcumin dose in combination with bile acid *in vitro* was determined through cell proliferation and viability testing. Curcumin- and bile acid-induced alterations of cyclooxygenase-2 (*COX-2*) gene expression were also studied as an additional mechanism of esophageal adenocarcinoma prevention. The aim of this study was to determine whether curcumin has effects on esophageal cell gene expression consistent with a chemopreventative in the setting of bile acid exposure.

MATERIALS AND METHODS

Chemicals

Curcumin was obtained from LKT Laboratories, Inc. (St. Paul, MN). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) and cDNA Trizol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). 5-bromo-2'-deoxyuridine (BrdU) ELISA assay kits were purchased from Roche Applied Science (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell culture

HET-1A cells, a SV-40 immortalized human esophageal epithelial cell line, were acquired from the American Type Culture Collection and grown in bronchial epithelial growth media (BEGM) and recommended supplements (BEGM Bullet Kit) obtained from Lonza Bio Science (Walkersville, MD). The growth media was also supplemented with 10% fetal bovine serum. Cells were grown in a monolayer and incubated at 37℃ in 5% CO_2 and 90% relative humidity.

For cellular proliferation and viability studies, HET-1A cells were plated in 96-well microtiter plates at a density of 1×10^4 cells per well and allowed to attach and grow for 48 h prior to treatment. The cells were treated with varying doses (100 nmol/L-1 mmol/L) of curcumin dissolved in ethanol and added to fresh media for a total ethanol concentration that did not exceed 2.5%. Treatment with deoxycholic acid (in ethanol) at a concentration of 100 μmol/L combined with curcumin was also performed. A vehicle control of media and 2.5% ethanol was used to study the effect of ethanol, and all treated groups were compared to this control. Treatments were conducted for 24 h.

For mRNA expression studies, HET-1A cells were plated in 6-well plates at a density of 1.5×10^5 cells per well and grown for 48 h. Curcumin and deoxycholic acid dissolved in ethanol were added with fresh media at varying concentrations. The final ethanol concentration was maintained at 0.2%, and 0.2% ethanol solution was used as a vehicle control. All studies were performed in triplicate to confirm reproducibility.

Cell viability and cell proliferation assays

MTT assays were conducted as described previously[19]. Briefly, treated media was aspirated without disrupting the cells at the bottom of the plate. MTT at a concentration of 5 mg/mL was dissolved in phosphate buffered saline (PBS) and added to each well. After incubation at 37℃ for 2 h, the MTT solution was aspirated and the wells were air-dried. DMSO was added to the wells and the absorbance was read at 570 nm in a spectrophotometer.

Cell proliferation assay based on incorporation of BrdU was used to investigate the effect of the various treatments. After the 24 h treatments, the BrdU ELISA was performed according to the manufacturer's directions.

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

Gene	Forward	Reverse
COX-2	5'-CAGGGTTGCTGGTGGTAGGA-3'	5'-CGTTTGCGGTACTCATTAAAAGACT-3'
SOD-1	5'-GTGGCCGATGTGTCTATTGAAG-3'	5'-CGTTTCCTGTCTTTGTACTTTCTT-3'
SOD-2	5'-TGGCCAAGGGAGATGTTACAG-3'	5'-CTTCCAGCAACTCCCCTTTG-3'
β-actin	5'-TTCAACTTCATCATGAAGTGTGACGTG-3'	5'-CTAAGTCATAGTCCGCCTAGAAGCATT-3'

COX: Cyclooxygenase; SOD: Superoxide dismutase.

RNA extraction and real-time polymerase chain reaction

RNA was isolated using the Trizol® method (Invitrogen, Carlsbad, CA). All procedures were carried out in an RNase free environment. The quality of the RNA was ascertained by gel electrophoresis and quantitated using NanoDrop® (NanoDrop Technologies, Wilmington, DE). The RNA was then diluted to 5 ng/μL concentration and stored at -80℃ until use. Equal amounts of RNA (0.1 μg) were reverse transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Forster City, CA) according to the manufacturer's instructions.

Primers for quantitative real-time polymerase chain reaction (RT-PCR) were designed across exon boundaries to avoid amplification of genomic DNA, using Primer express® 3.0 software (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies, Inc., (Coralville, IA). The sequences of the forward and reverse primers for each gene tested are listed in Table 1.

The PCR amplification was carried out in a final reaction volume of 20 μL containing 1 × Power SYBR® Green PCR master mix (Applied Biosystems, CA); 10 nmol/L each of forward and reverse primers specific for each gene and 40 ng of cDNA. Quantitative PCR was performed using a 7300 Prizm (Applied Biosystems, Foster City, CA) using the relative quantification protocol. The PCR conditions were: 50℃ for 2 min; DNA polymerase activation at 95℃ for 10 min; followed by 40 cycles at 95℃ for 15 s and 60℃ for 1 min. The $2^{-\Delta\Delta Ct}$ method was used to determine gene quantification with human β-actin used as an endogenous reference gene. Results were expressed as fold change in gene expression compared to the vehicle control.

Statistical analysis

Relative fold changes in each group were compared using one-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison post test. A *P*-value < 0.05 was considered significant. All statistical analyses were performed using SPSS Version 17 (*SPSS* Inc., Chicago, IL).

RESULTS

Dose response effects of curcumin on cell viability and proliferation

In order to establish a sub-toxic dose of curcumin, the influence of curcumin on HET-1A cell viability and proliferation was assessed using both MTT and BrdU assays, respectively. Similar trends were seen for both BrdU and MTT assays (Figure 1A and B). Compared to the vehicle control, all concentrations of curcumin tested showed significant reduction in cell viability as demonstrated by the MTT assay (33% to 58% reduction, $P < 0.01$). The cytotoxicity and reduction in proliferation were higher at concentrations greater than 100 μmol/L when compared to the vehicle control (MTT: 1 mmol/L - 48%, 100 μmol/L - 58%, $P < 0.001$; BrdU: 1 mmol/L - 80%, 100 μmol/L - 73%, $P \leq 0.01$) (Figure 1A). However, less cytotoxicity was seen at curcumin concentrations less than 10 μmol/L (MTT: 30%-40%, $P < 0.01$), and curcumin did not ad-

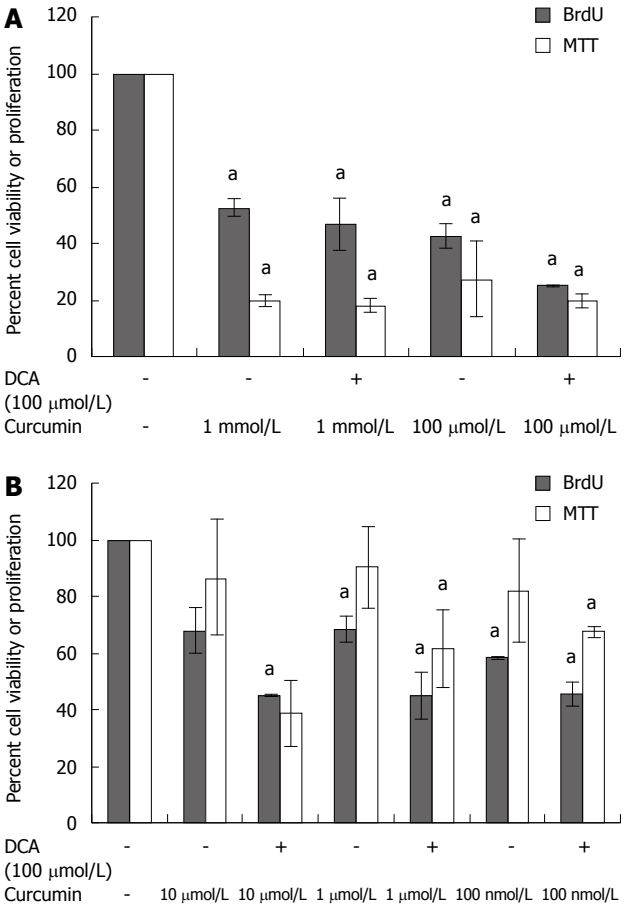

Figure 1 Effect of curcumin concentration on cell viability and proliferation at concentration ⩾ 100 μmol/L (A) and ⩽ 10 μmol/L (B). HET-1A cells incubated in 96-well plates at a density of 10 000 cells/well for 24 h demonstrated decreased cell viability and proliferation in the presence of curcumin at a concentration ⩾ 100 μmol/L or ⩽ 10 μmol/L. The viability and proliferation were also reduced in the presence of curcumin combined with deoxycholic acid (DCA) when compared to vehicle control (2.5% ethanol). $^aP < 0.05$ *vs* vehicle control. MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium; BrdU: 5-bromo-2'-deoxyuridine; DCA: Deoxycholic acid.

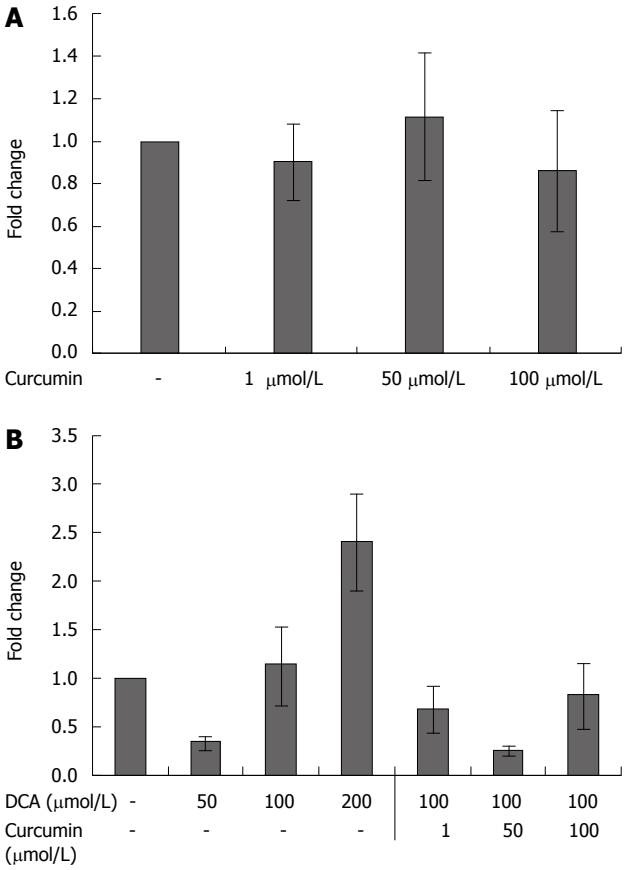

Figure 2 Effect of curcumin and deoxycholic acid on *cyclooxygenase-2* gene expression. A: Effect of curcumin on cyclooxygenase-2 (*COX-2*) gene expression. HET-1A cells in 6-well plates at a density of 1.5×10^5 cells per well incubated with curcumin for 24 h showed unaltered gene expression of COX-2 when compared to vehicle control (0.2% ethanol). Error bars: mean ± SD (n = 4); B: Effect of both deoxycholic acid and curcumin on *COX-2* gene expression. HET-1A cells in 6-well plates at a density of 1.5×10^5 cells per well incubated with deoxycholic acid (DCA) for 24 h showed increased gene expression of COX-2 with increasing doses of DCA when compared to vehicle control (0.2% ethanol). The addition of curcumin diminished the increase in gene expression. Error bars: mean ± SD (n = 4).

versely effect cell proliferation at these concentrations, as demonstrated by the BrdU assay (10%-14% reduction, $P > 0.05$) (Figure 1B). When cells were treated with deoxycholic acid (100 μmol/L) combined with varying curcumin concentrations, increased cytotoxicity was seen at each concentration of curcumin tested (Figure 1A and B). Again, the greatest cytotoxicity was demonstrated at doses of curcumin greater than 100 μmol/L ($P < 0.001$). All doses of curcumin tested caused synergistic cytotoxicity in the presence of deoxycholic acid (100 μmol/L).

Combined effects of deoxycholic acid and curcumin on COX-2 gene expression

We investigated the effects of curcumin on *COX-2* gene expression in HET-1A cells (Figure 2A and B). It was observed that 24 h curcumin treatment alone had minimal impact on COX-2 mRNA production. The degree of COX-2 mRNA production was unaltered even at doses of 100 μmol/L. Increased levels of COX-2 mRNA were observed in HET-1A cells treated with bile acid alone. Deoxycholic acid at 200 μmol/L caused a 2.4-fold increase in COX-2 mRNA levels compared to vehicle control. The increased expression of *COX-2* genes induced by deoxycholic acid was partially reversed by the addition of curcumin. It was observed that treatment of HET-1A cells with deoxycholic acid and curcumin reduced COX-2 mRNA levels 3.3- to 1.3-fold. Curcumin inhibition of bile acid-induced *COX-2* expression was observed at doses as low as 1 μmol/L curcumin. This effect was not dose-dependent, with 50 μmol/L curcumin showing the greatest effect.

Combined effects of deoxycholic acid and curcumin on SOD-1 and SOD-2 gene expression

Measurements were also made of *SOD* gene expression following curcumin treatment of HET-1A cells for 24 h. Two human gene forms of superoxide dismutase were tested: Cu/ZnSOD (*SOD-1*) and MnSOD (*SOD-2*). Results revealed that curcumin treatment led to a non-significant 36% to 52% reduction in *SOD-1* expression at all concentrations of curcumin tested. Curcumin alone also had a non-significant impact on *SOD-2* expression (Figure 3A). Compared to controls, bile acid treatment of HET-1A cells yielded consistent reduction of SOD-1 and SOD-2 mRNA production, with the exception that high dose deoxycholic acid at 200 μmol/L led to a 3-fold increase in *SOD-2* expression. Deoxycholic acid inhibited the expression of *SOD-1* (2.8- to 4.0-fold) at concentrations ⩾ 50 μmol/L (Figure 3B). The addition of curcumin treatment reversed this reduction in *SOD-1* expression induced by bile acid at all concentrations of curcumin tested (Figure 3B). The combined treatment of deoxycholic acid and curcumin resulted in reduced expression of *SOD-2* at concentrations ⩾ 50 μmol/L (Figure 3C).

DISCUSSION

The purpose of this study was to investigate the potential of curcumin as a chemopreventative in the development of reflux-induced esophageal adenocarcinoma using an *in vitro* model of HET-1A human esophageal epithelial cells. This study demonstrates that curcumin can attenuate certain effects of bile acid on HET-1A cell gene expression. Specifically, this analysis reveals that curcumin is able to partially reverse bile acid suppression of gene expression of the free radical scavenger superoxide dismutase in the form of SOD-1; however, the impact toward SOD-2 was variable. In addition, curcumin was noted to inhibit bile acid induction of *COX-2* gene expression in esophageal epithelial cells.

Several factors were taken into consideration to establish the *in vitro* model. Of the potentially available bile acids, the cells were exposed to deoxycholic acid because it has been commonly detected in aspirates of patients with Barrett's esophagus[20], and deoxycholic acid has been shown to induce DNA damage in esophageal cells through oxidative injury[21]. Also important to the model was establishing an *in vitro* therapeutic dosage range for curcumin, as curcumin is known to induce apoptosis in

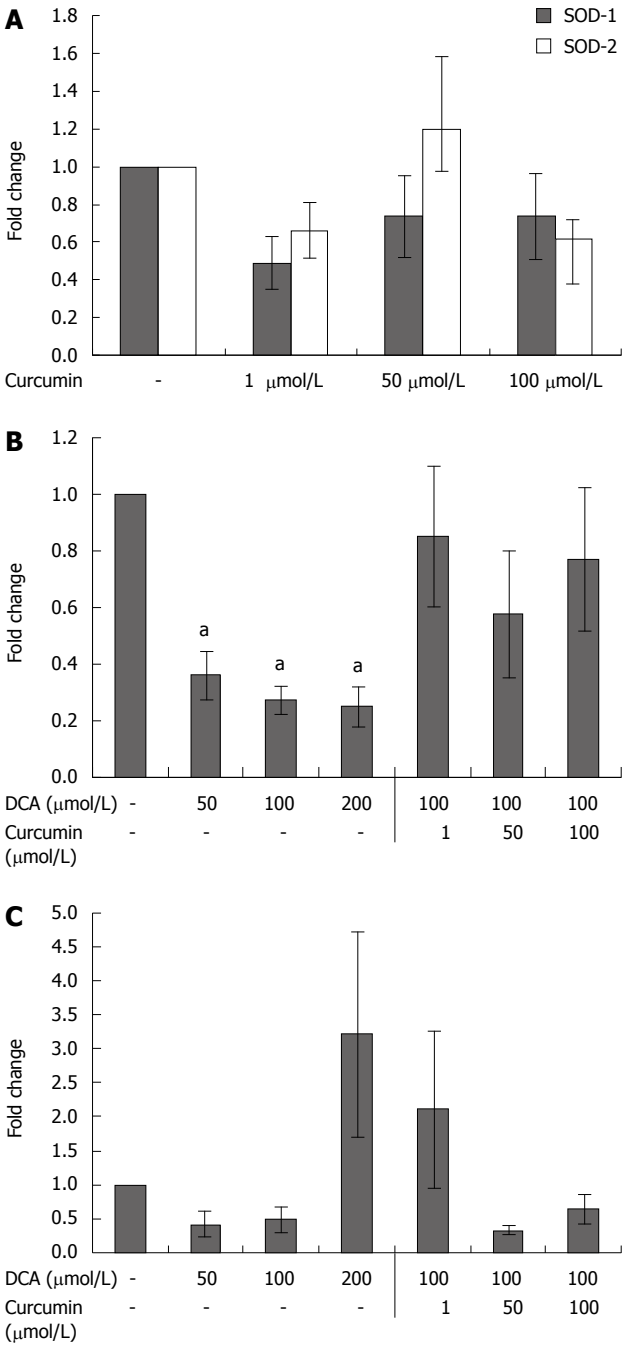

Figure 3 Effect of curcumin and bile acid on superoxide dismutase gene expression. A: Effect of curcumin on superoxide dismutase (*SOD*)-*1* and *SOD-2* gene expression. HET-1A cells in 6-well plates at a density of 1.5 × 10^5 cells per well incubated with curcumin for 24 h did not show a significant impact on *SOD-1* or *SOD-2* gene expression. Error bars: mean ± SD (n = 4); B: Effect of curcumin and bile acid on *SOD-1* gene expression. HET-1A cells in 6-well plates at a density of 1.5 × 10^5 cells per well incubated with deoxycholic acid (DCA) for 24 h showed a significant decrease in SOD-1 expression. When DCA treatment at 100 μmol/L was combined with curcumin the suppression in SOD-1 expression was alleviated. Error bars: mean ± SD (n = 4). $^aP < 0.05$ *vs* vehicle control; C: Effect of curcumin and bile acid on *SOD-2* gene expression. HET-1A cells in 6-well plates at a density of 1.5 × 10^5 cells per well incubated with deoxycholic acid (DCA) for 24 h showed a significant increase in SOD-2 expression at a DCA concentration of 200 μmol/L. The combined treatment of DCA and curcumin showed reduced expression of SOD-2 at concentrations ⩾ 50 μmol/L. Error bars: mean ± SD (n = 4).

both malignant and non-malignant cell types. Curcumin concentrations less than 10 μmol/L had the least impact on esophageal cell viability and proliferation in the presence of bile acid, and concentrations in the range of 50 μmol/L had the most dramatic impact on esophageal cell gene expression. This dosage range was valid because only curcumin concentrations greater than 100 μmol/L showed significant toxicity. The greatest toxicity was seen in curcumin doses higher than 100 μmol/L in combination with bile acid, which led to a 2- to 5-fold decrease in the proliferation and viability of HET 1-A cells.

The results revealed that curcumin did not inhibit the baseline expression of *COX-2* genes by HET-1A cells, but curcumin did show suppression of bile acid-induced expression of *COX-2* genes. Curcumin has been previously noted to inhibit both the activity and expression of COX-2 in colon cancer cells[22,23], and it has been shown that curcumin inhibits bile acid-induced COX-2 expression and activity in esophageal adenocarcinoma and squamous cell carcinoma cell lines[24]. Animal models have shown that COX-2 inhibition can potentially prevent the development of esophageal adenocarcinoma and Barrett's esophagus[25,26]. Epidemiologic studies have also suggested that the use of COX-2 inhibitors leads to lower rates of esophageal adenocarcinoma[27]. The use of selective COX-2 inhibitors has recently fallen out of favor due to their cardiotoxic side effects[28], and non-selective cyclooxygenase inhibitors such as aspirin have potential gastrointestinal effects that make them unappealing therapeutic agents in patients already suffering from reflux. Therefore, curcumin may represent a safe alternative means of COX-2 inhibition in patients with GERD.

Curcumin and bile acid treatments of HET-1A cells revealed variable alterations of *SOD* gene expression. Superoxide dismutase is the primary scavenger of superoxide anion which has been implicated as the primary reactive oxygen species involved in reflux-induced oxidative damage[29-32]. Impairment of cellular antioxidant mechanisms that manage superoxide anions may contribute to the processes underlying esophageal mucosal injury by bile acids. Supplementation with SOD has been found to be protective against reflux-induced damage of the esophagus in rats[33]. Also, decreased activity of MnSOD has been measured in esophageal tissue of patients with esophagitis and Barrett's esophagus[29,34]. Using the esophageal duodenal anastomosis-based rat model, we have previously demonstrated a decreased incidence of Barrett's esophagus and esophageal adenocarcinoma accompanied by decreased oxidative injury in rats treated with Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP), an SOD mimetic[34].

Curcumin alone had minimal impact on either *SOD-1* or *SOD-2* expression. Bile acid induced *SOD-2* gene expression, but inhibited *SOD-1* gene expression. Curcumin ameliorated the effect of bile acid on *SOD-1*, causing a decrease in the degree of suppression. These findings are in contrast to previous work using a rat model of bile reflux demonstrating that esophagitis was associated with decreased SOD-2 enzyme production and activity and had no association with the activity and expression of SOD-1[35]. Other work by Jiménez *et al*[29] found increased

SOD-1 and SOD-2 expression in esophageal biopsies of patients with Barrett's and reflux esophagitis as compared to normal controls. However, the overall SOD activity was decreased in patients with esophagitis and Barrett's esophagus compared to normal epithelium. In another investigation using human esophageal biopsy specimens, Sihvo *et al*[36] found that only specimens of Barrett's associated with dysplasia showed increased SOD activity. These conflicting data illustrate that complex mechanisms related to both the timing and extent of bile acid exposure likely play a role in altering SOD expression and its impact on the development of esophageal adenocarcinoma. Curcumin as a preservative of SOD-1 expression in the early phase of bile acid exposure may provide a mechanism for the chemoprevention of esophageal adenocarcinoma.

A major difficulty of *in vitro* studies of reflux and Barrett's esophagus is the inability to replicate the timing and nature of acid exposure in the distal esophagus. We were limited as to the concentration of bile acid that could be used due to the degree of cellular toxicity in the presence of curcumin. As a result, the concentrations of bile acid tested were lower than those that can occur in the distal esophagus of patients with gastroesophageal reflux, which have been measured as high as 1 to 2 mmol/L[3]. Therefore, the bile acid levels tested may have been too low to induce the degree of gene expression changes that are experienced *in vivo*. Adjustments were also not made for varying pH levels of the experiments, and our study looked at 24 h exposure times to bile acids and curcumin. Longer exposure times and different pH levels may yield different results with respect to gene expression.

Curcumin has previously been investigated as a chemopreventive agent in a small number of phase I and phase Ⅱ trials and found to be safe and well tolerated[37-39]. However, a primary impediment to the use of curcumin has been its poor bioavailability as a result of extensive metabolization in the human gut[40]. This may be less of a drawback for curcumin use in the esophagus, as initial exposure to curcumin would occur prior to intestinal metabolism. Also, current work on developing lipolized or heat-solubilized forms of curcumin may help overcome this issue[38,41]. The current study provides a basis for possible use of curcumin for chemoprevention of esophageal adenocarcinoma through its effects on bile acid-induced alterations of *COX-2* and *SOD* gene expression. Curcumin should be further investigated as a chemopreventative agent in the setting of reflux esophagitis and Barrett's esophagus.

COMMENTS

Background

The incidence of esophageal adenocarcinoma has increased dramatically over the past few decades. Oxidative injury resulting from the reflux of bile acids is thought to play a role in the development of esophageal adenocarcinoma. Curcumin is a naturally occurring antioxidant derived from the herb turmeric, that might potentially counteract the effects of bile acids on esophageal cells.

Research frontiers

Curcumin has demonstrated anticancer properties in a variety of tumor types. Curcumin is also known to impact the gene expression of a variety of enzymes. This study analyzes the possible effects of curcumin on bile acid-induced alterations of gene expression by esophageal cells.

Innovations and breakthroughs

This study is one of the first to report that curcumin may have chemopreventative effects on esophageal cells in the presence of bile acids. The results of this research also suggest a dosage range of curcumin that is effective in altering esophageal cell gene expression. Understanding this therapeutic dosage range has important implications for future *in vivo* studies of curcumin, as it is known to have limited bioavailability.

Applications

By demonstrating that curcumin effects the production of cyclooxygenase-2 (COX-2) and antioxidant enzymes, this study suggests a possible use for curcumin as a chemopreventative against esophageal adenocarcinoma.

Terminology

HET-1A cells are an epithelial cell line derived from human esophagus. Superoxide dismutase (SOD) is an important enzyme responsible for managing the reactive oxygen species, superoxide. COX-2 is an enzyme involved in the formation of certain inflammatory mediators.

Peer review

In this manuscript, the authors determined a basis for possible use of curcumin for chemoprevention of esophageal adenocarcinoma through its effects on bile acid-induced alterations of *COX-2* and *SOD* gene expression. This is a very interesting paper for the scientific community involved in Barrett's esophagus and Barrett's cancer research. Good design, good technical performance and critical discussion.

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BRIEF ARTICLE

ESWL for difficult bile duct stones: A 15-year single centre experience

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Abstract

AIM: To evaluate the efficacy of extracorporeal shock wave lithotripsy (ESWL) for the management of refractory bile duct cholelithiasis in a third level referral centre.

METHODS: The clinical records of all patients treated with a second generation electromagnetic lithotripter (Lithostar Plus, SIEMENS) from October 1990 to April 2005 were evaluated. All patients were monitored during the procedure and antibiotics were administered in case of cholangitis. The χ^2 test and logistic regression analysis were performed as appropriate.

RESULTS: Two hundred and fourteen patients (102 males, 112 females; mean age 74.8 ± 0.84 years - single stone 97, multiple stones 117) underwent ESWL. The mean number of sessions and shock waves were 3.5 ± 0.13 and 3477.06 ± 66.17, respectively. The maximum stone size was 5 cm. Complete stone clearance was achieved in 192 (89.7%) patients. Of the remaining patients 15 required surgery, 2 a palliative stent and in 5 patients stone fragmentation led to effective bile drainage with clinical resolution despite incomplete clearance. Age, sex and stone characteristics were not related to treatment outcome. Major complications occurred in two patients (haemobilia and rectal bleeding) and minor complications in 25 (3 vomiting, 22 arrhythmias). No procedure-related deaths occurred.

CONCLUSION: ESWL is a safe and effective technique for clearance of refractory bile duct stones.

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Key words: Difficult bile duct stones; Extracorporeal shock wave lithotripsy

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic sphincterotomy (EST) with or without mechanical lithotripsy followed by stone extraction is the treatment of choice for bile duct stones, and clearance can be achieved in over 90% of cases[1,2].

Ductal clearance failure by this method (5%-10%) is usually due to characteristics of the stones (shape, size,

number and position, i.e. impacted or proximal to a ductal stenosis) or due to anatomic variants of the biliary tree[3].

In these cases, alternative therapeutic approaches include surgical exploration, contact dissolution, electrohydraulic and laser lithotripsy, stenting and extracorporeal shock wave lithotripsy (ESWL)[4].

The prevalence of bile duct stones increases with age and their treatment in older patients is difficult. Common bile duct stones are very common in the elderly and surgical treatment in these patients involves a significant degree of morbidity and mortality. In fact, surgical exploration of common bile duct stones is associated with a mortality of 1% or less in young, fit patients and a mortality of approximate 9%-10% in patients 80 years or older[5-7].

In many studies it has been demonstrated that ESWL combined with endoscopic procedures represents a safe and effective option for removal of difficult bile duct stones, especially in patients with high surgical risk[8-12].

In this study, we describe our 15 years of experience with ESWL in the treatment of difficult bile duct stones and evaluate its safety and effectiveness, particularly in elderly patients.

MATERIALS AND METHODS

ESWL treatment is considered for all patients with difficult bile duct stones, defined as stones which did not clear from the papilla after EST and which could not be removed during ERCP (Dormia basket, balloon catheter or mechanical lithotripsy).

From October 1990 to April 2005, 214 patients with lithiasis of the extrahepatic biliary tract not suitable for endoscopic removal underwent ESWL (using a second generation electromagnetic generator, Lithostar Plus, Siemens). The Lithostar Plus generates electromagnetic shockwaves which are focused on the target. Targeting was performed under fluoroscopic control. The injection of contrast medium was carried out through a *naso-biliary* drainage tube, previously fixed during the last ERCP, or through percutaneous transhepatic catheter (PTC) or with a T-tube. Occasionally, targeting was performed under ultrasonography guidance.

All patients who underwent ESWL treatment were selected on the basis of inclusion and exclusion criteria. Before treatment, preliminary tests were performed: blood sampling for hematology, chemistry and coagulation; urine sampling for pregnancy test; chest radiography and abdominal ultrasound. A positive pregnancy test and severe irreversible coagulopathy were absolute contraindications to ESWL treatment, while cirrhosis, portal hypertension, arrhythmias, pancreatitis, thrombosis, abdominal aneurysm, renal failure, voluminous angiomas, gut or lung interposition and voluminous cysts were relative exclusion criteria. All patients gave their written informed consent to the procedure.

The patients with cholangitis and those with associated intrahepatic stones were treated with antibiotic therapy. Patients were fasted for at least 12 h before treatment.

Table 1 Population and stone characteristics

Study population	
n	214
Age	74.8 ± 0.9
Sex (M:F)	102:112
Stone characteristics (%)	
Single	45
Multiple	55
⩾ 1.5 cm	94
< 1.5 cm	6

All patients were monitored with pulse oximetry during the procedure. A continuous ECG was also performed in cardiopathic patients.

During treatment the prone position was adopted in the majority of cases. Routine laboratory tests were performed in all patients 6 h after the procedure and the following day, according to the protocol.

When multiple sessions were required, the time interval between sessions was always more than 24 h. During the study period, 214 patients underwent ESWL (102 men and 112 women). The mean age was 74.8 ± 0.84 years (range 29-96); the maximum dimension of the treated gallstones was 5 cm. Ninety seven patients had single stones and 117 had multiple stones (Table 1).

Seven hundred and fifty one sessions were performed (3.5 ± 0.13 sessions for each patient, range 1-14), on average the number of hits per session and per patient was 3477.06 ± 66.17 (range 400-6000) and 13 032.41 ± 667.49, respectively.

Every session ended when optimal fragmentation of the stones was obtained or if the maximum number of hits was reached, in order to avoid possible tissue damage (the established limit was 6000 hits per session).

Medium high power (range 1-8) energy was used per session, according to the pain tolerance of each patient. No patient received anesthesia, while 70 patients were sedated with Fentanil and 12 patients received analgesia with Ketorolac.

Statistical analysis was performed using the MedCalc package. Data are expressed as mean ± SE. The χ^2 test was performed to evaluate differences between the group of patients who were cleared of stones and the group who were not. Logistic regression analysis was used to evaluate the variables correlated with treatment outcome. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 214 patients underwent 751 ESWL procedures during the study period (mean 3.5 ± 0.13, range 1-14 for a single patient). The treatment was stopped in only 8 patients because of pain. Eleven patients (5%) required 6 sessions, 31 patients (14%) 5 sessions, 52 patients (24%) 4 sessions, 47 patients (22%) 3 sessions, 31 patients (14%) 2 sessions and 32 patients (15%) only 1 session; between 7 and 14 sessions were necessary in the remaining patients (Table 2).

Table 2 No. of extracorporeal shock wave lithotripsy sessions required to achieve stone clearance

ESWL sessions required	No. of patients (n = 214)
1	32
2	31
3	47
4	52
⩾ 5	52

ESWL: Extracorporeal shock wave lithotripsy.

Table 3 Clearance and causes of treatment failure n (%)

Outcome	No. of patients (n = 214)
Clearance	192 (89.7)
Spontaneous or after 1 ERCP	178
⩾ 2 ERCP	14
Treatment failure	22 (10.3)
No cause	15
Discontinued	7

ERCP: Endoscopic retrograde cholangiopancreatography.

In 205 patients a naso-biliary tube was positioned during a previous ERCP. In 6 patients targeting was performed through percutaneous drainage, in 1 case through a Kehr drainage tube and in 2 cases by ultrasonography guidance.

Endoscopic clearance of the biliary duct was successful in 182 cases, while in 10 cases spontaneous clearance was observed. Fifteen patients required surgery and 7 suspended treatment, in 5 of these cases stone fragmentation led to effective bile drainage with clinical resolution despite incomplete clearance, and 2 received a palliative stent.

Clearance

Clearance of the biliary ducts (both spontaneous or through drainage or ERCP) was observed in 192 of the 214 patients treated (89.7%).

In the vast majority of patients (178, 93%) duct clearance was achieved spontaneously or with only 1 ERCP post-ESWL. Only 14 patients (7%) required two or more ERCP sessions. Most of the patients needed 3 or 4 ESWL sessions (48 and 46 patients, respectively). 15 patients underwent surgery and 7 discontinued treatment (Table 3).

The majority of patients (57%) with clearance had stones with a maximum diameter of 2 cm (range 0.8-5 cm). Usually, stones smaller than 1.5 cm are amenable to mechanical lithotripsy. In fact, in the vast majority of cases (94%) the stones were larger than that. However, in a few cases smaller stones were present together with large stones, and in two patients with single stones (dating before 1997) the endoscopist was unsuccessful in retrieving them. Stone size, number (multiple 55.1% or single 44.9%), sex (52.4% women; 47.6% men) and age did not seem to influence treatment outcome.

Among those without clearance, the majority (51%) had stones with a diameter over 2 cm (range 1-3.5 cm).

Furthermore, in this group none of the variables were correlated with a negative outcome: sex (54.5% women, 45.5% men), mean age (71 years) and number of stones (11 patients with multiple gallstones; 12 with a single stone).

Table 4 Procedure-related complications n (%)

Complications	No. of patients (n = 27)
Major	2 (0.9)
Haemobilia	1
Rectal bleeding	1
Minor	25 (12)
Vomiting	3
Arrhythmias	22

Complications

Complications related to the treatment occurred in only 27 patients. Among these, 25 patients (12%) reported minor complications such as vomiting and arrhythmias (mainly extrasystoles and bradycardia). We observed only 2 cases of major complications: haemobilia and rectal bleeding. The haemobilia was mild, without ultrasonographic signs and haematology alterations. The patient with rectal bleeding lost 2 g of haemoglobin which did not necessitate blood transfusion (Table 4). We had to suspend ESWL in only four cases because of minor complications (twice for arrhythmia and twice for pain intolerance).

DISCUSSION

In about 5%-10% of patients with common bile duct stones, duct clearance can not be achieved with traditional techniques such as ERCP and sphincterotomy. These approaches usually fail for reasons related to the stone characteristics: large size, impacted or proximal to a stricture. ESWL is an alternative therapy for difficult duct stones and is also a support to ERCP when it fails to achieve stone fragmentation[13].

We report our 15 years of experience in the treatment of difficult duct stones with the Lithostar Plus.

Our population was similar to that reported in previous studies. In fact, the vast majority of patients were older than 65 years of age.

Our results suggest that ESWL is a safe, well tolerated and effective technique for the treatment of difficult bile duct stones. Of note, we performed the procedure without the need for anesthesia or flat analgesia in almost all patients. This suggests that careful targeting with the correct focus of the lithotripter allows the execution of ESWL without the need for drugs that may cause side effects in an elderly population possibly already taking different drugs for co-morbidities.

We did not observe any differences in the baseline characteristics between patients with and without stone clearance, probably because of the high success rate. In fact, we did not find any patient or stone characteristics related to treatment failure.

In our population, some smaller stones were present with large stones. It should be noted that in these cases

ESWL was performed on the largest stone in order to achieve subsequent bile duct clearance. However, with our lithotripter, the targeting of stones 1-1.5 cm is easily performed and subsequent stone fragmentation suggests that stone size is not a limiting factor in ESWL treatment. In fact, in two cases, where the endoscopist failed to retrieve the small stones, ESWL was successful.

In our study, no deaths occurred and the few major complications which occurred were immediately treated and resolved rapidly.

We must emphasize that the median age of our patients was 74, and ESWL was performed in 63 patients (29%) aged more than 80 years. For such patients, the mortality rate after common duct bile exploration is still high (around 10%). Furthermore, urgent surgery for biliary stones in elderly subjects has a very high mortality rate ranging from 12% to 21%. Therefore, ESWL is the procedure of choice for difficult bile duct stones in elderly subjects[5,7,14,15].

In addition ESWL, even when multiple sessions and longer hospitalization are required, has lower costs due to the decreased rate of minor complications compared to surgical treatment.

Other techniques may be employed for the treatment of difficult bile duct stones but they have a lower efficacy or more side effects.

Contact dissolution therapy has been abandoned because of the number of complications and low success rate[16-18].

Electrohydraulic and laser lithotripsy require a more invasive approach, including the use of a choledochoscope for direct visualization of the stones. They are alternative therapeutic options but have more complications and require more expensive equipment compared with ESWL[19-21].

ESWL is a valid technique with low cost compared to other therapeutic options for difficult bile duct stones and its utilization can be extended to urology and orthopaedics. Therefore, the same device can be used by different medical staff in the same hospital thus reducing management costs.

Newer generations of lithotripters delivering higher energies and allowing smaller focusing on the target stone are now available; therefore, it is expected that even better results may be achieved.

In conclusion, ESWL is an effective and safe treatment which improves the outcome of biliary duct lithiasis. In association with endoscopy, it is successful in the vast majority of cases and allows surgery sparing in high risk patients, especially in the elderly.

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COMMENTS

Background

Extracorporeal shock wave lithotripsy (ESWL) uses electromagnetic waves to fragment difficult biliary stones when Endoscopic Retrograde Cholangiopancreatography fails. This may prevent surgery which still carries significant morbidity and mortality when the common bile duct is involved.

Research frontiers

The results obtained with second generation electromagnetic lithotripters such as ours are already optimal; however, technological improvements in lithotripters now delivering higher energies and allowing smaller focusing on the target stone may further enhance our performance.

Innovations and breakthroughs

The authors' work emphasizes, in a wide patient population, that ESWL is safe and effective which was also true in elderly patients. In fact, elderly patients are more difficult to treat due to coexisting co-morbidities that frequently contraindicate surgery.

Applications

The study is of interest for physicians dealing with the biliary tree and particularly those managing bile duct stones. Based on these results, ESWL was confirmed to be the first choice for the treatment of difficult biliary stones even in cases outside standard treatment guidelines.

Peer review

Overall, the study helps to evaluate a role of ESWL in difficult common bile duct stones.

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BRIEF ARTICLE

Outcome of surgical treatment of intestinal perforation in typhoid fever

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Abstract

AIM: To represent our clinical experience in the treatment of intestinal perforation arising from typhoid fever.

METHODS: The records of 22 surgically-treated patients with typhoid intestinal perforation were evaluated retrospectively.

RESULTS: There were 18 males and 4 females, mean age 37 years (range, 8-64 years). Presenting symptoms were fever, abdominal pain, diarrhea or constipation. Sixteen cases were subjected to segmental resection and end-to-end anastomosis, while 3 cases received 2-layered primary repair following debridement, one case with multiple perforations received 2-layered primary repair and end ileostomy, one case received segmental resection and end-to-end anastomosis followed by an end ileostomy, and one case received segmental resection and end ileostomy with mucous fistula operation. Postoperative morbidity was seen in 5 cases and mortality was found in one case.

CONCLUSION: Intestinal perforation resulting from *Salmonella typhi* is an important health problem in Eastern and Southeastern Turkey. In management of this illness, early and appropriate surgical intervention is vital.

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Key words: Intestinal perforation; Typhoid fever; Treatment

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INTRODUCTION

Typhoid fever is a febrile disease caused by *Salmonella typhi*, a Gram-negative bacillus, which does not present as a significant health issue in developed countries, but continues to be an important problem in tropical regions[1,2]. It is generally transmitted by the fecal-oral route and may occasionally lead to an epidemic. Typhoid fever remains a notable public health issue in regions having no adequate and proper infrastructure[3].

Although intestinal hemorrhage is the most common complication of typhoid fever, intestinal perforation continues to be the most frequent reason behind high morbidity and mortality[2]. Generally, hemorrhage and

perforation occur in the terminal ileum secondary to necrosis of Peyer's patches at 2-3 wk after the onset of the disease[4,5]. Frequency of perforation varies between 0.8% and 18%[3,6,7]. Mortality rates of typhoid intestinal perforation (TIP) cases are reported to be between 5% and 62%. Perioperative mortality rates are noted to rise up to 80% in patients who received surgery due to late perforations[2,6-9].

Studies focusing on TIP in large series, are generally reported from endemic regions[3]. In a study including 229 cases, Asefa[10] notes TIPs as one of the most important causes underlying the acute abdomen.

While early surgical procedures are regarded as definitive treatments along with preoperative resuscitation and postoperative intensive care, the methods that should be used in surgery are still contentious.

The aim of the present study is to retrospectively review TIP cases and evaluate the outcomes of this complication among patients treated in the Department of General Surgery, Faculty of Medicine, Yuzuncu Yil University of Van, which has provided healthcare services since 1994 to a wide region of Turkey encompassing many provinces and districts.

MATERIALS AND METHODS

The study included 22 cases admitted with an acute abdomen profile who were diagnosed with TIP and treated in the Department of General Surgery between 1994 and 2010. By retrospectively reviewing the patient records, the cases were analyzed in terms of demographic, medical, and surgical personal data. The cases were evaluated with regard to age, gender, number of perforations, localization of the perforation, type of operation, and morbidity and mortality rates. With the exception of 3 patients who were admitted to the Department of Internal Diseases and Department of Infectious Diseases and diagnosed with typhoid fever before being transferred to the Department of Surgery upon development of acute abdomen, all cases initially presented to the Emergency Department because of abdominal pain. The cases who were considered to be prediagnosed with acute abdomen secondary to medical history and physical examination results, were subjected to erect abdominal plain film, posterior to anterior lung film, complete blood count, complete urinalysis, and biochemical analysis including amylase. None of our patients, even those who were reported to have intraabdominal free fluid by ultrasonography, received paracentesis.

RESULTS

There were 18 males (81.8%) and 4 females (18.2%), with an age range of 8-64 years (mean, 37 years). Common symptoms were fever, abdominal pain, and vomiting. Physical examination revealed generalized peritonitis in all cases. Each patient received urinary and nasogastric catheters prior to the operation. Fluid/electrolyte imbalance was corrected and antibiotherapy was started. Three cases transferred from the Departments of Internal Diseases and Infectious Diseases were diagnosed and recorded as intestinal perforation cases, whereas the remaining patients were operated on with the prediagnoses of peptic ulcer perforation, perforated appendicitis, and generalized peritonitis. In all cases, laparotomy was performed by midline incision. Six cases demonstrated multiple perforations, while 16 cases showed perforation on the antimesenteric side, appearing similar to a staple hole. One of the cases with multiple perforations had 7 perforation foci. The location of the perforations was the jejunum in 3 cases (located at an average distance of 63 cm from the Treitz ligament), the ileum in 18 cases (located at an average distance of 50 cm from the ileocaecal valve), and the cecum in one case. Based on the preference of operating surgeons and the extent of peritoneal contamination, 15 cases (68.2%) were subjected to segmental resection and end-to-end anastomosis, while 3 cases (13.6%) received 2-layered primary repair following debridement, one case (4.55%) with multiple perforations received 2-layered primary repair and end ileostomy, one case (4.55%) received segmental resection and end-to-end anastomosis, followed by an end ileostomy, one case (4.55%) received right hemicholectomy and end-to-end anastomosis, and one case (4.55%) received segmental resection and end ileostomy with mucous fistula operation.

Table 1 Preferred surgical methods

Preferred surgical methods	Patients (n = 22)
Segmental resection + anastomosis	16
Jejunum	3
Ileum	12
Cecum (right hemicholectomy)	1
Debridement + 2-layered primary repair	3
2-layered primary repair + end ileostomy	1
Segmental resection + anastomosis followed by an end ileostomy	1
Segmental resection + end ileostomy with mucous fistula	1

Pezzer drains were inserted into both subhepatic and retrovesical spaces of 4 cases, while placing 2 Pezzer drains in the retrovesical space in one case, and one Pezzer drain in the retrovesical spaces of 17 cases. The preferred surgical methods are outlined in Table 1. In all the 19 patients who had no serological or bacteriological diagnosis, typhoid fever diagnosis was verified by isolation of *Salmonella typhi* serologically and/or from stool. The case who presented with a sepsis profile and was subjected to ileum resection and anastomosis treatment, died postoperatively at 10 h. Five cases, including a patient who received debridement and primary repair before formation of an ileal fistula that closed spontaneously during the postoperative period, and 4 patients who exhibited wound infection, developed morbidity.

Histopathologic results

Macroscopic view: In patients who received resection, there were ulcers in the jejunum, ileum, and cecum, which had a perforated appearance, extending parallel to the axis of the intestines and displaying a length varying between 0.2 and 2 cm. Mesenteric lymphadenomegaly was determined.

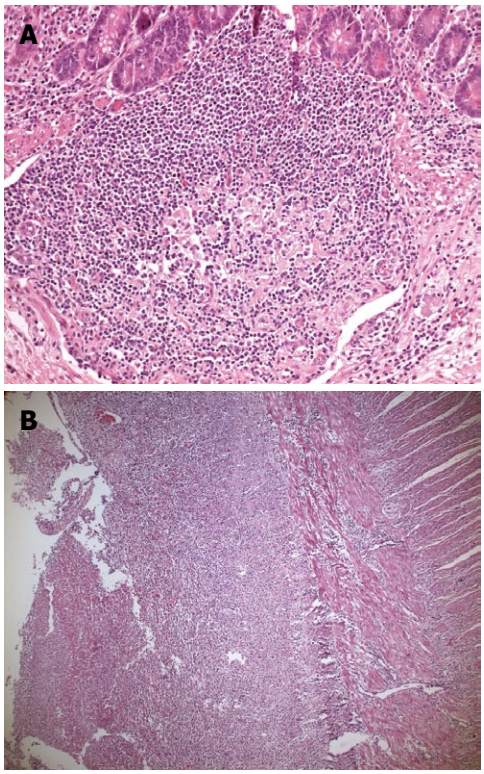

Figure 1 Histopathologic view of typhoid lesions. A: Typhoid nodule, there are macrophages containing bacteria, red blood cells, and nuclear debris from small nodular aggregates in Peyer's patches (HE stain, × 20 objective); B: Typhoid ulceration (HE stain, × 5 objective).

Microscopic view: The sections acquired from the ulcerated areas showed loss of mucosal integrity, enclosure of the muscular mucosa by the ulcers which also appeared to have destroyed the inner circular muscle layer, a predominance of macrophages beneath the mucosa and in the adjacent areas, and infiltrating mononuclear inflammatory cells. The typhoid nodule (Figure 1A) had macrophages containing bacteria, red blood cells, and nuclear debris from small nodular aggregates in Peyer's patches. In typhoid ulceration (Figure 1B) macrophages, which are also defined as typhoid cells, were observed to form clusters in the mesenteric lymph ganglia.

DISCUSSION

In the current study 22 surgically treated patients with TIP were evaluated. The most common surgical intervention was segmental resection with end-to-end anastomosis.

Most fatal complications of typhoid fever are intestinal hemorrhage and enteric perforation. Those complications occur secondary to necrosis of Peyer's patches[3,7,8]. Typhoid fever leads to hyperplasia in the reticuloendothelial system, necrosis, and ulceration limited to Peyer's patches[1,9]. The frequency of TIP is reported to vary between 0.8% and 39%, depending on the geographic region[7-9]. Butler[11] reviewed 15 980 typhoid fever cases from the world literature and found the frequency of TIP to be 2.8%. In our country, the frequency of typhoid complications is around 20%[3]. Hosoglu *et al*[4] reported the frequency of TIP as 10.5%.

Those complications generally occur during the second or third week of the disease[3-5]. While typhoid fever often affects the terminal ileum, in rare cases the jejunum and cecum may also be involved[9]. TIP in appendicitis cases has been mentioned as a case report in the literature[12]. Typhoid fever is known to cause spontaneous gall bladder perforation among cases with no cholelithiasis[1]. Cecal ulcers are smaller than the ones occurring in the jejunum, and they seldom demonstrate perforation. Generally, TIP occurs as a single perforation similar to a stapler hole, and is localized on the antimesenteric side[9]. In the present study, perforations were localized in the jejunum in 3 patients, in the ileum in 18 patients, and in the antimesenteric side of the caecum in one patient. The length of the perforations localized in the jejunum and ileum, varied between 0.5 and 2 cm, while the size of the perforation in the cecum was found to be smaller, thereby being consistent with the relevant literature.

The number and size of ulcers do not have any relationship with the severity of the symptoms. Characteristically, those ulcers do not cause symptoms of peritoneal irritation before being perforated, and the peritoneal response following perforation is observed to be delayed. Unlike other perforations, in cases with TIP, the omentum does not migrate to the perforation site[9].

In the study of Ameh *et al*[13], fever and abdominal pain were found to be the most common symptoms, whereas guarding was observed to be the most common physical examination finding. All our 22 cases had fever, abdominal pain and peritoneal irritation signs in the physical examination. However, none of our cases demonstrated a sign of synchronous intestinal hemorrhage. Relative bradycardia is an important finding for enteric fever and it is seen more commonly among adult and adolescent patients[14]. In this series, none of the cases displayed that finding. In addition, among typhoid fever cases, hepatosplenomegaly is known to be frequent, and while it is reported to be the most common physical finding in one study[4], we did not determine even a single splenomegaly case among our patients.

TIP is encountered rarely among people under the age of 5 years and over the age of 50 years. More than 50% of the cases are seen during the second and third decades of life. Its prevalence in men is 3 times higher than in women[7,9]. Saxe *et al*[2] conducted a study of 112 cases with typhoid perforation and found the mean age was 20 years (range, 3-75 years) and the male/female ratio was 1.73. In another 2 similar studies, the male/female ratio was found to be 2.5 and 4[15,16]. In the study of Atamanalp *et al*[7], mean age was found to be 36.3 years (range, 7-68 years). In the current study, mean age was 37 years (range, 8-64 years) and the male/female ratio was 4.5. Risk factors for perforation among hospitalized patients were determined to be short symptomatic period prior to presentation, leukopenia, inadequate treatment, and being male[4]. Although the exact underlying mechanism of TIP among men is not yet known, spending longer time and consuming more food outdoors may lead to more frequent contact with the bacillus[7].

TIPs started to be treated surgically towards the end of the 1800s. As a result of understanding the pathogenesis of typhoid fever and using more effective antibiotherapies, early surgery has become the optimal treatment option for perforations[1]. However, the method to be applied in surgical treatment of TIP cases, is still contentious. From a practical point of view, the perforation site should be closed and the peritoneal cavity should be irrigated in the surgical treatment. In multiple perforations, segmental resection and anastomosis can be performed safely[2]. Rahman *et al*[17], found no correlation between the applied surgical procedure and the reduction in mortality. In a majority of the cases, TIP affects the ileum and primary repair is appropriate. Shah *et al*[18] found the rates of complications and mortality in resection-anastomosis patients were lower than in other intervention groups. Therefore, they advocated resection-anastomosis as the ideal surgical method for typhoid enteric perforations.

Similarly, Athié *et al*[19] performed surgery on 352 cases with typhoid ileal perforation, and found the rates of mortality and morbidity in the resection-anastomosis group were lower than in the primary closure group. They recommended a 10 cm resection from the upper and lower ends of the perforation and anastomosis (even if there is only one perforation) in cases with a perforated ileum. However, Beniwal *et al*[20] suggested primary closure as the first choice of treatment. Similarly, Shukla *et al*[21], reported a reduction in mortality rate from 35% to 10.8%, secondary to using a single-layer primary closure method. Adesunkanmi *et al*[22], advocated the 2-layer closure technique as the most successful surgical method regardless of the application of an omental patch. In the study of Saxe *et al*[2], which included 112 typhoid enteric perforation cases, 77% of the cases received primary repair for single perforation, while 19% of the cases were subjected to segmental resection because of multiple perforations. Atamanalp *et al*[7] performed surgery on 82 patients with typhoid ileal perforation: primary repair after debridement in 32 cases, wedge resection and primary closure in 9 cases, resection and anastomosis in 9 cases, end ileostomy after resection in 28 cases, and exteriorization in 4 cases. In multiple perforation cases where short bowel syndrome development is likely, primary repair is recommended instead of resection[3,23]. Several authors suggest ileostomy in cases with delayed multiple perforation and diffuse peritoneal contamination[22,24,25].

Recently, laparoscopic treatment methods have also been employed in TIP cases. Ramachandran *et al*[26] reported 6 successful laparoscopic primary closure cases. Sinha *et al*[27] treated 25 cases laparoscopically with a port-site infection rate of 8%.

The mortality rate in TIP cases is reported to vary between 5% and 60%[7,9,28]. Saxe[2] found a postoperative mortality rate of 16%. In the studies of Ameh[13] and Meier *et al*[24], the mortality rates were 20% and 39%, respectively. Atamanalp *et al*[7] determined a mortality rate of 11%. Although, mortality rates have shown a decrease lately, they are still at important levels. The significant differences in reported mortality rates have revealed the need to investigate the underlying reasons. Young age, inadequate medical treatment, late presentation, number of perforations and sepsis are mentioned among the factors influencing mortality[1]. Some authors claim that the number of perforations might affect prognosis[20,22]. On the other hand, Rahman *et al*[17] and Atamanalp *et al*[7] determined no significant correlation between the number of perforations, and prognosis and mortality. We found no study reporting a relationship between the localization of perforation and prognosis in the literature. In the present study, among our 22 cases, only one 62-year-old patient with sepsis died in the postoperative period. The low mortality rate in our study might be secondary to factors such as early and appropriate surgical intervention, effective perioperative resuscitation, postoperative intensive care procedures, safe anesthesia, and delivery of wide-spectrum antibiotics with low resistance.

The most common complication of TIP cases is wound infection, while the most serious is formation of a fecal fistula. Wound dehiscence, intestinal obstruction, intraabdominal abscess, empyema, bleeding diathesis, and psychosis may occur[7]. In the present study, one case developed an ileal fistula which closed spontaneously in the postoperative period and 4 cases developed wound infection (5 complications in total).

In conclusion, the treatment of TIP consists of appropriate early surgical intervention, effective resuscitation in the preoperative period, postoperative care, and use of proper antibiotics. Although primary closure is the most frequently recommended procedure, segmentary resection and end-to-end anastomosis may be reserved for patients with multiple perforations. Segmentary resection and end-to-end anastomosis has low mortality and morbidity rates. Thus resection-anastomosis should be used as a surgical treatment method for TIP. Ileostomy is associated with high mortality and morbidity. However, it may be life-saving in patients with severe abdominal contamination.

COMMENTS

Background

Typhoid fever is a febrile disease caused by *Salmonella typhi* and is an important problem in tropical regions. Progression of disease is associated most commonly with hemorrhage and intestinal perforation.

Research frontiers

Typhoid perforation is an important complication of typhoid fever. It is seen rarely, but shows a high mortality and morbidity. The mainstay of treatment of typhoid intestinal perforation is surgery.

Innovations and breakthroughs

Recent reports have highlighted the importance of surgical treatment. Pathogenesis of typhoid perforation indicates the need for more effective antibiotic therapies and early surgery.

Applications

Although primary closure is the most frequently recommended procedure, this research opens the way for new surgical options such as segmentary resection and end-to-end anastomosis.

Peer review

After reviewing the literature on surgical aspects, authors should recommend the surgery of choice and should be included in conclusions.

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BRIEF ARTICLE

Non-alcoholic steatohepatitis and influence of age and gender on histopathologic findings

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Abstract

AIM: To characterize the histopathologic specifications of non-alcoholic steatohepatitis (NASH) according to age and gender.

METHODS: An analytical cross-sectional study was conducted in two private gastroenterology clinics on biopsy proven patients suffering from NASH. Biopsy histopathologic findings as well as demographic and laboratory data of the patients at the time of biopsy were gathered retrospectively from clinical records. The grading and staging of histopathologic findings were performed according to the Brunt method after reevaluation of the slides by a pathologist. Patients were divided into two groups according to age (below and above 55 years). Mean quantitative grade of all pathologic findings were also calculated according to Brunt scoring values.

RESULTS: A total number of 77 NASH patients, consisting of 58 males (75.3%) and 19 (24.7%) females with a mean age of 41.99 ± 11.80 years (range, 18-70 years), were enrolled. The mean age (48.72 ± 13.99 years *vs* 39.74 ± 10.16 years, $P = 0.004$) and aspartate aminotransferase level (75.11 ± 29.68 U/L *vs* 52.78 ± 25.00 U/L, $P = 0.002$) was significantly higher in female patients. Mean quantitative grade of hepatosteatosis was significantly higher in females (2.00 ± 0.82 *vs* 1.59 ± 0.68, $P = 0.031$) compared to males. Fifty four percent (34/65) of young patients had mild hepatosteatosis (Grade Ⅰ) while only one patient (11.2%) in the older group had grade Ⅰ hepatosteatosis. Patients aged ⩾ 55 had significantly more severe hepatosteatosis (Grade Ⅲ) (44.4% *vs* 9.5%, $P = 0.007$) and the mean quantitative grade of hepatosteatosis was significantly higher among them (2.33 ± 0.71 *vs* 1.56 ± 0.67, $P = 0.002$). Multivariate analysis after omitting the confounding role of age revealed a higher grade of hepatosteatosis in female patients ($P = 0.010$).

CONCLUSION: These findings point toward the possible influence of age in the severity of steatohepatitis, portal and lobar inflammation in patients suffering from NASH while gender independently might contribute to the level of steatohepatitis.

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Key words: Non-alcoholic steatohepatitis; Age; Gender; Histopathologic findings

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is a common clinicopathological entity with a worldwide distribution[1] which is defined as a cryptogenic form of liver disease that is often observed in obese patients in the absence of identifiable causes such as alcohol abuse, drug toxicity or viral infection[2]. Although the disease is often indolent, 15%-20% of patients may progress to fibrosis and 7%-17% may insidiously develop cirrhosis which is a strong warning finding in patients' liver biopsies. Additionally, NASH is responsible for the majority of cryptogenic cirrhosis in patients who are at risk of complications, such as portal hypertension and hepatocellular carcinoma[3-7]. According to survival rates, population-based studies in the United States revealed lower overall survival for patients with nonalcoholic fatty liver disease compared to the general population[8].

Although the etiology of NASH is unknown, a number of co-morbid conditions have been suggested as predisposing factors for the development of NASH such as obesity, type 2 diabetes mellitus, and hyperlipidemia[6,7,9-13]. It is assumed that the emergence of NASH closely resembles the epidemiology of obesity and diabetes mellitus[14].

Recently, there was a parallel increase in the prevalence of obesity, metabolic syndrome (hyperglycemia, visceral obesity, hyperlipidemia and hypertension) and NASH. As a result, NASH is considered part of metabolic syndrome[15]. Pathogenesis of metabolic syndrome is based on insulin resistance. Aging and reduced physical activity, which cause hyperinsulinemia and insulin resistance, result in obesity and initiate metabolic syndrome which in turn further increases insulin resistance[16]. Considerable difference in the prevalence of metabolic syndrome between genders has been attributed to sex hormones. Many studies have shown that women tend to gain weight following the menopause and distribution of their body fat changes toward visceral adiposity[17]. There is a considerable increase in the prevalence of metabolic syndrome in women after menopause.

Despite the high prevalence of NASH, its natural history is not well understood. To the best of our knowledge, only about 30 cases of NASH have been described with sequential liver biopsies[3,6,7]. Because different sections of metabolic syndrome show significant differences that are related to age and gender, there is speculation that the clinicopathological features of NASH may also vary in relation to these factors. The following study was performed to evaluate the relationship between age and gender with pathologic findings on liver biopsies of NASH patients.

MATERIALS AND METHODS

Subjects

This analytic cross-sectional study was conducted in two private gastroenterology clinics in Tehran, Iran during 2005-2009. Data were retrospectively collected using the medical records of all eligible subjects who were biopsy proven NASH patients that met the following inclusion criteria: (1) Histopathologic confirmation of nonalcoholic steatohepatitis; (2) Non-alcohol consumers or intake of less than 100 *g* ethanol per week which was confirmed by the attending physician and family members who were in close contact with the patient; (3) Negative serologic markers of viral or autoimmune hepatitis, including hepatitis B surface antigen, hepatitis C virus antibody (ELISA), human immunodeficiency virus antibody (ELISA), antinuclear antibodies, anti-smooth muscle antibodies, anti-liver/kidney microsomes type 1 antibodies and negative α-1 antitrypsin; and (4) Not having any other liver disorders including metabolic liver diseases (e.g. Wilson's disease and hemochromatosis), drug induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, and biliary obstruction. Moreover, patients with any history of jejunoileal bypass surgery, having received hormone replacement therapy for menopause, any usage of other drugs known to result in steatosis (e.g. glucocorticoids, synthetic estrogens, aspirin, tamoxifen, amiodarone, calcium-channel blockers, and methotrexate), pregnancy or incomplete medical records were all excluded. Finally, 77 NASH patients were eligible to enroll in this study.

Laboratory and histopathologic assessments

Levels of aminotransferases, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (Alk-P), total serum cholesterol and triglycerides, low density lipoprotein, high density lipoprotein, fasting blood sugar, partial thromboplastin time, serum platelet count and serum level of γ-glutamyl transpeptidase were measured using standard techniques. In addition, other baseline characteristics such as patients' age and gender and body mass index were recorded for all patients.

According to the guidelines concerning the indications for liver biopsy, all patients who show no improvement of liver function after several months of diet and exercise treatment are recommended to undergo a liver biopsy. Due to the retrospective nature of the study, all biopsy specimens were recovered from the laboratory and reevaluated for histopathologic findings. In order to definitely diagnose NASH, all liver biopsy specimens were examined for fibrosis, steatosis, hepatocyte ballooning, lobar and portal inflammation. The grading and staging of all biopsy specimens were determined based on the method proposed by Brunt *et al*[18]. The categorization of the histopathologic findings regarding this method is presented in Table 1. Mild and moderate steatohepatitis were defined as conditions in which < 33% and 33%-66% of hepatocytes were affected, respectively; whereas, severe

Table 1 Histopathologic grading (Brunt)[18] of different finding in liver biopsies of non-alcoholic steatohepatitis patients

Finding	Grading
Hepatosteatosis	
Grade Ⅰ	< 33% of hepatocytes affected
Grade Ⅱ	33%-66% of hepatocytes affected
Grade Ⅲ	> 66% of hepatocytes affected
Fibrosis	
No fibrosis	No fibrosis
Grade Ⅰ	Zone 3 perisinusoidal pericellular fibrosis, focally or extensively present
Grade Ⅱ	Zone 3 perisinusoidal pericellular fibrosis, with focal or extensive periportal fibrosis
Grade Ⅲ	Zone 3 perisinusoidal pericellular fibrosis and portal fibrosis with extensive or focal bridging fibrosis
Cirrhosis	Cirrhosis
Hepatocyte ballooning	
No ballooning	No ballooning
Grade Ⅰ	Sometimes, zone 3
Grade Ⅱ	Evident, zone 3
Grade Ⅲ	Symptomatic, more dominant in zone 3
Lobar inflamation	
No change	No change
Grade Ⅰ	Diffuse neutrophils, monocytes at 1 or 2 points in a 20 × microscopic field
Grade Ⅱ	PMN with ballooning hepatocytes, chronic inflammation at 2 to 4 points in a 20 × microscopic field
Portal inflamation	
No change	No change
Grade Ⅰ	Mild, some portal areas
Grade Ⅱ	Mild to moderate, most portal areas
Grade Ⅲ	Moderate to severe, most portal areas

PMN: Polymorphonuclear leukocyte.

steatohepatitis was characterized with > 66% involvement of hepatocytes. Additionally, advanced fibrosis consisted of bridging fibrosis (grade Ⅲ) and cirrhosis. In order to restrict the information bias, a single pathologist reported all histopathologic findings. The mean grade of all pathologic findings were also calculated according to Brunt scoring values.

All laboratory and histopathologic features of enrolled NASH patients were evaluated and compared between two gender (male *vs* female) and age (< 55 years as younger *vs* ⩾ 55 years as older patients) groups.

Statistical analysis

The data were analyzed using SPSS v.15 software for Windows (Chicago, USA). Descriptive data were reported using parameters such as frequency, mean, mode and SD. Kolmogorov-Smirnov test was performed to evaluate normal distribution of the quantitative variables. To test the differences between parametric and non-parametric variable means in the two study groups, independent *T*-test and Mann-Whitney *U*-test were used.

To compare the differences between the frequency of qualitative variables between study groups Chi square test was performed. Moreover, in order to evaluate the confounding effects of age on the association between histopathologic findings and gender, univariate analysis was used. Receiver operating characteristics (ROC) curve analysis was also performed to assess the predictability of advances in histopathologic findings with patients' age, and the area under curve (AUC) and the best cutoff value were determined. The appropriate diagnostic values of each cutoff point were reported, including sensitivity and specificity.

A 5% probability of a type I error (two-tailed), and a power of 80% were considered in the analysis. All reported *P*-values are two-tailed.

RESULTS

Baseline characteristics

A total of 77 NASH patients were enrolled in this study, consisting of 58 males (75.3%) and 19 (24.7%) females with a mean age of 41.99 ± 11.80 years (range, 18-70 years). Baseline characteristics including paraclinical data are listed in Table 2. As shown, the mean serum levels of liver enzymes including ALT, AST and Alk-P were 117.73 ± 237.52 U/L, 58.29 ± 27.77 U/L and 176.48 ± 96.95 U/L, respectively.

As mentioned before, all patients underwent liver biopsy and the results are shown in Table 3. The most common grade of hepatosteatosis was grade I in 36 patients (46.8%) and liver cirrhosis was detected in 5 patients (6.7%).

Characteristic and histopathologic features of NASH patients regarding different sex groups

Baseline and clinical characteristics of NASH patients are listed in Table 2. As shown, the mean age of female patients was significantly higher than male patients (48.72 ± 13.99 years *vs* 39.74 ± 10.16 years, $P = 0.004$). In addition, the mean serum level of AST was also significantly higher in females (75.11 ± 29.68 U/L *vs* 52.78 ± 25.00 U/L, $P = 0.002$). Other baseline and clinical characteristics were not significantly different between the two gender groups.

Different histopathologic findings of the patients are presented in Table 3. The most common grade of hepatosteatosis in males was grade I in 51.7% (30/58); whereas, the commonest grade in females was grade Ⅱ with a prevalence of 36.8% (7/19). Moreover, the mean quantitative grade of hepatosteatosis was significantly higher in females (2.00 ± 0.82 *vs* 1.59 ± 0.68, $P = 0.031$). Advanced fibrosis (consisting of grade Ⅲ and cirrhosis) was reported in 8.8% (5/58) and 16.7% (3/19) of males and females, respectively. The mean grade of fibrosis was also higher in females (1.22 ± 1.35 *vs* 0.88 ± 1.05). However, these differences were not statistically significant ($P = 0.563$ and 0.263). As shown in Table 3, other histopathologic findings were not statistically different between the two gender groups of the NASH patients.

Characteristic and histopathologic features of NASH patients regarding different age groups

Based on the data in Table 2, the mean serum level of ALT in patients aged < 55 years and ⩾ 55 years was 123.21 ± 260.49 U/L and 100.44 ± 91.57 U/L, respectively, which showed no significant difference ($P = 0.772$).

Table 2 Baseline and clinical characteristics of non-alcoholic steatohepatitis patients regarding different sex and age groups (mean ± SD)

Variable	Total (*n* = 77)	Sex groups		*P*-value	Age groups		*P*-value
		Male (*n* = 58)	Female (*n* = 19)		< 55 yr (*n* = 65)	≥ 55 yr (*n* = 12)	
Age (yr)	41.99 ± 11.80	39.74 ± 10.16	48.72 ± 13.99	0.004[a]	-	-	-
Gender, *n* (%)							
Female	19 (24.7)	-	-	-	14 (21.5)	5 (41.6)	0.214
Male	58 (75.3)				51 (78.5)	7 (58.4)	
BMI (kg/m^2)	28.62 ± 3.42	28.86 ± 3.25	27.81 ± 4.00	0.336	28.74 ± 3.55	26.87 ± 2.52	0.186
Fasting blood sugar (mg/dL)	107.37 ± 47.60	103.16 ± 47.39	121.14 ± 47.14	0.195	107.88 ± 52.06	114.12 ± 25.85	0.742
Total cholesterol (mg/dL)	197.29 ± 47.81	192.07 ± 45.52	212.68 ± 52.26	0.105	197.07 ± 46.98	200.67 ± 37.82	0.827
Triglyceride (mg/dL)	202.26 ± 118.13	201.72 ± 97.26	203.89 ± 169.65	0.947	206.40 ± 121.92	202.33 ± 100.36	0.876
LDL-cholesterol (mg/dL)	119.09 ± 39.20	119.87 ± 35.00	117.13 ± 49.64	0.822	118.27 ± 38.74	134.43 ± 40.13	0.313
HDL-cholesterol (mg/dL)	46.61 ± 24.53	44.97 ± 25.20	50.87 ± 22.97	0.301	47.86 ± 26.45	40.12 ± 13.68	0.891
PTT (s)	12.47 ± 1.50	12.39 ± 1.66	12.72 ± 0.86	0.846	12.45 ± 1.58	12.40 ± 1.52	0.842
Platelet count (/L)	358 505 ± 48 852	357 505 ± 52 403	361 125 ± 39 954	0.122	395 325 ± 55 385	237 385 ± 63 753	0.964
ALT (U/L)	117.73 ± 237.52	92.05 ± 60.72	196.11 ± 466.82	0.943	123.21 ± 260.49	100.44 ± 91.57	0.772
AST (U/L)	58.29 ± 27.77	52.78 ± 25.00	75.11 ± 29.68	0.002[a]	57.28 ± 28.17	57.89 ± 20.52	0.951
Alk-P (U/L)	176.48 ± 96.95	171.14 ± 92.19	192.21 ± 111.00	0.417	176.11 ± 94.50	183.44 ± 128.40	0.837
γ-GTP (U/L)	29.55 ± 28.40	28.69 ± 30.95	31.42 ± 24.47	0.852	28.74 ± 3.55	15.17 ± 3.76	0.333

[a]Statistically significant. BMI: Body mass index; LDL: Low density lipoprotein; HDL: High density lipoprotein; PTT: Partial thromboplastin time; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Alk-P: Alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase.

Table 3 Histopathologic characteristics of non-alcoholic steatohepatitis patients regarding different sex and age groups (mean ± SD) *n* (%)

Finding	Total (*n* = 77)	Sex groups		*P*-value	Age groups		*P*-value
		Male (*n* = 58)	Female (*n* = 19)		< 55 yr (*n* = 65)	≥ 55 yr (*n* = 12)	
Hepatosteatosis							
Grade Ⅰ	36 (46.8)	30 (51.7)	6 (31.6)		34 (54)	1 (11.2)	
Grade Ⅱ	29 (37.7)	22 (37.9)	7 (36.8)	0.068	23 (36.5)	4 (44.4)	0.007[a]
Grade Ⅲ	12 (15.6)	6 (10.3)	6 (31.6)		6 (9.5)	4 (44.4)	
Mean grade	1.69 ± 0.73	1.59 ± 0.68	2.00 ± 0.82	0.031[a]	1.56 ± 0.67	2.33 ± 0.71	0.002[a]
Fibrosis							
No fibrosis	31 (41.3)	24 (42.1)	7 (38.9)		27 (44.3)	2 (22.2)	
Grade Ⅰ	29 (38.7)	24 (42.1)	5 (27.8)		22 (36.1)	4 (44.4)	
Grade Ⅱ	7 (9.3)	4 (7)	3 (16.7)	0.563	6 (9.8)	1 (11.1)	0.307
Grade Ⅲ	3 (4)	2 (3.5)	1 (5.6)		3 (4.9)	-	
Cirrhosis	5 (6.7)	3 (5.3)	2 (11.1)		3 (4.9)	2 (22.2)	
Mean grade	0.96 ± 1.13	0.88 ± 1.05	1.22 ± 1.35	0.263	0.91 ± 1.09	1.56 ± 1.51	0.115
Hepatocyte ballooning							
No ballooning	52 (72.2)	41 (74.5)	11 (64.7)		42 (71.2)	6 (75)	
Grade Ⅰ	12 (16.7)	8 (14.5)	4 (23.5)	0.224	11 (18.6)	1 (12.5)	0.935
Grade Ⅱ	7 (9.7)	6 (10.9)	1 (5.9)		5 (8.5)	1 (12.5)	
Grade Ⅲ	1 (1.4)	-	1 (5.9)		1 (1.7)	-	
Mean grade	0.40 ± 0.72	0.36 ± 0.68	0.53 ± 0.87	0.448	0.41 ± 0.72	0.37 ± 0.74	0.864
Lobar inflammation							
No change	25 (36.8)	23 (42.6)	2 (14.3)		18 (31.6)	6 (100)	
Grade Ⅰ	37 (54.4)	27 (50)	10 (71.4)	0.138	34 (59.6)	-	0.005[a]
Grade Ⅱ	6 (8.8)	4 (7.4)	2 (14.3)		5 (8.8)	-	
Mean grade	0.72 ± 0.62	0.65 ± 0.62	1.00 ± 0.55	0.052	0.77 ± 0.60	-	0.004[a]
Portal inflammation							
No change	41 (55.3)	33 (58.9)	8 (44.4)		35 (58.3)	3 (33.3)	
Grade Ⅰ	25 (33.8)	19 (33.9)	6 (33.3)		19 (31.7)	5 (55.6)	0.045[a]
Grade Ⅱ	6 (8.1)	3 (5.4)	3 (16.7)	0.192	5 (8.3)	-	
Grade Ⅲ	2 (2.8)	1 (1.8)	1 (5.6)		1 (1.7)	1 (11.1)	
Mean grade	0.59 ± 0.81	0.52 ± 0.76	0.83 ± 0.92	0.163	0.55 ± 0.79	0.89 ± 0.93	0.200

[a]Statistically significant.

Similarly, the mean serum level of AST was not statistically different between the two age groups of patients (57.28 ± 28.17 U/L *vs* 57.89 ± 20.52 U/L, $P = 0.951$). Other baseline and clinical characteristics were not significantly different between the two age groups.

Table 3 shows the histopathologic findings of NASH patients regarding different age groups. While 54% (34/65) of younger patients had mild hepatosteatosis (Grade Ⅰ), only one patient (11.2%) in the older group had grade Ⅰ hepatosteatosis. On the other hand, the older

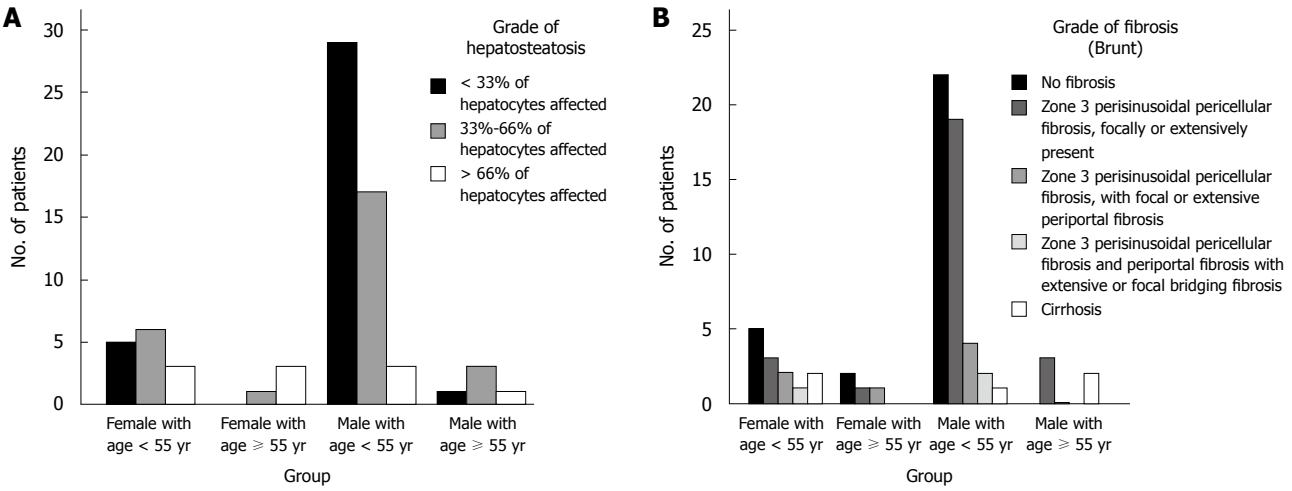

Figure 1 Frequency of different grades of hepatosteatosis (A) and fibrosis (Brunt) (B) in non-alcoholic steatohepatitis patients regarding their gender and age groups.

patients had suffered significantly more severe hepatosteatosis (Grade Ⅲ) (44.4% *vs* 9.5%, $P = 0.007$). Moreover, the mean quantitative grade of hepatosteatosis was significantly higher among patients with an age of ⩾ 55 years (2.33 ± 0.71 *vs* 1.56 ± 0.67, $P = 0.002$). Although the prevalence of any grade of fibrosis was also higher in older patients, this difference was not statistically significant (77.8% *vs* 54.7%, $P = 0.307$). By contrast, lobar and portal inflammation were reported significantly more often in histopathologic findings of younger NASH patients ($P = 0.005$ and 0.045, Table 3).

More detailed analysis was performed to evaluate the best cutoff points of patients' age to predict advances in histopathologic findings. First, the results of ROC curve analysis showed that the values of patients' age could significantly differentiate NASH patients with or without advanced fibrosis (AUC = 0.734, $P = 0.032$). Additionally, the cutoff value of 50 years had 62.5% sensitivity and 78.1% specificity to predict advanced fibrosis; whereas, the cutoff point of 45.5 years had a sensitivity of 75% and specificity of 69.7%.

Age and sex interactions

According to the above, female patients were significantly older than males ($P = 0.004$); therefore, multivariate analysis was performed to evaluate the confounding role of age in the association between gender and histopathologic findings (especially the mean grade of hepatosteatosis). The results emphasized the higher grade of hepatosteatosis in female patients even when considering patients' age as a fixed covariate between two genders ($P = 0.010$).

After omitting the patients aged > 50 years, the mean age of two gender groups were matched (36.87 ± 11.11 years in females *vs* 36.40 ± 7.25 years in males); and again the mean grade of hepatosteatosis was higher in female NASH patients (1.87 ± 0.83 *vs* 1.49 ± 0.62).

Furthermore, when both age and sex were considered (by dividing the patients into four groups), the more severe hepatosteatosis were reported in female patients aged ⩾ 55 years (Figure 1A) while the mildest histopathologic findings were found in younger males (Figure 1A and B).

DISCUSSION

It has been suggested that some patients with NASH may manifest a benign course where others follow a more aggressive course which results in cirrhosis and final liver failure. The basis for this unusual course in patients suffering from the identical situation proposes that the disease might have a benign and non-aggressive course in some patients whereas, in patients with more aggressiveness some factors may contribute and aggravate the consequent liver damage and fibrosis which can finally result in cirrhosis. For the last two decades, several studies have considered different issues as possible risk factors for NASH such as hyperlipidemia, hypertension and insulin resistance. Unlike these studies, we did not focus on metabolic syndrome because these risk factors have been studied in many previous reports[19,20] but we have tried to demonstrate some of the factors which are believed to play a role in disease severity and pathological findings in Iranian patients suffering from NASH. However, it is noted that the prevalence of the known risk factors of metabolic disorders were not significantly different between the two age and gender groups in our study (Table 2).

This study has revealed some important points which need to be discussed further. First, the severity of hepatosteatosis and portal inflammation increases with age. Second, female patients being affected are older. Third, hepatosteatosis is more severe in female patients even after age-adjustment and finally, female patients have higher AST levels.

Early studies had considered female gender as a risk factor for NASH but this was not identified by recent studies[9,21]. Angulo *et al*[22] reported that there is a trend toward higher levels of fibrosis in female patients with NASH but in another study on children suffering from NASH such a relationship was not reported[23]. Ratziu

et al[24] studying the clinical and pathological aspects of NASH in Japanese patients concluded that older patients have more severe fibrosis compared to younger adults where the older group consisted of significantly more females. According to these findings they considered female gender as a possible risk factor for NASH severity but according to multivariate analysis on both age groups, gender had no role in the severity of the liver involvement. Likewise, level of fibrosis was higher in female patients in this study, but there was not a significant difference among different genders. The only significant difference considering age and pathological findings was the level of hepatosteatosis. In contrast to the study of Yatsuji *et al*[25], our findings highlighted the significantly higher grade of hepatosteatosis in female patients despite the elimination of the confounding role of patients' age in this association.

Patients aged ≥ 55 years of age had significantly higher levels of hepatosteatosis compared to younger patients. Although the level of fibrosis was not significantly different, older patients had considerably more severe portal inflammation and less lobar inflammation. In addition, results of ROC curve analysis showed that the cutoff values of 50 or 45.5 years for patients' age could significantly differentiate NASH patients with or without advanced fibrosis.

Previous studies suggested a number of factors associated with more severe and progressive liver fibrosis. In one study focusing on obese patients, age ≥ 50 years [odds ratio (OR) 14.1], a body mass index ≥ 28 kg/m^2 (OR 5.7), triglycerides ≥ 1.7 mmol/L (OR 5), and an ALT concentration ≥ 2 × normal (OR 4.6) were associated with more severe fibrosis[24]. In another study on 733 patients, the authors presented a model consisting of age, body mass index, platelet count, albumin, and AST/ALT ratio which had a good predictive value for advanced fibrosis[26].

As mentioned before, in a similar study on Japanese patients, the older patients had much more advanced fibrosis and worse deterioration of liver function compared to the younger group[25]. Age related mitochondrial dysfunction has been suggested recently as a contributing factor in developing insulin resistance[27]. Pathophysiological considerations, laboratory investigations and clinical association studies have supported the central role of mitochondrial dysfunction in developing insulin resistance and subsequent liver disease[20,28-30].

More recently, a systematic review was performed for assessment of factors associated with fibrosis progression in NASH patients by Argo *et al*[31]. They involved patients with one initial liver biopsy and one or more biopsies during the course of disease and revealed that age and initial level of fibrosis are the only predictors of fibrosis progression. Compared to their findings our results also showed that advanced age contributed to higher grades of hepatosteatosis and portal inflammation but such a relationship was not found with gender. However, the most severe grade of hepatosteatosis was found in older female patients.

Unfortunately previous studies comparing different components of Brunt classification of liver pathology according to independent risk factors are scarce and more precise studies with a greater study population are needed to correctly evaluate the relationship between these factors and liver pathology.

In conclusion, our data points toward the possible influence of age in the severity of steatohepatitis, portal and lobar inflammation in patients suffering from NASH and also indicates that gender independently contributed to the level of steatohepatitis. Moreover, according to the lower cutoff values of 50 or 45.5 years of age for advanced fibrosis in Iranian patients, it might be possible to consider progression toward cirrhosis earlier (before 55 years) in an Iranian population.

COMMENTS

Background

Prevalence of obesity, metabolic syndrome and non-alcoholic steatohepatitis (NASH) has increased recently in a parallel manner. NASH has been considered as a possible part of metabolic syndrome. Insulin resistance is the basis of metabolic syndrome pathogenesis. Hyperinsulinemia and insulin resistance occurs with aging and reduced physical activity. Additionally, due to sex hormones the prevalence of metabolic syndrome is different between genders.

Research frontiers

NASH is an important clinicopathological entity which is usually distributed among obese patients who show considerable insulin resistance. This study has investigated the possible difference in pathological findings of patients suffering from NASH according to age and gender.

Innovations and breakthroughs

These findings point toward the possible influence of age in the severity of steatohepatitis, portal and lobar inflammation in patients suffering from NASH while gender independently might contribute to the level of steatohepatitis. Gender might contribute to the level of steatohepatitis. Severity of steatohepatitis, portal and lobar inflammation is higher in older patients suffering from NASH.

Applications

Physicians should be more cautious while treating older and female patients as these groups might have more severe pathologic findings in their liver biopsies.

Peer review

This manuscript is interesting. The number of patients included in the study is small and may underestimate the frequency of NASH.

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BRIEF ARTICLE

Age-dependent eradication of *Helicobacter pylori* in Japanese patients

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Abstract

AIM: To determine the general risk factors affecting the failure rate of first-line eradication therapy in Japanese patients with *Helicobacter pylori* (*H. pylori*) infection.

METHODS: The present study enrolled 253 patients who had an *H. pylori* infection, underwent gastroendoscopy, and were treated with *H. pylori* eradication therapy. Eradication therapy consisted of 30 mg lansoprazole plus 750 mg amoxicillin and 400 mg clarithromycin twice daily for 7 d. All of the patients underwent a ^{13}C urea breath test at least 1 mo after the completion of eradication therapy. The current study investigated the independent factors associated with successful *H. pylori* eradication using a multiple logistic regression analysis.

RESULTS: The overall success rate in the patients was 85.8%. Among the general factors examined in the multivariate analyses, only having an age less than 50 years was found to be significantly associated with a poor response to *H. pylori* eradication. Moreover, side effects were the only clinical factors in the patients who were under 50 years of age that significantly influenced the poor response to *H. pylori* eradication.

CONCLUSION: *H. pylori*-positive elderly patients should undergo eradication therapy. In addition, it is necessary to improve *H. pylori* eradication therapy in younger patients.

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Key words: *Helicobacter pylori*; Eradication; Treatment; Age; Side effect

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infections cause chronic gastritis and are associated with an increased risk of major upper gastrointestinal diseases, such as peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma[1]. Proton-pump inhibitor (PPI)-based triple therapy, with a PPI, clarithromycin (CAM), and either amoxicillin (AMPC) or metronidazole, is a widely recommended eradication therapy[2]. In addition, this triple therapy has been approved by the medical insurance system in Japan, and the eradication therapy is administered by physicians in general practice.

Consensus conferences have recommended therapeutic regimens that achieve *H. pylori* cure rates of over 80% on an intent-to-treat basis[3-5]. However, several large-scale clinical trials and meta-analyses have demonstrated that the most common first-line therapies fail in up to 20% of patients[6,7], and in the clinical setting, the actual treatment failure rate may be even higher[8]. Few studies have examined the causes of patient failure in these therapies among the general clinical factors in Japan. The aims of the current study were to determine the general risk factors that affect the failure rate of first-line eradication therapy in Japanese patients with *H. pylori* infection using a multivariate analysis.

MATERIALS AND METHODS

The current series included 253 patients who underwent a gastro-endoscopy between January 2006 and September 2007 in the Shinko hospital in Kobe, Japan, and who were diagnosed with an *H. pylori* infection by the presence of the bacterium at endoscopy, detection of the bacteria or an antibody in patient urine, or a positive ^{13}C urea breath test (UBT; with a cut-off value of 2.5‰; Ubit, Otsuka Pharmaceuticals, Tokyo, Japan). All patients were treated with *H. pylori* eradication therapy, consisting of 30 mg lansoprazole, 750 mg amoxicillin, and 400 mg clarithromycin twice daily for 7 d. Patient compliance and treatment-related side effects were assessed at the end of the treatment period. The patients underwent a UBT at least 1 mo after the completion of eradication therapy. Successful *H. pylori* eradication was defined as a negative result on the UBT. Independent factors that may have been associated with successful *H. pylori* eradication were studied using multiple logistic regression analysis. The following variables were evaluated as independent factors: sex, age (generation), diagnosis, and side effects. The StatView system software package (version 5.0) was used for the statistical analysis. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Demographic and clinical features of the patients who underwent first-line eradication therapy

Table 1 lists the demographic and clinical features of the patients who underwent *H. pylori* eradication therapy. Adverse events were observed in 41 patients within the first week after the initiation of the eradication therapy. The most common adverse events included diarrhea and gustatory dysfunction. None of the patients withdrew from the treatment course due to eradication therapy-related side effects.

Success rate of first-line eradication therapy

The overall success rate in the patients was 85.8%. Figure 1A shows the success rate of the first-line eradication therapy in patients with *H. pylori* infection when correlated with the patients' sex, diagnoses, and side effects. The success rate tended to be higher in patients with peptic ulcers than in those without peptic ulcers (87.9% *vs* 81.8%). Figure 1B shows the success rates according to the patient's age. A good success rate was observed, even among elderly patients.

Table 1 Demographic and clinical characteristics of patients undergoing eradication therapy for an *Helicobacter pylori* infection

	n = 253
Sex	
Male	174
Female	79
Generation (s)	
20	10
30	15
40	50
50	92
60	54
70	27
> 80	5
Diagnosis	
Ulcer	165
Non-ulcer	88
Side effect	
No	212
Yes	41

Ulcer: A peptic ulcer was detected by gastroscopy; Non-ulcer: No peptic ulcer was detected by gastroscopy (instead, for example, atrophic gastritis, among others); Side effects: Diarrhea (n = 23), gustatory dysfunction (n = 10), among others.

Multivariate analysis of independent factors for the patient response to first-line eradication therapy

The independent factors associated with the response to first-line *H. pylori* eradication therapy were evaluated by a multivariate analysis. The only factor (among sex, age, diagnosis, and side effects) examined in the multivariate analysis that was found to be significantly associated with a poor response to first eradication therapy was patient age of less than 50 years of age (P = 0.015, Table 2). Moreover, side effects were the only independent factor significantly associated with a poor response in patients under 50 years of age (P = 0.033, Table 3).

DISCUSSION

The current study showed that first-line eradication treatment failures occurred significantly more frequently in patients aged less than 50 years of age. Elderly patients are frequently infected with *H. pylori* for a longer period of time, present with enlarged atrophic gastritis, and progress to intestinal metaplasia. These patients may experience easier *H. pylori* eradication with first-line eradication treatment. In addition, the gastric mucosa becomes more atrophic in elderly patients in comparison to younger patients, and elderly patients also display gastric acid hyposecretion. This can compromise their ability to inactivate the AMPC and CAM. These conditions may contribute to the current results.

Pretreatment antibiotic resistance is the primary rea-

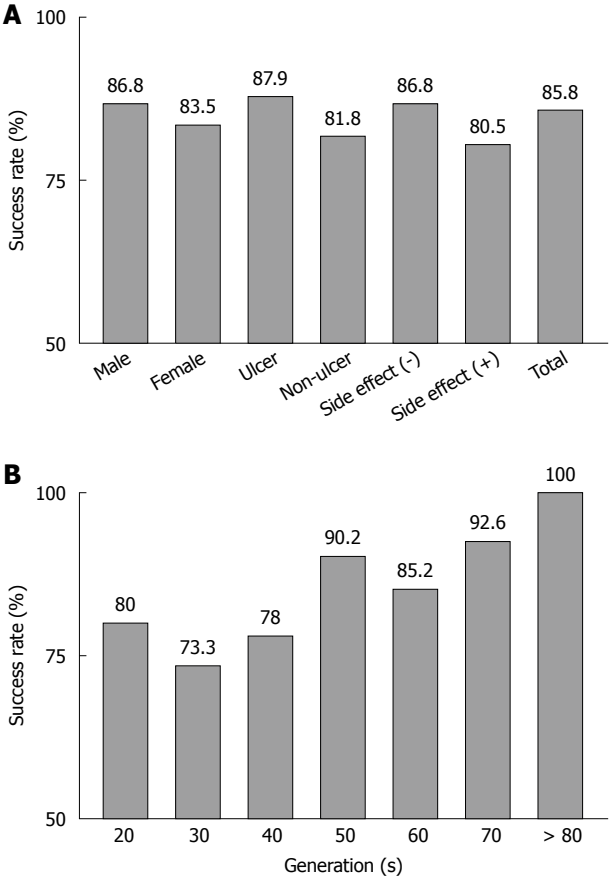

Figure 1 Success rate of first-line eradication therapy. A: Success rates according to sex (male, female), diagnosis (ulcer or non-ulcer), side effects [no (-) or yes (+)], and the total patient success rate of eradication therapy for the treatment of *Helicobacter pylori* (*H. pylori*); B: Success rates according to the patient generation of eradication therapy for *H. pylori*.

son some patients do not respond to initial treatment[9-13]. In particular, antibiotic CAM resistance has been identified as a major factor affecting the ability to cure *H. pylori* infection, and the rate of resistance to this antibiotic is increasing in many geographical areas[14,15]. A similar tendency is believed to be occurring in Japan. The efficacy of PPI-based regimens is decreasing, and several studies have reported intention-to-treat eradication rates lower than 75%[16-24]. The overall success rate in patients was greater than 85% in the current series. This high success rate may be due to patients having been diagnosed with an *H. pylori* infection during the medical examination, and the fact that few patients were receiving any medication. Future studies will examine whether patients with *H. pylori* infections display antibiotic resistance.

The only factors that significantly influenced the response to eradication therapy in patients were being under 50 years of age and diarrhea, which was the most common side effect for half of the patients. It is necessary to determine how to increase the treatment success rate among younger patients. A previous study from Japan reported that the use of the probiotic bacterium *Clostridium butyricum* MIYAIRI 588 strain reduced fluctuations in the intestinal flora and decreased the incidence of gastrointestinal side effects[25]. Supplementation with probiotics may have several beneficial effects on *H. pylori* eradication, especially in younger patients.

Table 2 A multivariate analysis of all patients (n = 253)

Variable	Multivariate OR	95% CI	*P*-value
Sex (male *vs* female)	1.29	0.62-2.71	0.50
Age (under 50 yr *vs* over 50 yr)	0.41	0.20-0.84	0.015[a]
Disease (ulcer *vs* non-ulcer)	1.61	0.79-3.30	0.19
Side effect (no *vs* yes)	1.59	0.67-3.80	0.29

[a]Statistically significant. OR: Odds ratio; CI: Confidence interval.

Table 3 Multivariate analysis of under 50-yr-old patients (n = 75)

Variable	Multivariate OR	95% CI	*P*-value
Sex (male *vs* female)	1.46	0.49-4.33	0.50
Disease (ulcer *vs* non-ulcer)	0.79	0.24-2.57	0.70
Side effect (no *vs* yes)	3.97	1.12-14.16	0.033[a]

[a]Statistically significant. OR: Odds ratio; CI: Confidence interval.

Elderly patients also exhibited a good success rate in the current series. A previous report showed that approximately 53%-73% of elderly peptic ulcer patients are *H. pylori*-positive; however, the percentage of *H. pylori*-positive elderly patients who are treated for an *H. pylori* infection remains low. One-week PPI-based triple therapy regimens are highly effective and well tolerated in elderly patients[26]. Therefore, it is recommended that *H. pylori*-positive elderly patients be treated with eradication therapy.

In conclusion, the current study was conducted to determine the general risk factors that affect the failure rate of first-line eradication therapy in Japanese patients with an *H. pylori* infection. The overall success rate in patients was 85.8%. Among the general factors examined in the multivariate analyses, a patient age of less than 50 years significantly influenced the response to *H. pylori* eradication. Moreover, among the other clinical factors in patients under 50 years of age, only the presence of side effects was found to be significantly associated with the response to *H. pylori* eradication. It is necessary to determine how to increase the eradication success rate among younger patients.

COMMENTS

Background

Proton-pump inhibitor (PPI)-based triple therapy with PPI, clarithromycin, and amoxicillin is a widely recommended eradication therapy for *Helicobacter pylori* (*H. pylori*) in Japan.

Research frontiers

Few studies have described the causes of patient failure with these therapies with regard to general clinical factors in Japan.

Innovations and breakthroughs

Among the general factors examined in the multivariate analyses, only a patient age of less than 50 years was significantly related to the response to *H. pylori* eradication treatments. Moreover, among the other clinical factors in patients under 50 years of age, only the presence of treatment side effects was found to be significantly associated with the patient's response to *H. pylori* eradication.

Applications

H. pylori-positive elderly patients should undergo eradication therapy, and it is necessary to determine how to increase the success rate among younger patients in Japan.

Peer review

The subject of this article to examine the results of *H. pylori* eradication is important. There are geographic differences in antibiotic resistance affecting the eradication results and the antimicrobial resistance may vary in time. Also, reports of other factors affecting the clinical treatment results are welcome.

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BRIEF ARTICLE

Efficient hemostatic method for endoscopic submucosal dissection of colorectal tumors

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Abstract

AIM: To evaluate a new hemostatic method using hemostatic forceps to prevent perforation and perioperative hemorrhage during colonic endoscopic submucosal dissection (ESD).

METHODS: We studied 250 cases, in which ESD for colorectal tumors was performed at the Kyoto Prefectural University of Medicine or Nara City Hospital between 2005 and 2010. We developed a new hemostatic method using hemostatic forceps in December 2008 for the efficient treatment of submucosal thick vessels. ESD was performed on 126 cases after adoption of the new method (the adopted group) and the new method was performed on 102 of these cases. ESD was performed on 124 cases before the adoption of the new method (the unadopted group). The details of the new method are as follows: firstly, a vessel was coagulated using the hemostatic forceps in the soft coagulation mode according to the standard procedure, and the coagulated vessel was removed using the forceps in the "endocut" mode without perioperative hemorrhage. Secondly, the partial surrounding submucosa was dissected using the forceps in the endocut mode. In the current study, we evaluated the efficacy of this method.

RESULTS: Coagulated vessels were successfully removed using the hemostatic forceps in all 102 cases without severe perioperative hemorrhage. Moderate perioperative hemorrhage occurred in five cases (4.9%); however, it was stopped by immediately reuse of the hemostatic forceps. The partial surrounding submucosa was dissected using the forceps in all 102 cases. In the adopted group, the median operation time was 105 min. The proportion of endoscopic *en bloc* resection was 92.8% ($P < 0.01$) compared to 80.6% in the unadopted group. The postoperative hemorrhage and perforation rates were 2.3% and 2.3%. The rate of perforation was significantly lower than that in the unadopted group (9.6%, $P < 0.01$). We evaluated the ease of use of this method by allowing our three trainees to performed ESD on 46 cases, which were accomplished without any severe hemorrhage.

CONCLUSION: The new method effectively treated submucosal thick vessels and shows promise for the prevention of perforation and perioperative hemorrhage in colonic ESD.

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Key words: Endoscopic submucosal dissection; Colorectal tumor; Hemostatic forceps; Perforation; Perioperative hemorrhage

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INTRODUCTION

Endoscopic submucosal dissection (ESD) has emerged as a standard therapy for the treatment of large gastric tumors in Japan[1]. However, ESD is not a standard procedure for treating colorectal tumors due to technical difficulties[2-10] that arise from the winding nature and thin wall of the colon. Perioperative hemorrhage is a frequent complication of ESD for gastric and colorectal tumors. Severe hemorrhage hampers the speedy dissection of the submucosa and can increase the risk of perforation. Therefore, submucosal thick vessels detected during ESD are clamped and coagulated using hemostatic forceps to prevent hemorrhage. However, this causes coagulation in the surrounding submucosa. Moreover, coagulation of the submucosal vessel and surrounding submucosa decreases submucosal elevation and hinders speedy submucosal dissection. If the submucosa is not properly elevated, the risk of perforation, which is the most severe complication associated with ESD for colorectal tumors, significantly increases. The frequency of perforation has been reported to be in the range of 1.5%-10.4%[2-10] in ESD for colorectal tumors. In the current study, we developed a new hemostatic method using hemostatic forceps for the treatment of submucosal thick vessels. We assessed the safety and efficacy of this method with respect to the prevention of perforation and perioperative hemorrhage in ESD for colorectal tumors.

MATERIALS AND METHODS

We studied 250 colorectal tumor cases that were treated with ESD at the Department of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine or at the Center for Digestive and Liver Disease, Nara City Hospital, between April 2005 and March 2010. We developed a new hemostatic method using a Coagrasper (Olympus Medical System Co., Tokyo, Japan) in December 2008 and performed ESD on 126 cases, which was defined as the adopted group. The new method was used to remove submucosal thick vessels and to dissect the partial submucosa in 102 of these cases. We had performed ESD on 124 cases before the adoption of the new method, thus this group was defined as the unadopted group. We used hemostatic forceps in 96 cases to coagulate the thick vessel according to the standard procedure in the unadopted group.

ESD was indicated if the colorectal tumor measured more than 20 mm in diameter, had a protruding or superficial, but not pedunculated, morphology, and the carcinoma was suspected to have invaded the superficial submucosa, as determined with endoscopic findings or histopathological examinations as previously reported[8]. The method of ESD was approved by the institutional review boards in our institutions. The patients provided their written informed consent for undergoing ESD. Two endoscopic surgeons specializing in endoscopic colorectal treatment and three trainees performed ESD on all the patients.

Preparation for ESD

We used a lower gastrointestinal endoscope with a single-channel endoscope (PCF-Q260AI; Olympus Medical System Co., Tokyo, Japan or EC-590MP; Fuji Film Medical, Tokyo, Japan) and an automatically controlled high-frequency generator (VIO300D; Erbe Elektromedizin Ltd., Tubingen, Germany). A sodium hyaluronate solution (MucoUp; Johnson & Johnson, Tokyo, Japan) and a glycerin solution (Glycerol; Chugai Pharmaceutical Co., Tokyo, Japan) were injected into the submucosa, according to a previously described method[11,12]. According to standard colonoscopic examinations, an isotonic polyethylene glycol electrolyte solution (Niflec; Ajinomoto Pharma Co., Ltd., Tokyo, Japan) was used to achieve good bowel preparation. We used an obtuse-edged, short-tipped Flush knife (Fuji Film Medical, Tokyo, Japan) that can also be used for injections[9,10].

ESD strategy

ESD was performed as previously reported[5-7]. In brief, the submucosa below the tumor was injected with a sodium hyaluronate solution and glycerin using a 25 G needle (TOP Co., Tokyo, Japan). Subsequently, a partial mucosal incision was performed on the anal side of the tumor. Thereafter, the submucosa below the tumor was dissected from the anal side. A circumferential mucosal incision was added appropriately through submucosal dissection and finally, *en bloc* resection of the tumor was accomplished.

New hemostatic method using hemostatic forceps

The new hemostatic method using hemostatic forceps was performed to remove submucosal thick vessels and to dissect the partial submucosa (Figure 1A-D). From the many available hemostatic forceps, we used a Coagrasper (FD-410LR or FD-410QR; Olympus, Tokyo, Japan) that has a rotation function and small teeth (Figure 2). The procedure was as follows: when a vessel with a diameter greater than 1 mm was found in the submucosa, it was slightly pulled out using the hemostatic forceps. It was then coagulated with the forceps in the soft coagulation mode (output, 60 W; effect 5) as previously reported[5-7] (Figure 3A). The vessel became whitish, the submucosa became less elevated, and the muscularis propria was close

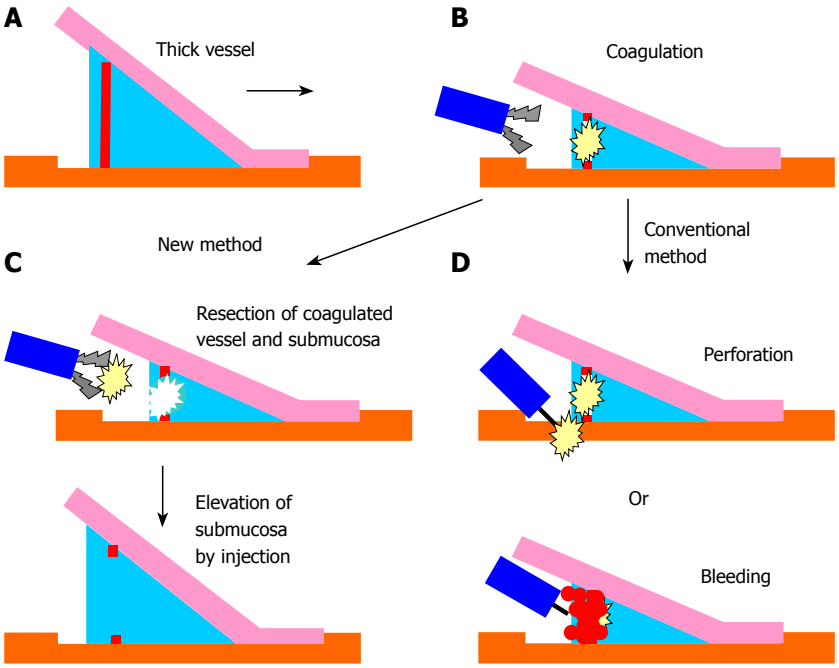

Figure 1 Schema of the efficacy of the new hemostatic forceps method. A: A thick vessel is detected during the submucosal dissection; B: The thick vessel is coagulated using the hemostatic forceps. The vessel and surrounding submucosa become whitish, and the submucosa becomes less elevated; C: In the new method, the coagulated vessel is removed using the hemostatic forceps and the surrounding submucosa is also dissected. After the treatment, the submucosa is elevated by injecting it with hyaluronic acid and glycerin; D: In the conventional use of hemostatic forceps, perforation can occur because of inadequate submucosal elevation; moreover, hemorrhage can occur from the coagulated vessel while dissecting it with a knife.

Figure 2 The hemostatic forceps, coagrasper. These forceps have a rotation function and small teeth.

to the coagulated vessel (Figure 3B). The coagulated vessel was once again grasped using the hemostatic forceps and removed in the "endocut" mode (effect 2, duration 2, interval 1) (Figure 3C). Subsequently, the coagulated submucosa surrounding the vessel was dissected using the hemostatic forceps in the endocut mode (effect 2, duration 2, interval 1) (Figure 3C). In the situation of hemorrhage, the brownish coagulated submucosa was dissected using the hemostatic forceps in the endocut mode (effect 2, duration 2, interval 1) (Figure 4A-C and Figure 5).

We analyzed the feasibility and safety of this new method with respect to the removal of coagulated vessels and the dissection of the surrounding submucosa. We also assessed the frequency of perforation and postoperative hemorrhage. We also examined whether this procedure would result in the excess burning of the histopathological findings of the resected specimen. Excess burning was defined as the state in which glands and muscularis mucosae at the margin of the resected specimen were not determined clearly. Furthermore, we evaluated the ease of use this method by allowing our three trainees to perform the method.

We compared the outcome of 126 cases in the adopted group to that of 124 cases in the unadopted group, with respect to tumor size, tumor location, operation time, proportion of *en bloc* resection, frequency of histopathological curative resection, histopathological diagnosis, and the incidence of complications. Histopathological curative resection was determined as being free of cancer cells in both the vertical and lateral margins. Tumor location was divided into three groups: Right side colon (Cecum to Transverse colon), Left side colon (Descending colon to Sigmoid colon), and Rectum. We used the World Health Organization classification system for histopathological diagnosis[13]. In view of the risk of metastasis of lymph nodes, submucosally invaded cancers were divided into slightly invaded submucosal cancer (sSM) whose invasion length into submucosa was less than 1000 microns) or massively invaded submucosal cancer (mSM) whose invasion length was more than 1000 microns, according to previous report[14]. Complications of perforation and postoperative hemorrhage were assessed. A perforation was defined as a hole in the proper muscle layer that could be detected during ESD and from which released air could be detected using computed tomography. A postoperative hemorrhage was defined as the occurrence of hematochezia requiring endoscopic treatment to stop the hemorrhage and were divided into moderate type and severe type: the former was without significant decrease in hemoglobin (Hb), the latter was with significant decrease in Hb (more than 2 g/dL). Statistical analysis was performed using the chi-square test and the Mann-Whitney *U* test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

The new hemostatic method using hemostatic forceps was employed in 102 cases. In 97 cases, the whitish coagulated vessel could be successfully removed using the hemostatic forceps without severe perioperative hemor-

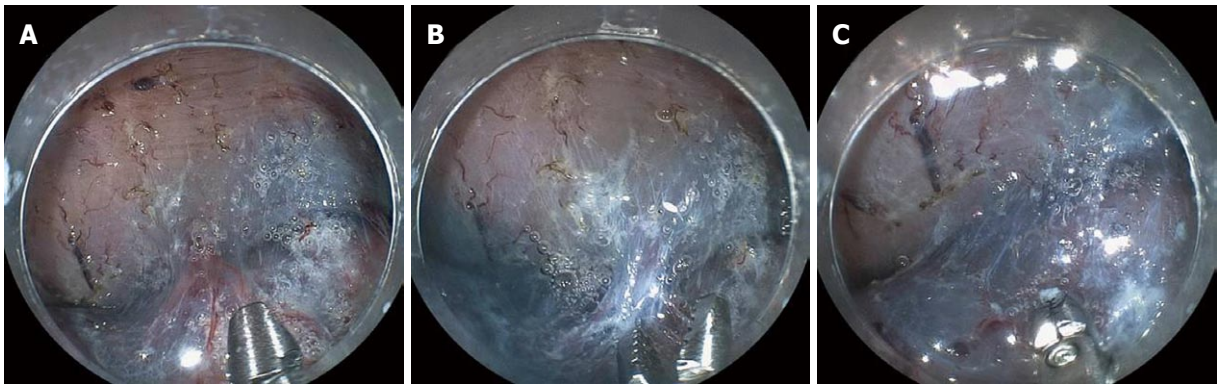

Figure 3 Clinical use of the new method in endoscopic submucosal dissection for colorectal tumors. A: A vessel with a diameter of 1 mm was detected; B: It was coagulated in the soft coagulation mode. Thereafter, the vessel and the surrounding submucosa became whitish, and the submucosa became less elevated. Submucosal dissection with a knife was slightly difficult because of the obscured view of the submucosa and the proximity of the muscularis propria; C: The whitish coagulated vessel was removed using the hemostatic forceps in the "endocut" mode. Subsequently, the whitish surrounding submucosa was dissected using the forceps. Thereafter, the view of the submucosa was improved.

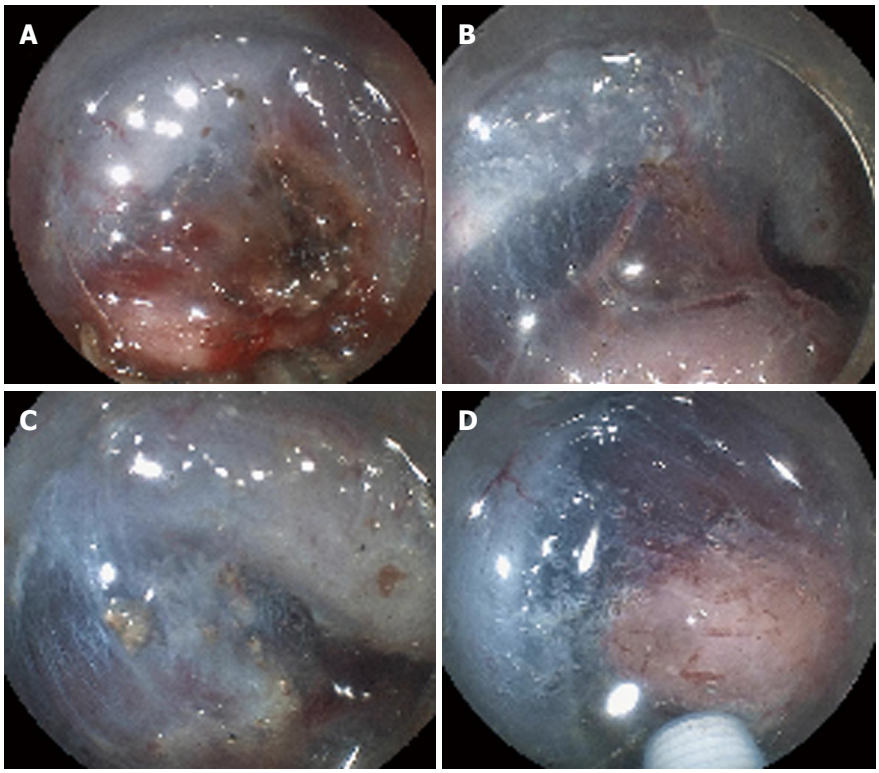

Figure 4 Clinical use of the new method for hemorrhage from the submucosal vessel. A: Coagulation of the hemorrhagic vessels made the vessels and the surrounding submucosa brownish; B: The brownish vessel was removed and the surrounding submucosa was dissected. The view of the submucosa became clear and the other vessel could be detected; C: The vessel was coagulated using the forceps to prevent perioperative hemorrhage; D: The coagulated vessel was removed and the surrounding submucosa was dissected. Thereafter, the view of the submucosa was improved.

rhage. Moderate perioperative hemorrhage occurred in five cases (4.9%); however, in all 5 cases, hemorrhage was arrested by immediately coagulating the affected vessel by reusing the hemostatic forceps. In all cases, the whitish or brownish coagulated submucosa surrounding the coagulated vessel was safely and easily dissected using the hemostatic forceps. After the dissection of the whitish or brownish coagulated vessel and submucosa, subsequent submucosal dissection using a Flush knife was easily and safely performed because the view of the surrounding submucosa was improved (Figure 4C and Figure 5C).

The patient characteristics in the two groups, the adopted group and unadopted group, are summarized in Table 1. Tumors in the Right side colon in the adopted group were significantly higher than that in the unadopted group. In the adopted group, the median operation time was 106 min and the frequency of endoscopic *en bloc* resection was 92.8%. Any lesions after EMR were not performed in the unadopted group. On the other hand, three lesions were resected in the adopted group. Histopathological complete resection was achieved in 89.6% of the adopted group. There were no significant differences between two groups in histopathological diagnosis. In sSM cancer, all eight cases were well-differentiated adenocarcinoma; venous infiltration was not detected in any cases of both groups. However, lymphatic infiltration was detected in one case in the adopted group and the patient received an additional surgical operation. In mSM cancer, nine cases were well-differentiated adenocarcinoma, and one case was moderately differentiated adenocarcinoma. Venous

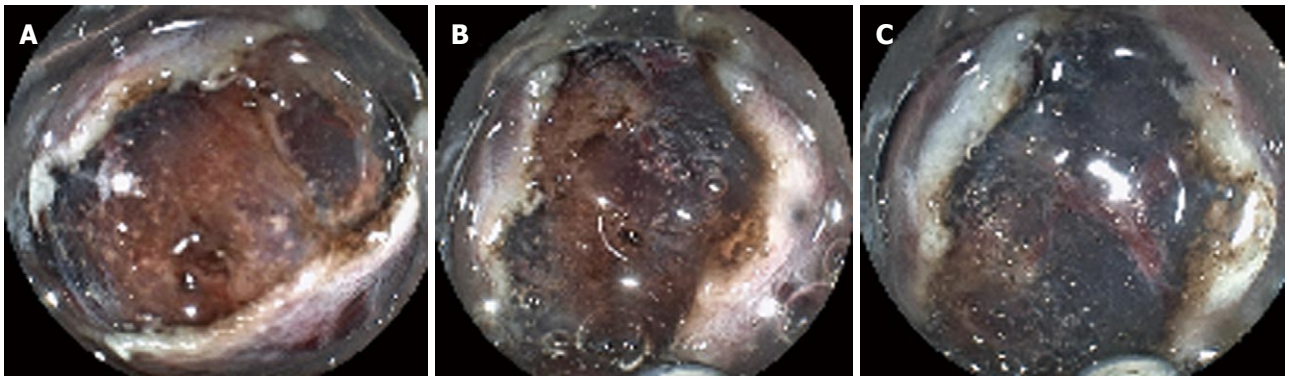

Figure 5 Clinical use of the new method for brownish coagulated submucosa. A: Brownish coagulated submucosa was detected; however, the view of the submucosa was obscured; B: The brownish submucosa was dissected using the new method; C: The view of the submucosa was restored.

Table 1 Comparison of endoscopic submucosal dissection data for colorectal tumors between the new and previous method of using hemostat forceps

	The adopted group	The unadopted group	*P*-value
No. of cases	126	124	
Median age (yr, range)	68.4 (55-87)	64.9 (45-85)	NS
Tumor size (mm, range)	29.6 (15-130)	28.6 (12-70)	NS
Location: Right side colon/ left side colon/rectum	72/26/28	52/21/51	< 0.01
En bloc resection, *n* (%)	117 (92.8)	100 (80.6)	< 0.01
Histopathological complete resection, *n* (%)	113 (89.6)	90 (72.5)	< 0.01
Operation time (min, range)	106 (40-330)	105 (20-270)	NS
Histopathological diagnosis adenoma/M/sSM/mSM/MP	57/60/4/5/0	55/59/3/5/2	NS
Perforation, *n* (%)	3 (2.3)	12 (9.6)	< 0.01
Postoperative hemorrhage, *n* (%)	3 (2.3)	3 (2.4)	NS

M: Mucosa; sSM: Slightly invaded submucosa; mSM: Massively invaded submucosa; MP: Muscularis propria; NS: Not significant.

infiltration was detected in one case in the adopted group and in one case in the unadopted group. Lymphatic infiltration was detected in one case in the unadopted group. One case of sSM cancer and eight cases of 10 mSM cancer received additional surgical operations and there were no metastasis of the lymph nodes in any of these cases. The frequencies of postoperative hemorrhage in the adopted group and the unadopted group were 2.3 % and 2.4%, respectively. All cases were moderate hemorrhage. The frequencies of perforation in the adopted group and the unadopted group were 2.3% and 9.6% ($P < 0.01$). One case in the unadopted case received an urgent surgical operation and was discharged in 14 d. The other 14 cases of perforation were cured with endoscopic clipping and fast. They discharged 5 to 10 d after ESD.

Excess burning of the vertical margin associated with histopathological diagnosis was detected in three out of 102 cases (2.9%) using the new method. Conversely, it was detected in three out of 96 cases (3.1%) in the group using hemostatic forceps according to the conventional approach. There was no significant difference between these two groups.

In relation to the ease of the new method, three trainees performed ESD in 62 cases in the adopted group. The new method was performed on 46 cases. The characteristics of these cases were described as follows: average tumor size: 28.2 mm, Location: Right side colon 25 cases: Left side colon: 6 cases Rectum 15 cases. ESD was successfully accomplished in all cases without severe hemorrhage and perforation. Their average operation time was 110 min.

DISCUSSION

In the current study, we developed and assessed the efficacy of a new hemostatic method using hemostatic forceps for the treatment of submucosal thick vessels. Using the new method, coagulated vessels were successfully removed without severe hemorrhage. The dissection of the surrounding partial submucosa improved the view of the submucosa, whose subsequent dissection with a knife could be performed safely. This method enables us to perform the safe dissection of the submucosa without hemorrhage, and to prevent perforation and perioperative hemorrhage during ESD of colorectal tumors.

Moderate hemorrhage was experienced using this method, because of the inadequate coagulation of the vessel; however, the immediate reuse of the hemostatic forceps stopped the hemorrhage. When vessels that have been coagulated using the hemostatic forceps are dissected with a knife following the conventional approach, severe hemorrhage sometimes occurs because of inadequate coagulation. In such a case, the knife needs to be replaced with hemostatic forceps to arrest the hemorrhage; however, continued hemorrhage during this replacement might obscure the view from the endoscope. In our new method, the need for switching instruments is eliminated: the hemostatic forceps can be immediately reused in the event of hemorrhage during vessel removal. Moreover, this method could also shorten the operation time required for ESD.

The cases in the adopted group showed a significant bias towards tumors in the right side colon. This seemed to be caused by the increase of difficult cases that were introduced by other institutions. The proportion of *en bloc*

resection in the adopted group (92.8%) was significantly better than that in the unadopted group (80.6%). Moreover, the rate of perforation was significantly lower in the adopted group (2.4%) than in the unadopted group (9.6%). These improvements are probably due to easier and speedy submucosal dissection using the new method. This new method provided efficient at removing vessels and gave better view of the submucosa during ESD. However, a learning curve was also associated with these improvements. Increasing experience will lead to the choice of a safe strategy for ESD and a suitable choice of knife, which are important in preventing perforation[6].

Three trainees performed ESD with this new method in 46 cases. Although a lack of experience with ESD for colorectal tumors is associated with an increased risk of perforation[5-7], perforation did not occur in these 46 cases. The new method decreased the occurrence of difficult submucosal dissections that can increase the risk of perforation. With regards to the ease of use this new method, the trainees could easily perform this new method without any difficulties; thus, we consider that the new method can be safely and effectively performed in other institutions.

It was possible that this new method could cause excess burning of the colorectum, because the head of the hemostatic forceps was larger than the Flush knife; however, histopathological analysis revealed that the rate of excess burning in the resected specimens using the new method was similar to the rate observed in the cases that underwent the standard procedure. Moreover, delayed perforation due to excess burning was not detected in any cases using the new method. The location of the vessel grasped by the forceps was important. When it was located on the near side of the muscularis propria, the effect of burning might invade the muscularis propria. Conversely, when it was located on the near side of the mucosa, the effect of burning might invade the resected specimen. This made histopathological diagnosis difficult. Therefore, we recommend that the vessel grasped by the forceps should be in the middle of the elevated submucosa. A wide range of hemostatic forceps manufactured by various medical companies is currently available. In our new method, we used the Coagrasper that has a rotation function and small teeth. To safely remove vessels, the hemostatic forceps should be positioned parallel to the short axis of the vessel. The teeth on these forceps enabled us to firmly grasp the affected vessel; it is tremendously difficult to grasp the coagulated vessel using toothless forceps. However, care should be taken during the procedure to avoid grasping the muscularis propria.

This new method was also associated with certain cost benefits. In our institution, hemostatic forceps were used in 96 of 124 cases (77.4%) for the coagulation of the submucosal vessel in the unadopted group. In such cases, difficult submucosal dissections could be made using this method. This implies that other knives such as the Hook knife (Olympus Medical Systems, Tokyo, Japan), which are sometimes used for difficult submucosal dissections, are not needed. Therefore, it is expected that there will be a decrease in cases requiring multiple knives using the new method and this will reduce the cost of performing ESD.

Standardization of the ESD procedure for colorectal tumors has not been possible because of technical difficulties. New medical equipment has been developed to overcome the technical difficulties associated with this procedure[15-17]; however, perforation still represents a considerable problem. Perforation might result in a difficult submucosal dissection. Difficult submucosal dissections can sometimes occur due to perioperative hemorrhage. Our method was effective at preventing perioperative hemorrhage and, thus, makes the dissection of the submucosa easier. This new method is likely to be useful for the standardization of ESD for colorectal tumors.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is not a standard procedure for treating colorectal tumors because of technical difficulties. The frequency of perforation has been reported to be in the range of 5%-14% in ESD for colorectal tumors.

Research frontiers

In general, perioperative severe hemorrhage hampers the speedy dissection of the submucosa and can increase the risk of perforation. Therefore, submucosal thick vessels detected during ESD are clamped and coagulated using hemostatic forceps to prevent hemorrhage. However, this sometimes hinders speedy submucosal dissection. In the current study, the authors developed a new hemostatic method using hemostatic forceps for the treatment of submucosal thick vessels.

Innovations and breakthroughs

The new method was effective for the treatment of submucosal thick vessels. Moreover, this method showed promise for the prevention of perforation and perioperative hemorrhage in colonic ESD.

Applications

This new method was effective at preventing perioperative hemorrhage and, thus, makes the dissection of the submucosa easier. This new method is likely to be useful for the standardization of ESD for colorectal tumors.

Terminology

ESD was developed as a method using special knife for *en bloc* resection for large epithelial gastric tumors and has become a standard therapy for large gastric tumors in Japan. On the other hand, endoscopic mucosal resection (EMR) has been generally performed for colorectal tumors. However, it is difficult to perform *en bloc* resection by EMR for a colorectal tumor whose size is larger than 20 mm. ESD for colorectal tumor was an expected procedure for large colorectal tumor.

Peer review

The authors described a new technique using hemostatic forceps to coagulate submucosal vessels and to divide these vessels during ESD for colon mucosal lesions. The manuscript would be of interest to endoscopists performing ESD. It can, however, be substantial improved before its final publication.

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BRIEF ARTICLE

Diagnostic sensitivity of imaging modalities for hepatocellular carcinoma smaller than 2 cm

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Abstract

AIM: To compare the imaging results with histology and to evaluate the diagnostic sensitivity of imaging modalities for hepatocellular carcinoma (HCC) smaller than 2 cm.

METHODS: Nodules smaller than 2 cm (n = 34) revealed by ultrasonography (US) in 29 patients with liver cirrhosis were analyzed. Histological diagnosis of HCC was performed by ultrasonographic guidance: moderately-differentiated HCC (n = 24); well-differentiated HCC (n = 10). The patterns disclosed by the four imaging modalities defined the conclusive diagnosis of HCC: (1) contrast-enhanced computed tomography (CECT), hypervascularity in the arterial phase and washout in the equilibrium phase; (2) Sonazoid contrast-enhanced US (CEUS), hypervascularity in the early vascular phase and defect in the Kupffer phase; (3) gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI), hypervascularity in the arterial phase and/or defect in the hepatobiliary phase; and (4) CT arterioportal angiography: hypervascularity by CT during arteriography and/or perfusion defect by CT during arterial portography.

RESULTS: Overall, the sensitivity of diagnosing HCC smaller than 2 cm was 52.9% (18/34) (95% CI: 35.1-70.2) by CECT; 67.6% (23/34) (95% CI: 49.5-82.6) by Sonazoid CEUS; 76.5% (26/34) (95% CI: 58.8-89.3) by Gd-EOB-DTPA MRI; and 88.2% (30/34) (95% CI: 72.5-96.7) by CT arterioportal angiography. The diagnostic sensitivity of detecting moderately-differentiated HCC by CECT, Sonazoid CEUS, Gd-EOB-DTPA MRI and CT arterioportal angiography was 62.5% (15/24) (95% CI: 40.6-81.2), 79.2% (19/24) (95% CI: 57.8-92.9), 75.0% (18/24) (95% CI: 53.3-90.2) and 95.8% (23/24) (95% CI: 78.9-99.9), respectively. A significant difference (P < 0.05) was observed between CECT and CT arterioportal angiography in all nodules. There was no difference between Sonazoid CEUS, Gd-EOB-DTPA MRI, and CT arterioportal angiography. The combined sensitivity of Sonazoid CEUS and Gd-EOB-DTPA MRI was 94.1% (32/34).

CONCLUSION: Changing the main diagnostic modality for HCC smaller than 2 cm from CT arterioportal angiography to Sonazoid CEUS and Gd-EOB-DTPA MRI is recommended.

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Key words: Computed tomography arterioportal angiography; Contrast-enhanced computed tomography; Diagnostic sensitivity; Gd-EOB-DTPA-enhanced magnetic

resonance imaging; Hepatocellular carcinoma smaller than 2 cm: Sonazoid contrast-enhanced ultrasonography

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INTRODUCTION

The definitive diagnosis of nodular lesions, detected by imaging techniques in the liver with cirrhosis, remains a critical challenge for clinicians. The issue is particularly complicated for small (1-2 cm) nodules, many of which may be preneoplastic with uncertain malignant potential[1], such as macroregenerative nodules, low-grade dysplastic nodules (LGDN) or high-grade dysplastic nodules (HGDN), or more rarely, hemangiomas that are found in up to 42% of explanted livers[2-4].

Recently, clinicians have been able to conduct computed tomography (CT) scanning during angiography, thereby acquiring data on lesions and intranodular blood flow simultaneously[5,6]. To resolve the areas of uncertainty, we have previously reported on the superiority of CT arterioportal angiography [including CT during arteriography (CTA) and CT during arterial portography (CTAP)], concluding that it is superior to contrast-enhanced CT (CECT) and magnetic resonance imaging (MRI) in the diagnosis of hepatocellular carcinoma (HCC) nodules smaller than 2 cm[7].

Moreover, development of the newly introduced diagnostic imaging techniques, Sonazoid contrast-enhanced ultrasonography (CEUS) and gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MRI have provided higher degrees of detectability for small HCC. In this study, we compared the diagnostic sensitivity of CECT, Sonazoid CEUS, Gd-EOB-DTPA MRI, and CT arterioportal angiography in diagnosing HCC in nodules smaller than 2 cm.

MATERIALS AND METHODS

Patients

From April 2008 to December 2009, we analyzed 34 nodules smaller than 2 cm [8-20 mm; mean ± SD 12.7 ± 3.71 mm; the interquartile range (IQR) 10-15 mm] detected by US in 29 patients (13 men and 16 women; aged 55-84 years; mean ± SD 70.5 ± 7.96 years; IQR 67-76 years) with liver cirrhosis related to hepatitis B virus in 1, hepatitis C virus (HCV) in 24, and alcohol in 4. α-fetoprotein (AFP) measured less than 20 ng/mL in 21 patients and was above 21 ng/mL in 8 (Table 1). In this study, one nodule that was not histologically diagnosed as HCC irrespective of compatibility by imaging studies was excluded, and two nodules were excluded because of inconsistency between readers in the imaging results. The study was approved by the Ethics Committee in Kobe Asahi Hospital.

Table 1 Data and characteristics of 29 patients and 34 nodules

Age (yr), range (mean ± SD)	55-84 (70.5 ± 7.96) IQR 67-76
Sex (M/F)	13/16
Cause	
HBV	1
HCV	24
Alcohol	4
AFP (ng/mL)	
< 20	21
> 21	8
Nodule characteristics (mm), range (mean ± SD)	8-20 (12.7 ± 3.71) IQR 10-15
Histological diagnosis of the 34 nodules	
Moderately-differentiated HCC	24
Well-differentiated HCC	10

IQR: Interquartile range; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α-fetoprotein; HCC: Hepatocellular carcinoma.

CECT

CECT was conducted with the use of helical CT (Siemens, Germany) with precontrast and postcontrast triple-phase (arterial, portal, venous, and equilibrium phases) scans, after the injection of 120 mL of nonionic contrast medium at 3 mL/s; the scans were carried out in a craniocaudal direction with a 5 mm collimation in the other phases. Acquisition of the arterial and equilibrium phases was automatically started at 30 and 180 s, respectively, after the intravenous injection.

CEUS

Ultrasonography was performed using a SSA-660A (Toshiba Medical Systems, Tochigi, Japan). The vascular findings on phase-inversion harmonic US were shown as tumor vessel flow in the early vascular phase about 15-40 s after injection of Sonazoid (GE HealthCare, Piscataway, NJ, USA). The real-time replenishing images were obtained during the vascular phase (< 2 min after the injection) by release burst imaging. Images of the liver parenchyma were obtained in the postvascular Kupffer phase, at least 10 min after the intravenous injection of Sonazoid. Hepatic malignances were visualized as defects in the postvascular phase. An additional contrast agent was injected to confirm tumor vessel flow in the defect, a technique known as defect reperfusion imaging[8].

Gd-EOB-DTPA MRI

Images by MRI scans (Phillips, Netherlands) were obtained by the 1.0-T superconducting system (Gyroscan 10T-NT, Phillips, Netherlands). Enhanced MRI was used to obtain

Table 2 Imaging patterns for the conclusive diagnosis of hepatocellular carcinoma by the four modalities

Modality	Imaging pattern
Contrast-enhanced CT	Hypervascularity in the arterial phase and washout in the equilibrium phase
Sonazoid contrast-enhanced ultrasonography	Hypervascularity in the early vascular phase and defect in the Kupffer phase
Gd-EOB-DTPA magnetic resonance imaging	Hypervascularity in the arterial phase and/or defect in the hepatobiliary phase
CT arterioportal angiography	Hypervascularity by CTA and/or perfusion defect by CTAP

CT: Computed tomography; CTA: CT during arteriography; CTAP: CT during arterial portography; Gd-EOB-DTPA: Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid.

coronal images by the gradient-echo technique (FFG) at 150/3.5 ms TR/TE, 80° flip angle, and 168 × 256 matrix. In each sequence, the respiration suspension time was 20-30 s. Gd-EOB-DTPA (Primovist; Bayer HealthCare, Osaka, Japan) at a dose of 0.025 mmol/kg body weight was injected intravenously as a rapid bolus at 2 mL/s. Dynamic contrast-enhanced MRI was initiated at 30 s, 70 s, 2-3 min and 20 min after the start of the bolus injection to obtain multiphasic (arterial, portal, late, and hepatobiliary) images.

CT arterioportal angiography (CTA and CTAP)

CTA: At angiography, 45 mL of diluted contrast medium was injected through a catheter at 2 mL/s into the common hepatic artery. The whole liver was then scanned at intervals of 5 to 10 mm.

CTAP: At angiography, 115 mL of diluted contrast medium was injected through a catheter at 2 mL/s into the superior mesenteric artery, according to the scanning time of the entire liver using a power injector during sequential scanning of the liver with incremental changes in the position of the table. Infusion of contrast material was initiated 20 s before CTAP. The whole liver was then scanned at intervals of 5 to 10 mm.

US-guided biopsy

US-guided biopsy was carried out with the use of a 21 gauge Majima needle (Top, Japan). The diagnosis of HCC was made by two operators [a physician (K.S.) and a pathologist (Y.H.)] using the same specimen.

Histological diagnosis

Specimens were routinely processed and stained with hematoxylin and eosin and by the Masson trichromatic method. The diagnosis of HCC was made according to the criteria of the International Working Party[1].

Imaging patterns for the conclusive diagnosis of HCC by the four modalities

The following patterns disclosed by the four imaging modalities were defined as the conclusive diagnosis of HCC. (1) CECT: hypervascularity in the arterial phase and washout in the equilibrium phase; (2) Sonazoid CEUS: hypervascularity in the early vascular phase and defect in the Kupffer phase; (3) Gd-EOB-DTPA MRI: hypervascularity in the arterial phase and/or defect in the hepatobiliary phase; and (4) CT arterioportal angiography: hypervascularity by CTA and/or perfusion defect by CTAP (Table 2).

Imaging studies

To minimize differences in the results between the operators, imaging studies were carried out and reviewed by two operators [a physician (M.K.) and a radiologist (T.M.)] using the same examination protocol.

Statistical analysis

The sensitivity for detecting tumors was indicated by the 95% CI. The 95% CI was estimated by F distribution. The level of significance was set at $P < 0.05$.

RESULTS

The 34 nodules were histologically diagnosed as moderately-differentiated (24 nodules) and well-differentiated (10 nodules) HCC (Table 1). For HCC smaller than 2 cm, the overall diagnostic sensitivity was 52.9% (18/34) (95% CI: 35.1-70.2) by CECT; 67.6% (23/34) (95% CI: 49.5-82.6) by Sonazoid CEUS; 76.5% (26/34) (95% CI: 58.8-89.3) by Gd-EOB-DTPA MRI; and 88.2% (30/34) (95% CI: 72.5-96.7) by CT arterioportal angiography, with a significant difference ($P < 0.05$) between CECT and CT arterioportal angiography. The combined sensitivity of Sonazoid CEUS and Gd-EOB-DTPA MRI was 94.1% (32/34). In diagnosing moderately-differentiated HCC, the diagnostic sensitivity of CECT, Sonazoid CEUS, Gd-EOB-DTPA MRI and CT arterioportal angiography was 62.5% (15/24) (95% CI: 40.6-81.2), 79.2% (19/24) (95% CI: 57.8-92.9), 75.0% (18/24) (95% CI: 53.3-90.2) and 95.8% (23/24) (95% CI: 78.9-99.9), respectively. There was no difference between CECT, Sonazoid CEUS, Gd-EOB-DTPA MRI, and CT arterioportal angiography in moderately differentiated HCC. The sensitivity of well-differentiated HCC was not analyzed because of the paucity of cases (Table 3).

Representative cases

Case No. 1: Detection by Gd-EOB-DTPA MRI and arterioportal angiography: In a 67-year-old woman with HCV-related liver cirrhosis (AFP 9.0 ng/mL; PIVKA Ⅱ 21 mAU/mL), US revealed a 12 mm hyperechoic nodule in segment eight (Figure 1A). Sonazoid CEUS revealed no hypervascularity in the early vascular phase and no defect in the Kupffer phase. CECT revealed no hypervascularity in the arterial phase and washout in the equilibrium phase.

Table 3 Diagnostic sensitivity of hepatocellular carcinoma by the four modalities

Modality	Diagnostic sensitivity			
	All nodules (n = 34)		Moderately-differentiated HCC (n = 24)	
	n (%)	95% CI	n (%)	95% CI
Contrast-enhanced computed tomography	18 (52.9)	35.1-70.2	15 (62.5)	40.6-81.2
Sonazoid contrast-enhanced ultrasonography	23 (67.6)	49.5-82.6	19 (79.2)	57.8-92.9
Gd-EOB-DTPA magnetic resonance imaging	26 (76.5)	58.8-89.3	18 (75.04)	53.3-90.2
Computed tomography arterioportal angiography	30 (88.2)	72.5-96.7	23 (95.8)	78.9-99.9

Gd-EOB-DTPA: Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid; HCC: Hepatocellular carcinoma.

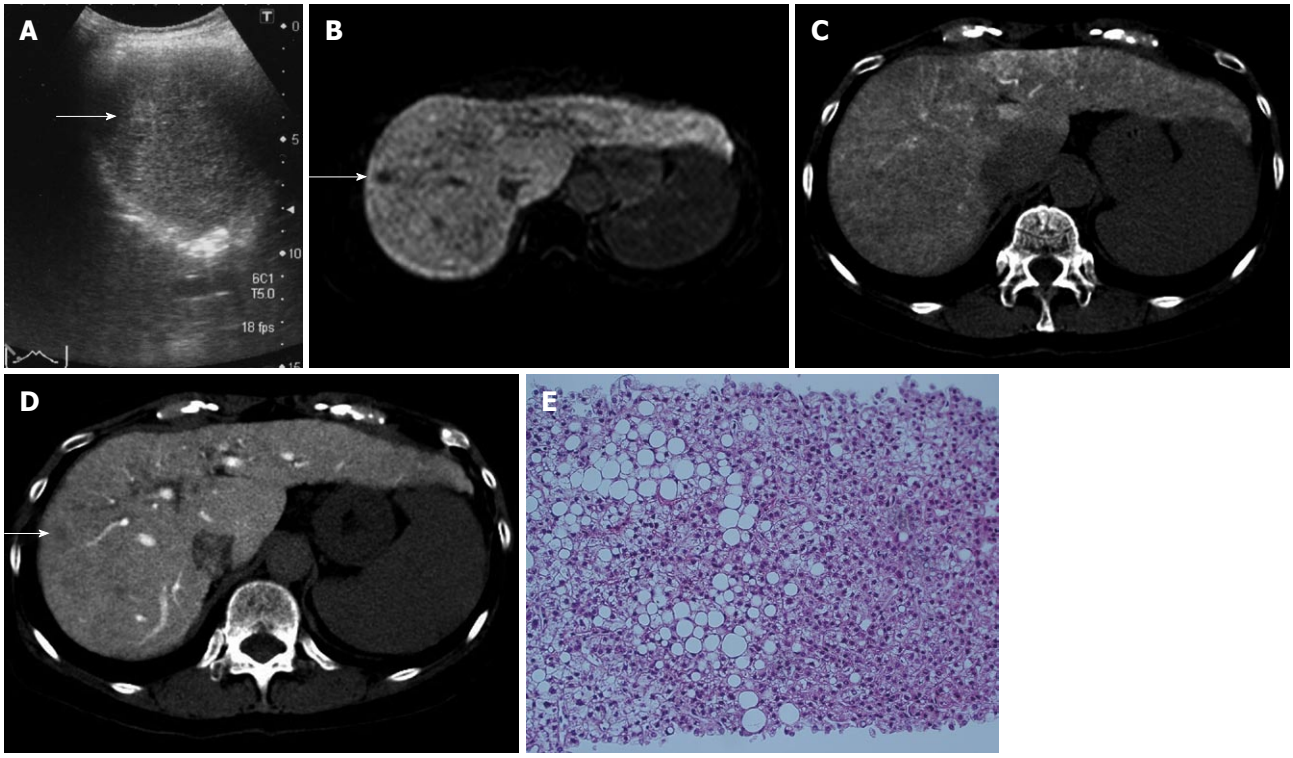

Figure 1 Case No. 1: detection by gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid magnetic resonance imaging and computed tomography arterioportal angiography. Imaging and histological findings of the nodule in segment eight. A: Ultrasonography (US) reveals a 12 mm hyperechoic nodule (arrow); B: Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid magnetic resonance imaging reveals a defect (arrow) in the hepatobiliary phase; C, D: Computed tomography during arteriography reveals isodensity (C) and computed tomography during arterial portography (D) reveals a perfusion defect (arrow); E: The nodule is diagnosed as moderately-differentiated hepatocellular carcinoma by US-guided biopsy.

Gd-EOB-DTPA MRI revealed no hypervascularity in the arterial phase, but a defect in the hepatobiliary phase (Figure 1B). CTA revealed isodensity (Figure 1C), and CTAP a perfusion defect (Figure 1D). US-guided biopsy revealed moderately-differentiated HCC (Figure 1E).

Case No. 2: Detection by Gd-EOB-DTPA MRI: In a 74-year-old woman with HCV-related liver cirrhosis (AFP 7.1 ng/mL, PIVKA Ⅱ 42 mAU/mL), US revealed an 8 mm hyperechoic nodule in segment six (Figure 2A). Sonazoid CEUS revealed no hypervascularity in the early vascular phase and no defect in the Kupffer phase. CECT revealed isodensity in both the arterial phase and the equilibrium phase. MRI revealed isointensity. Gd-EOB-DTPA MRI revealed no hypervascularity in the early phase, but disclosed a defect in the hepatobiliary phase (Figure 2B). CTA revealed no hypervascularity and CTAP no perfusion defect. US-guided biopsy revealed well-differentiated HCC (Figure 2C).

DISCUSSION

Confirmation of arterial hypervascularity by three imaging modalities (triphasic CT, triphasic MRI, and CEUS), even in the absence of a significant (> 400 ng/mL) rise in AFP, is recommended by the European Association for the Study of the Liver (EASL) as diagnostic criteria for HCC nodules larger than 2 cm in patients with cirrhosis[9]. These recommendations for the management of HCC provide a rational approach to the problem but leave some areas of uncertainty, particularly those regarding the interpretation of discordant vascularity, the use of imag-

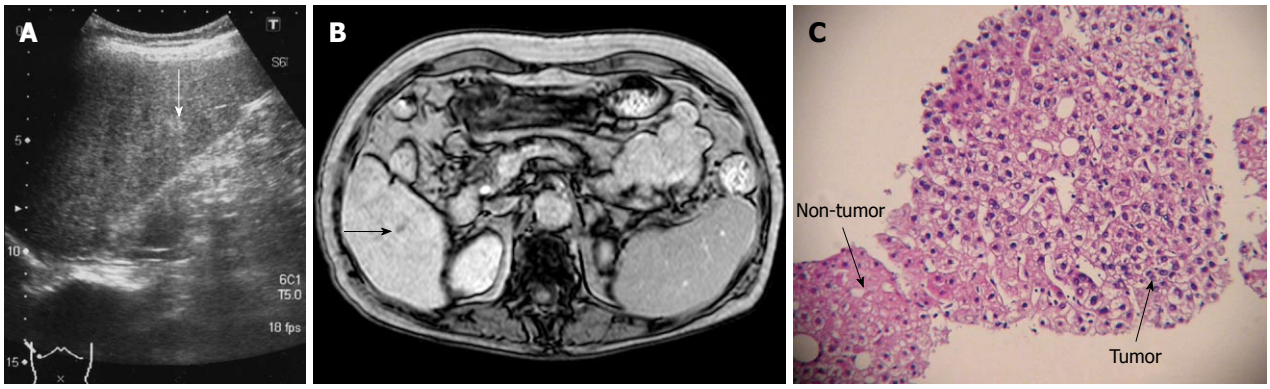

Figure 2 Case No. 2: detection by gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid magnetic resonance imaging. Imaging and histological findings of the nodule in segment six. A: Ultrasonography (US) reveals an 8 mm hyperechoic nodule (arrow); B: Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid magnetic resonance imaging reveals a defect (arrow) in the hepatobiliary phase; C: The nodule is diagnosed as well-differentiated hepatocellular carcinoma by US-guided biopsy, showing cellularity more than two-fold that of the non-tumorous area.

ing techniques in nodules smaller than 2 cm, the meaning of truly hypovascular nodules, and the management of those diagnosed with LGDN or HGDN at guided biopsy.

The American Association for the Study of Liver Diseases[2] recommends that the diagnosis of HCC should be made without biopsy when characteristic arterial vascularization and venous washout are observed on three imaging modalities: triphasic CT scan, triphasic MRI and contrast-enhanced harmonic US.

Nevertheless, these recommendations have not been tested and validated except by Bolondi *et al*[10] and Forner *et al*[11]. According to Bolondi *et al*[10], the noninvasive EASL criteria with CEUS and CECT for the diagnosis of HCC are satisfied in only 44% of nodules smaller than 2 cm in cirrhosis. Forner *et al*[11] reported that the diagnostic sensitivity of MRI and CEUS in the diagnosis of HCC (smaller than 2 cm) is 67%.

The main characteristics of Sonazoid, a newly introduced second-generation US contrast agent exclusively approved in Japan in 2007, are that it facilitates real-time blood flow images at low acoustic power and stable Kupffer phase imaging from 10 to 120 min after its injection. In vascular imaging, Sonazoid is considered more effective than Levovist and easy to use; it allows visualization, even with the use of non-high-end equipment and, therefore, reduces dependence on the operator's skills/equipment, all of which may promote the widespread use of CEUS. As stated earlier, Sonazoid CEUS provides very stable postvascular phase images for up to 60-120 min[12], which has resulted in the invention of the breakthrough method, defect reperfusion imaging that is an innovative technology that will greatly change the daily practices of HCC management. In our study, the diagnostic sensitivity of Sonazoid CEUS was 67.6% in all HCC, and 79.2% in moderately-differentiated HCC.

Kudo *et al*[8,13,14] have recently developed defect reperfusion imaging (using the properties of very stable Kupffer phase images and real-time fine blood flow images obtained with Sonazoid) for typical HCC, which is depicted by CT but not by B mode scanning. The method is a breakthrough for accurate localization and treatment guidance[8]: dramatic resolution of many limitations in the diagnosis and treatment of HCC, such as detection of small HCCs[15], evaluation of treatment response[16], and needle insertion guidance; additionally, detection is even more sensitive than with MDCT[15].

A newly introduced contrast agent, Gd-EOB-DTPA, approved in Japan in 2008, is a hepatocyte-specific MRI contrast medium with a different mechanism that utilizes neither dynamic nor Kupffer cell imaging. It is useful in cases which would be difficult to diagnose by techniques such as dynamic MRI or SPIO-MRI. Typical HCC shows high intensity with Gd-EOB-DTPA in the arterial-dominant phase and low intensity in the portal-dominant phase and thereafter. The imaging diagnosis of HCC can be made approximately 10-20 min after the injection of Gd-EOB-DTPA. In our study, the diagnostic sensitivity of Gd-EOB-DTPA MRI was 76.5% in all nodules and 75.0% in moderately-differentiated HCC.

Previously, we had concluded that CT arterioportal angiography was superior to CECT and Gadolinium-enhanced MRI for diagnosing HCC in nodules smaller than 2 cm[7]. In this study, the diagnostic sensitivity of CT arterioportal angiography was 88.2% in all nodules and 95.8% in moderately-differentiated HCC. We observed a significant difference between CECT and CT arterioportal angiography ($P < 0.05$) in all nodules. However, there was no difference between Sonazoid CEUS, Gd-EOB-DTPA MRI, and CT arterioportal angiography. The combined sensitivity of Sonazoid CEUS and Gd-EOB-DTPA MRI in all nodules was 94.1%, due to improvement in the diagnostic capabilities of Sonazoid CEUS and Gd-EOB-DTPA MRI. This improvement in these two imaging modalities with the use of the newly introduced contrast agents provided higher sensitivity for the diagnosis of nodules smaller than 2 cm with Sonazoid CEUS and Gd-EOB-DTPA MRI than with Sonovue CEUS and CECT reported by Bolondi *et al*[10], or with Sonovue CEUS and Gadolinium-enhanced MRI reported by Forner *et al*[11].

These results, considered together with the invasiveness of CT arterioportal angiography, suggest that the principal diagnostic modality for HCC smaller than 2 cm

should be changed from CT arterioportal angiography to Sonazoid CEUS and Gd-EOB-DTPA MRI.

COMMENTS

Background

In spite of the recent advances in imaging techniques, the definitive diagnosis of nodular lesions detected by imaging modalities in the liver with cirrhosis remains a critical challenge for clinicians. The issue is particularly complicated for small (1-2 cm) nodules, many of which may be preneoplastic with uncertain malignant potential. We undertook this study to evaluate the effectiveness of imaging techniques in the diagnosis of hepatocellular carcinoma (HCC) smaller than 2 cm on the basis of histologic findings. Four imaging modalities were compared: contrast-enhanced computed tomography (CECT), Sonazoid contrast-enhanced ultrasonography (CEUS), gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) magnetic resonance imaging (MRI), and CT arterioportal angiography.

Research frontiers

The authors compared the imaging results with histology and evaluated the diagnostic sensitivity of the 4 imaging modalities.

Innovations and breakthroughs

Previously, the authors had concluded that CT arterioportal angiography was superior to CECT and gadolinium-enhanced MRI for diagnosing HCC in nodules smaller than 2 cm. In this study, the sensitivity of diagnosing 34 HCCs smaller than 2 cm was 52.9% by CECT; 67.6% by Sonazoid CEUS; 76.5% by Gd-EOB-DTPA MRI; and 88.2% by CT arterioportal angiography. A significant difference was observed between CECT and CT arterioportal angiography ($P < 0.05$). There was no difference between Sonazoid CEUS, Gd-EOB-DTPA MRI, and CT arterioportal angiography, and the combined sensitivity of Sonazoid CEUS and Gd-EOB-DTPA MRI was 94.1%, due to improvement in the diagnostic sensitivity of Sonazoid CEUS and Gd-EOB-DTPA MRI. This improvement in these two imaging modalities with the use of the newly introduced contrast agents provided higher sensitivity for the diagnosis of nodules smaller than 2 cm with Sonazoid CEUS and Gd-EOB-DTPA MRI than with Sonovue CEUS and CECT reported by Bolondi *et al*, or with Sonovue CEUS and Gadolinium-enhanced MRI reported by Forner *et al*.

Applications

These results, considered together with the invasiveness of CT arterioportal angiography, suggest that the principal diagnostic modality for HCC smaller than 2 cm should be changed from CT arterioportal angiography to Sonazoid CEUS and Gd-EOB-DTPA MRI.

Peer review

The major strength of the study is that there are many patients with small tumors. The patients have also been applied to new equipment and new contrast substances. It's a very interesting paper.

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BRIEF ARTICLE

Effect of *Arctium lappa* L. in the dextran sulfate sodium colitis mouse model

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Abstract

AIM: To analyze the possible protective role of *Arctium lappa* L. (AL) in a murine model of ulcerative colitis (UC).

METHODS: BALB/c mice were administered 100 mg/kg AL powder orally each day. After 7 d, colitis was induced by administration of dextran sulfate sodium (DSS) (5% W/V) in drinking water for a further 8 consecutive days. Diarrhea and bloody stools as well as colonic histology were observed. The level of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in colonic sections were detected by immunohistochemistry.

RESULTS: There were significant differences in mean body weight values and disease activity indices between controls and AL-treated animals. Moreover, the histological findings showed that AL treatment can prevent mucosal edema, submucosal erosions, ulceration, inflammatory cell infiltration and colon damage. In addition, immunohistochemistry analysis showed that the levels of the inflammatory cytokines, IL-6 and TNF-α were also decreased in AL-treated groups.

CONCLUSION: We suggest that AL can prevent intestinal damage and decrease inflammatory cytokines in mice with DSS-induced colitis. Thus, AL could prove to be a useful food for UC.

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Key words: *Arctium lappa* L.; Colitis; Cytokine; Inflammatory bowel disease; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis and Crohn's disease are chronic inflammatory bowel diseases (IBD). Although these two diseases have some characteristics in common, the exact etiology and pathogenesis of these disorders remain unclear. How-

ever, in recent years, epidemiologic and genetic studies in man and particularly, in IBD-related animal models, have suggested that a combination of genetic susceptibility factors and altered immune response driven by microbial factors in the enteric environment contributes to the initiation and chronification of these diseases. On the other hand, there is substantial evidence that intestinal inflammation is likely to depend on cytokines including interleukin (IL)-1, IL-6, IL-12p40, IL-23p19, IL-10, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)[1]. When chosen appropriately, animal models can be used to investigate pathophysiological mechanisms and are valuable tools for testing emerging therapeutic strategies in the preclinical phase. Hence, animal models of colitis resemble some of the important immunological and histopathological aspects of IBD in humans.

The dextran sulfate sodium (DSS) challenge-induced ulcerative colitis (UC) model has been well characterized morphologically and biochemically. DSS induces an acute colitis characterized by bloody stools, ulcerations and infiltration of inflammatory cells[2]. Histologically, DSS produces submucosal erosions, ulceration, inflammatory cell infiltration and crypt abscesses as well as epithelioglandular hyperplasia. It is generally believed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier. The lumen bacteria induce the production of inflammatory cytokines, IL-6 and TNF-α, and cause colitis. Hence, the DSS-induced colitis model is particularly useful for studying the contribution of inflammatory mechanisms in colitis.

The "Gobo", the roots of edible burdock [*Arctium lappa* L. (AL)], is a food in Asia and contains a higher amount of polysaccharides and residues than other vegetables and is easily obtained all year round. AL has been extensively analyzed due to its reserve and cell-wall polysaccharides[3]. *Arctium lappa* has been reported to have antimicrobial activity[4] as well as antioxidant activity[5]. The chloroform extract fraction of the roots from AL protects animals from chronic gastric ulceration by reducing gastric acid secretion *via* inhibition of gastric H^+, K^+-ATPase[6]. However, the effects of AL on colitis are not fully understood.

The AL extract was able to significantly reduce the release of inflammatory mediators through inhibition of degranulation and cys-leukotriene release[7]. This research indicated that AL has anti-inflammatory activity and may have therapeutic or prophylactic effects on colitis. A mouse model of distal colitis induced by DSS that histologically resembles human UC was used in this study to evaluate the anti-colitis activity of AL powder.

MATERIALS AND METHODS

Preparation of burdock (*Arctium lappa* L.) powder

Fresh burdock (Cheer Mean Industrial Co., Ltd.) was sliced with a hand knife and spread in an oven (Gallenkamp, UK) at 60℃ for 9 h. The oven-dried AL was then crushed into tiny pieces in a mortar and then pulverized in an osterizer blender (Hitachi, Tokyo, Japan) to produce powdered AL.

Animals

BALB/c mice (12 wk old, 24-28 g) were bred under standard conditions and maintained in a 12-h light/12-h dark cycle at 22℃ plus or minus 1℃ and given food and tap water *ad libitum* in accordance with Taiwan Office Regulations.

Induction of colitis

Colitis was induced by modification of the method of DSS-induced colonic inflammation as previously described[2]. Colitis was induced in BALB/c mice by adding DSS (molecular weight: 36-50 kDa; MP Biomedicals) to drinking water at a level of 5% during the experiment period. Water consumption was comparable between the different groups. Mice were monitored daily for weight loss as well as signs of rectal bleeding and diarrhea. The animals received 200 μL of water (control group) or 100 mg/kg per 200 μL AL powder (treatment group) orally each day. After 7 d, colitis was induced by administration of DSS (5% W/V) in drinking water for a further 8 consecutive days. At day 8 of DSS administration, the mice were sacrificed and sections were taken from the colon for histological assessment.

Assessment of DSS colitis

Body weight loss, stool consistency, and blood in the stool were monitored daily to assess the severity of colitis. Body weight, rectal bleeding and stool consistency were monitored daily. Blood in the stool was scored as 0, normal; 2, slight bleeding; and 4, gross bleeding. Diarrhea was scored as 0, normal; 2, loose stools; and 4, watery diarrhea.

Histopathological analysis

For microscopic histological evaluation, formalin-fixed tissues were embedded in paraffin and 5 μm sections were stained with hematoxylin and eosin and evaluated using light microscopy by a pathologist in the Animal Hospital of NPUST who was blinded to the experimental protocol.

Immunohistochemistry staining

For immunohistochemistry evaluation, formalin-fixed tissues were embedded in paraffin and 5 μm sections were preincubated in PBS with 20% goat serum and 0.05% Saponin (Sigma) or 0.5% Triton X-100 for 1 h, and then incubated overnight with primary antibodies diluted in PBS with 2% goat serum at room temperature (10 μg/mL). The following antibodies were used: rat anti-mouse TNF-α (Southern Biotech, Birmingham, AL, USA), rat anti-mouse IL-6 (Southern Biotech, Birmingham, AL, USA). The second antibody step was performed using HRP-conjugated species-specific antibodies (Jackson ImmunoResearch, West Grove, PA, USA), 1:200 for 20-40 min developed in DAB for 10 min. Finally, sections were counter-stained with hematoxylin and mounted. Stained sections were examined and photographed using light microscopy by a pathologist in the Animal Hospital of NPUST who was blinded to the experimental protocol.

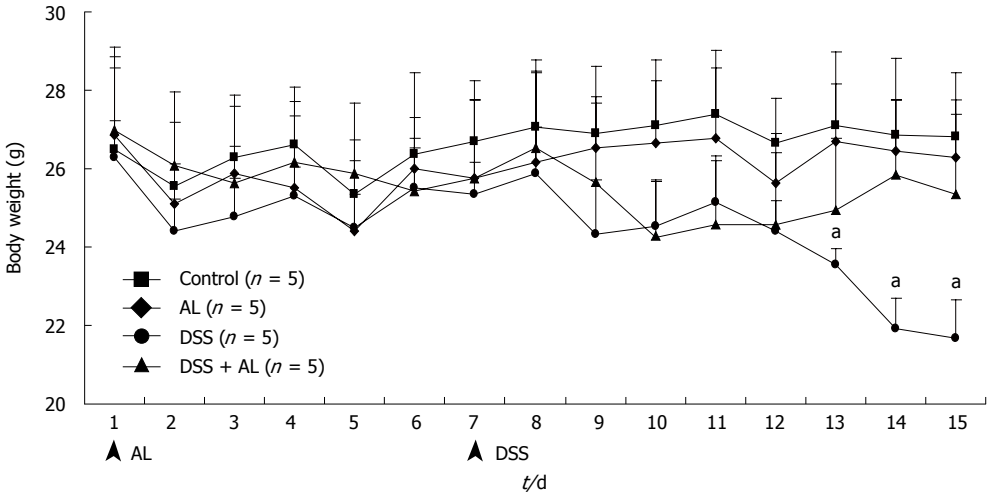

Figure 1 The effects of *Arctium lappa* L. in dextran sulfate sodium-induced colitis in mice. Weight lost in *Arctium lappa* L. (AL) or control mice exposed to 5% dextran sulfate sodium (DSS). Values are mean ± SD; $n = 5$ in each group. Data were analyzed by Student's *t* test. Statistical significance is based on the difference between DSS only-treated mice and DSS plus AL-treated mice ($^aP < 0.05$).

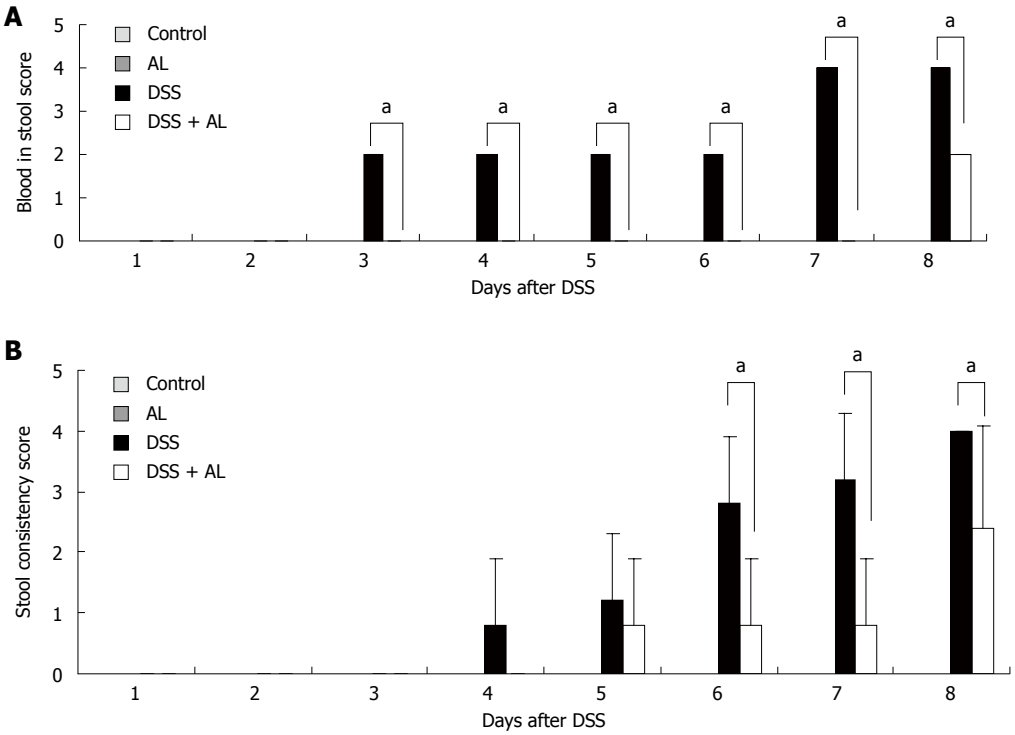

Figure 2 The disease activity index in mice. Colonic disease activity indices were scored as described in Materials and Methods. Data are expressed as mean ± SD; $n = 5$. Statistical comparison between the 2 groups was performed using the Student's *t* test. The level of significance was set to $P < 0.05$ ($^aP < 0.05$). AL: *Arctium lappa* L.; DSS: Dextran sulfate sodium.

Statistical analysis

Data are expressed as mean ± SD groups of data (histological scores, body weight) were analyzed using Student's *t* test.

RESULTS

Oral administration of AL prevents body weight loss in the DSS-induced colitis model

Symptomatic colitis parameters such as weight loss and disease activity index (DAI) score were monitored each day. Mice given 5% DSS in their drinking water for 7 d developed symptoms of colitis without mortality. Compared with vehicle-treated controls, the DSS-administered groups had significantly decreased body weight (Figure 1). Oral AL treatment obviously improved weight loss. Fecal characteristics and fecal occult blood were evaluated individually. Macroscopic examination revealed that no significant morphological changes were observed following water or AL administration. The DSS-administered

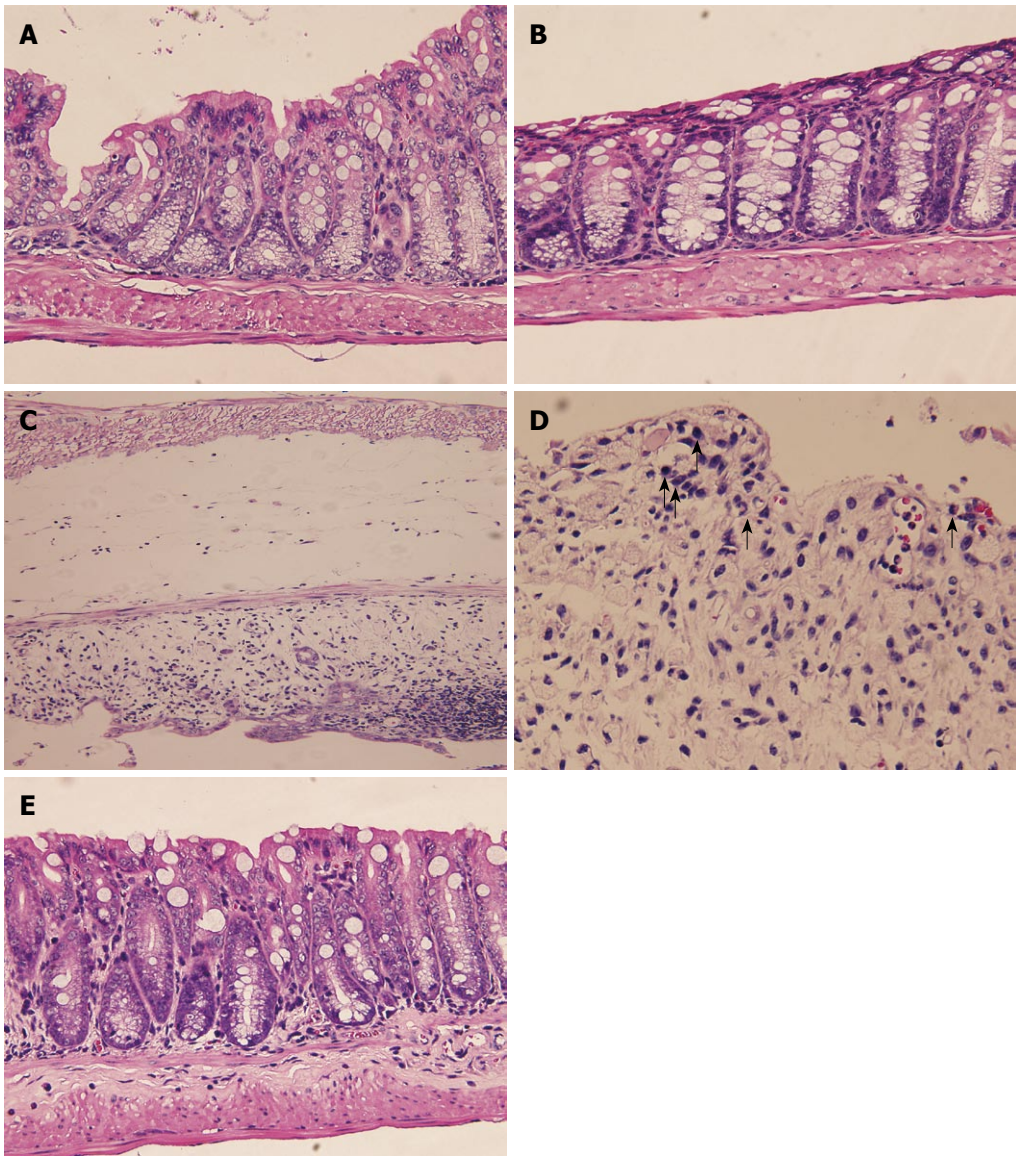

Figure 3 Histological analysis of mice. A: Colon section from control (water treated) animals showing normal colon tissue architecture (HE, × 400); B: Colon section from *Arctium lappa* L. (AL)-treated animals showing normal histological architecture with no inflammatory cell infiltration, edema or crypt abscesses (HE, × 400); C, D: Colon section from dextran sulfate sodium (DSS)-treated animals showing severe submucosal erosion with edema, ulceration, inflammatory cell infiltration (indicated with arrows) and crypt abscesses as well as epithelioglandular hyperplasia (C: HE, × 200, D: HE, × 400); E: Colon section from animals challenged with DSS after prior treatment with AL showing normal histological architecture with slight inflammatory cell infiltration and no submucosal edema or abnormality of crypt cells (HE, × 400). All the results are representative of 3 independent experiments.

groups had loose stools or diarrhea, occult or gross rectal bleeding which markedly increased the DAI scores (Figure 2A and B). Oral administration of AL before DSS obviously decreased colonic bloody diarrhea.

Microscopically, normal colonic tissue architecture is shown in Figure 3A. The control group given water showed no histological alterations as seen in Figure 3C. Similarly, mice given AL exhibited virtually the same normal histology with no inflammatory cell infiltration, edema or crypt abscesses (Figure 3B). Severe submucosal edema, erosion, ulceration, inflammatory cell infiltration and crypt abscesses as well as epithelial glandular hyperplasia were observed in the mucosa of DSS-treated animals (Figure 3C and D). Prior treatment with AL before the DSS challenge produced a slight inflammatory reaction in the colonic mucosa with no submucosal edema or crypt cell abnormalities (Figure 3E). On the other hand, many polymorphonuclear cells and mononuclear cells infiltrated the apical side of DSS-treated mouse intestine (Figure 3D arrows).

AL administration inhibited inflammatory mediator production in colonic tissues

IL-6 and TNF-α are considered important inflammatory mediators that play a key role in the pathogenesis of IBD. To determine the effect of AL on major inflammatory cytokines in the colon, we determined the levels of IL-6 and TNF-α (Figure 4). After 8 d of DSS administration, the levels of IL-6 and TNF-α increased significantly. AL administration prevented significant elevations in IL-6 and TNF-α at day 8. These results indicated that AL has anti-inflammatory effects in the DSS-induced colitis mouse model.

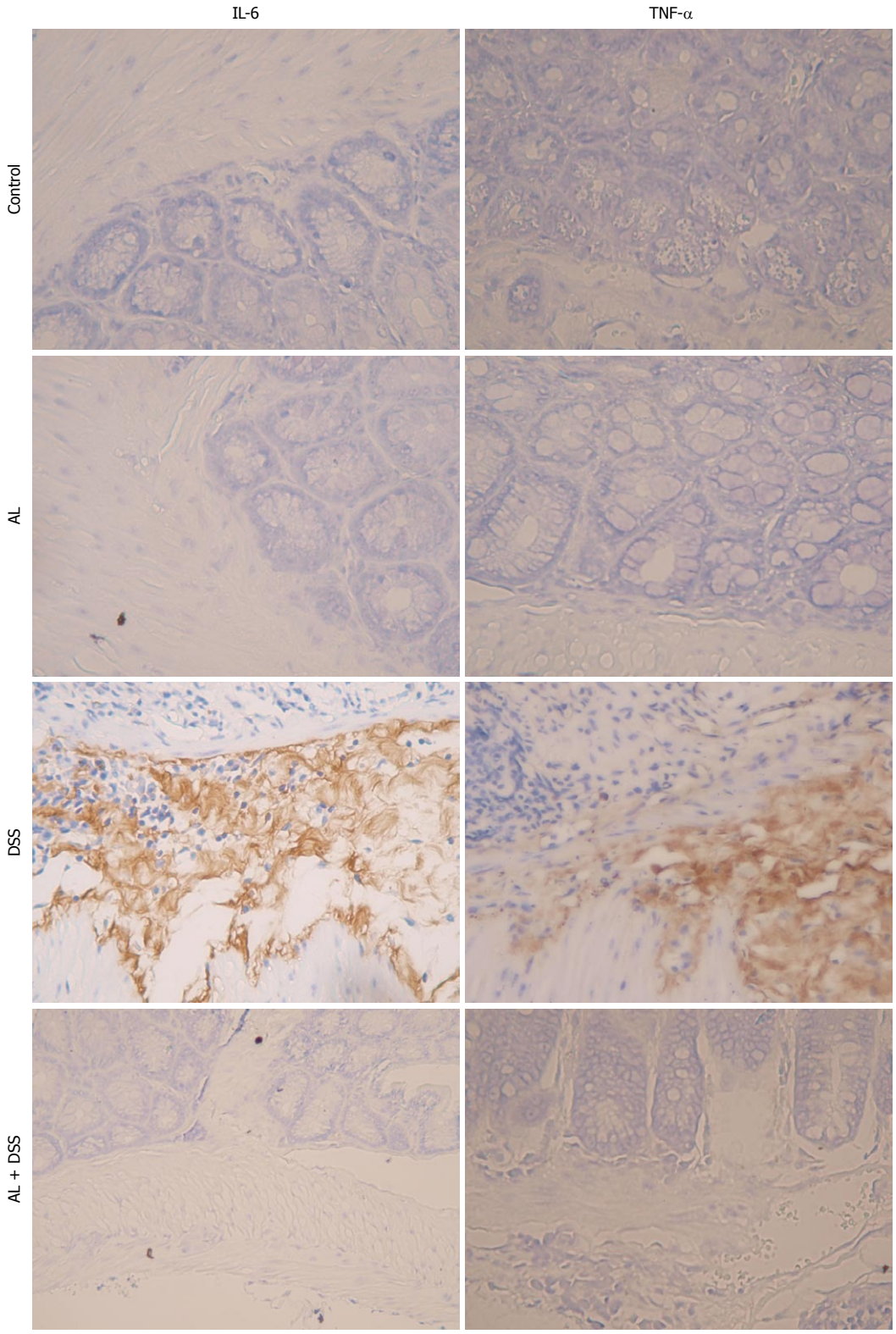

Figure 4 The level of inflammatory cytokine expression in mouse colon sections (HE, × 400). The expression level of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the colonic samples was determined using anti-IL-6 and TNF-α monoclonal antibody. AL: *Arctium lappa* L.; DSS: Dextran sulfate sodium.

DISCUSSION

The results presented herein clearly indicate that AL efficiently suppresses DSS-induced colitis in mice without any adverse effects. In this study, we showed that AL prevents body weight loss and an increase in DAI scores in mice with DSS-induced colitis. Furthermore, AL alleviates the symptoms of DSS-induced colitis and improves colitis-induced histological damage by reducing the infiltration of immune cells and the production of various inflammatory cytokines, such as IL-6 and TNF-α. These results suggest that AL has potentially

clinically useful anti-inflammatory and therapeutic effects in IBD.

The steroid agent, glucocorticoid, has been used clinically as an anti-inflammatory drug for some time for the treatment of IBD, but is frequently associated with serious side effects, such as liver damage, cancers, stroke, and growth inhibition, and there is a long-standing dilemma regarding the use of clinical steroid anti-inflammatory therapy[8,9]. New anti-inflammatory drugs, as well as prophylactic or therapeutic foods are important for IBD patients. Comprehensive data in previous reports show that IBD are fairly common chronic inflammatory conditions of the gastrointestinal tract. The main pathologic feature of IBD is an infiltration of polymorphonuclear neutrophils and mononuclear cells into the intestinal tissues. Both IL-6 and TNF-α play crucial roles in the DSS-mediated inflammatory response in mice and pigs[10,11]. Mice administered oral AL showed less infiltration of immune cells into the colon epithelium (Figure 3E). AL decreased inflammatory cytokine production and reduced elevated immune cell numbers. The therapeutic effect exhibited by AL may thus be explained by its ability to blunt inflammatory cytokine production in DSS-induced murine IBD.

The mouse model of distal colitis induced by DSS histologically resembles human ulcerative colitis[12]. The exact mechanism of DSS-induced mucosal injury is not fully understood, but a topical toxic effect of DSS on colonic epithelial cells has been proposed[13]. This breach of barrier function would likely result in increased uptake of luminal antigens (bacteria and bacterial products) as well as activation of lamina propria immune cells and the inflammatory response[1,12,14]. Recent studies pointed out the possible effect of inulins in plant materials on the functions of the immune system in relation to the regulation of differentiation and proliferation of intestinal epithelial cells[15-17]. AL is an inulin-rich food and is easily obtained all year round. Moreover, inulin can also stabilize the gut mucosal barrier[18]. In our data, the oral administration of AL powder maintained the architecture of colonic intestinal cells and the mucosal layer (Figure 3).

Oral (1% in drinking water, or 400 mg/d) administration of inulin in rats was found to ameliorate DSS-induced colitis[19]. It was found that daily administration of inulin by the oral route induced an acidic environment (pH < 7.0) from the cecum to the left colon and increased lactobacilli counts. Recently, an increase in the number of fecal bifidobacteria and lactobacilli in the cecal content of rats was reported[20]. These results indicated that a significant increase in propionic, succinic and butyric acid was observed in inulin-fed Sprague-Dawley rats. The authors postulated that oral inulin reduces the severity of DSS-induced colitis mediated by modification of the intracolonic milieu.

In addition to inulin, the role of chlorogenic acid should not be ignored. Chlorogenic acid, one of the most common polyphenols in the human diet, inhibits staphylococcal exotoxin-induced production of IL-1β, TNF, IL-6, INF-γ, monocyte chemotactic protein-l, macrophage inflammatory protein (MIP)-lα, and MIP-lβ in human peripheral blood mononuclear cells[21]. Chlorogenic acid also inhibits lipopolysaccharide (LPS)-induced inflammatory response in RAW 264.7 cells. Shan *et al*[22] (2009) indicated that chlorogenic acid significantly decreased LPS-induced up-regulation of cyclooxygenase-2 at the protein and mRNA levels resulting in the inhibition of prostaglandin E2 release from LPS-treated RAW 264.7 cells. Further studies showed that LPS-induced activation of nuclear factor-κB and c-JunN-terminal kinase-c-Jun-activator protein-1 pathway were significantly suppressed by chlorogenic acid.

According to these studies, we suggest that both inulins and chlorogenic acid in burdock powder play an important role in the AL-mediated prophylactic effect in DSS-induced colitis, however, the exact mechanisms still need to be investigated further. AL may have multiple functions including anti-inflammatory activity, modification of the content of colonic microorganisms, protection of epithelial cells and stabilization of the mucosal barrier.

In conclusion, oral AL improves body weight loss, histological scores, maintains the colon architecture, and decreases the release of inflammatory mediators in DSS-induced colitis in mice. Our findings may be relevant for future pharmacological or dietary interventions in patients with ulcerative colitis.

COMMENTS

Background

Ulcerative colitis and Crohn's disease (CD) are chronic inflammatory bowel diseases (IBD). Although these two diseases have some characteristics in common, the exact etiology and pathogenesis of these disorders remain unclear. Research has indicated that *Arctium lappa* L. (AL) has anti-inflammatory activity and may have therapeutic or prophylactic effects on colitis. However, the effects of AL on colitis are not fully understood.

Research frontiers

In this study, the authors examined whether AL had an effect on colitis. A mouse model of distal colitis induced by dextran sulfate sodium (DSS) that histologically resembles human ulcerative colitis (UC) was used in this study to evaluate the anti-colitis activity of AL powder.

Innovations and breakthroughs

The results presented herein clearly indicate that AL efficiently suppresses DSS-induced colitis in mice without any adverse effects. In this study, the authors showed that AL prevents body weight loss and an increase in disease activity index scores in mice with DSS-induced colitis. Furthermore, AL alleviates the symptoms of DSS-induced colitis and improves colitis-induced histological damage by reducing the infiltration of immune cells and the production of various inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α.

Applications

These findings may be relevant for future pharmacological or dietary interventions in patients with ulcerative colitis.

Peer review

This study investigated the effect of AL, a vegetable commonly used in Asian, on DSS-induced colitis in mice. It showed that pretreatment of the animals with AL exerted some protective effect on DSS-induced gut damage. This is a study with some practical value and clinical significance.

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BRIEF ARTICLE

Conservative management of chronic gastric volvulus: 44 cases over 5 years

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Abstract

AIM: To investigate clinical outcomes of patients with chronic gastric volvulus (GV) who were managed conservatively over a 5-year period.

METHODS: A total of 44 consecutive patients with chronic GV, as diagnosed by barium study between October 2002 and July 2008 were investigated. All of these patients received conservative management initially without anatomical correction. Their clinical manifestations, diagnostic work-ups, and clinical outcomes were analyzed. We sought to identify independent risk factors for poor outcome by using the Cox proportional hazards model.

RESULTS: The enrolled patients were predominantly male (n = 37, 84%) and of advanced age (median: 71 years old, interquartile range: 57.5-78 years). Abdominal pain and fullness were the most common presentations. During the follow-up period (median: 16 mo, up to 69 mo), there was no severe complication, but symptomatic recurrence was noted in 28 patients (64%). Only one patient turned to elective surgery for frequent symptoms. Peritoneal adhesion was the only independent risk factor associated with recurrence (hazard ratio: 2.58, 95% CI: 1.08-6.13, P = 0.033).

CONCLUSION: Symptomatic recurrence of chronic GV is very common although serious complications infrequently occur with conservative management. Peritoneal adhesion is independently associated with recurrence.

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Key words: Conservative treatment; Gastric volvulus; Upper gastrointestinal tract; Barium study; Peritoneal adhesion

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INTRODUCTION

Gastric volvulus (GV) is a rare condition that is defined as pathological rotation of the stomach. Laxity of gastric attachment constitutes the pathophysiological basis for the development of GV[1]. According to the axis of rotation, GV can be classified into organoaxial (long axis

connecting the gastroesophageal junction to the pylorus), mesenteroaxial (short axis bisecting the lesser and greater curvature), and combined type[2].

Although GV has been reported in all ages, it is more often diagnosed in elderly patients[3-6]. Clinical presentations of GV vary widely from incidental radiographic findings to life-threatening catastrophes, depending on its rapidity of onset, degree of rotation, and subsequent extent of obstruction[7-9]. Barium study may be considered as the diagnostic tool of choice, because of its accuracy in demonstrating the abnormal rotation and in estimating the amount of obstruction[4,6,10,11]. Definitive treatment of GV includes reduction of the twisted stomach, percutaneous endoscopic gastrostomy, gastropexy, and repair of the predisposing structural defects[4-6,12-14].

Acute GV usually presents with progressive abdominal (intra-abdominal volvulus) or chest (intra-thoracic volvulus) pain, severe vomiting, and epigastric distention[15]. The classical Borchardt's triad, which comprises severe epigastric pain, unproductive retching, and inability to pass a nasogastric tube, represent total gastric outlet obstruction[16]. Acute GV can lead to strangulation, necrosis, and perforation of the stomach, and should be regarded as a surgical emergency[7]. In contrast, chronic GV might be completely asymptomatic or manifest with recurrent non-specific symptoms such as vague abdominal pain, abdominal fullness, chest pain, retching, acid reflux, and dysphagia[8,9]. The natural history of chronic GV remains poorly understood. Although theoretically chronic GV can transform into acute volvulus, the actual incidence is unknown. Clinical outcomes of chronic GV patients managed conservatively without anatomical correction has not been investigated.

The aim of the present study was to investigate the manifestations, performance of diagnostic modalities, and specifically clinical outcomes of a large chronic GV cohort who received conservative management.

MATERIALS AND METHODS

Setting and patients

This was a retrospective study of consecutive chronic GV patients, which was conducted in a tertiary medical center that serves a metropolitan area of 11 million inhabitants in northern Taiwan (Taipei Veterans General Hospital). The Institutional Review Board of the hospital approved the study protocol. We reviewed the medical records and computerized database of all patients with a diagnosis of GV between October 2002 and July 2008. Eligible patients were identified by the following inclusion criteria: (1) unequivocal findings of a twisting stomach on upper gastrointestinal (GI) barium study; (2) age $\geqslant$ 20 years; (3) no urgent endoscopic or surgical intervention within 1 mo after diagnosis; and (4) no coexisting malignancy in the upper GI tract. An experienced radiologist (CKC) reviewed each barium study to confirm the diagnosis of GV.

Patients were excluded from analysis if they were not frequently followed up at least every 3 mo after diagnosis. We excluded those who received endoscopic or surgical management within 1 mo after diagnosis, to assure our study subjects were all chronic, instead of acute GV cases. In all enrolled subjects, upper GI endoscopy and computed tomography were performed for regular indication at the discretion of treating physicians. Conservative treatment of chronic GV was defined as no anatomical reduction or correction by endoscopic or surgical procedures, and comprised mainly prokinetic agents, anti-secretory therapy, and life style as well as diet modification.

Outcome measures and data collection

The primary endpoint of this study was clinical outcome during the follow-up period. We defined three categories of outcome: (1) severe complications including strangulation or perforation of the stomach, complete obstruction, and hypovolemic shock; (2) recurrence of symptoms as documented on the medical records; and (3) no or negligible recurrent symptoms throughout the follow-up period. Pertinent demographic and clinical data of the eligible patients were abstracted, which included age, sex, presenting symptoms, time interval between symptom onset and diagnosis, comorbidity, and findings of other diagnostic modalities (plain radiograph, sonography, and endoscopy). In addition, we determined if predisposing factors for secondary GV were present. Paraesophageal hernia was defined as a stomach protruding through the esophageal hiatus, with the gastroesophageal junction in a normal position at the level of the diaphragm[17], and eventration of the diaphragm as abnormal elevation of an intact hemidiaphragm[18]. Peritoneal adhesion was defined to be present if there was previous abdominal or thoracic surgery, or history of peritonitis[19].

Statistical analysis

Continuous variables were expressed with median and interquartile range (IQR) and categorical variables with proportions. Mann-Whitney test was used to compare continuous variables and χ^2 test was used for univariate analysis of proportions. Fisher's exact test was applied in the case of expectant values below 10. All statistical analyses were conducted using SPSS for Windows version 14 (SPSS Inc., Chicago, IL, USA), and SAS version 9.1 (Cary, NC, USA). Symptom recurrence was estimated using the Kaplan-Meier method and compared by the log-rank test from the time of diagnosis. Independent risk factors predictive for symptom recurrence were determined by the Cox proportional hazards model. For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Fifty-five chronic GV patients were identified by the inclusion criteria, but 11 were excluded from analysis because they were not closely followed up with an interval of at least 3 mo. We contacted these excluded patients or their family by telephone to document the ultimate clinical outcomes. Two had died from unrelated diseases, and no surgical intervention was reported in any of these

Table 1 Demographic and clinical characteristics of the study subjects

	Enrolled patients (*n* = 44)
Male sex, *n* (%)	37 (84)
Age, yr (IQR)	71 (57.5-78)
Duration between symptom onset and diagnosis, mo (IQR)	1 (0.5-6)
Symptoms, *n* (%)	
Abdominal pain	25 (57)
Abdominal fullness	24 (55)
Non-cardiac chest pain	18 (41)
Nausea	17 (39)
Heartburn	10 (23)
Dyspnea	9 (20)
Acid regurgitation	9 (20)
Dysphagia	8 (18)
Vomiting	5 (11)
Hematemesis	2 (5)
Axis of volvulus, *n* (%)	
Organoaxial	42 (95)
Mesenteroaxial	1 (2)
Mixed	1 (2)
Previous thoracic or abdominal surgery, *n* (%)	10 (22)
Secondary GV, *n* (%)	26 (59)
Duration of follow-up, mo (IQR)	16 (6-36)
Recurrence-free duration, mo (IQR)	3 (1-10.5)

IQR: Interquartile range; GV: Gastric volvulus.

Table 2 Probable etiology of secondary gastric volvulus *n* (%)

	Patients with secondary GV (*n* = 26)
Choledocholithiasis	9 (20)
Diaphragm defect	8 (18)
Diaphragm eventration	13 (30)
Gastric ulcer	9 (20)
Paraesophageal hernia	7 (16)
Peritoneal adhesion	11 (25)
Sliding hernia	10 (23)

Multiple predisposing factors for gastric volvulus (GV) might coexist in one patient.

Table 3 Performance of diagnostic modalities for gastric volvulus

	Ordered	Suggestive	Diagnostic	Unrevealing
Chest radiography	34	2	0	32
Abdominal radiography	30	2	0	28
Abdominal sonography	24	0	0	24
Upper GI endoscopy	25	6	2	17

GI: Gastrointestinal.

patients. Baseline demographics of these 44 enrolled patients are summarized in Table 1. They were predominantly male (84%), of advanced age (median: 71 years old, IQR: 57.5-78 years). Common presenting symptoms were abdominal pain (57%), abdominal fullness (55%), chest pain (41%), and nausea (39%). Secondary GV was present in 26 patients, most of whom had more than one etiology (Table 2). Twenty-eight patients (64%) experienced recurrence of symptoms during follow-up (median: 16 mo, IQR: 6-36 mo) (Figure 1). None developed acute complications in this study. Among the 44 enrolled patients, only one underwent elective open surgery for frequent symptoms, and the remaining 43 patients received conservative treatment throughout the study period.

The diagnosis of GV was confirmed by barium study in all patients. The performance of other diagnostic modalities is summarized in Table 3. Plain abdominal and thoracic radiography each suggested GV in one patient, but neither was diagnostic. Sonography failed to disclose clues of GV in any patient. Among the 25 patients who underwent upper GI endoscopy, six (24%) were suspected and two (8%) were confirmed to have GV.

Probable risk factors for recurrence, as determined by univariate analysis, are presented in Table 4. Factors associated with recurrence included longer duration between onset of symptoms and diagnosis (divided by 1.6 mo, $P = 0.065$), previous thoracic or abdominal operation ($P = 0.061$), and peritoneal adhesion ($P = 0.015$, Figure 2). The multivariate regression analysis (Cox proportional hazards model) indentified that peritoneal adhesion (hazard ratio: 2.58, 95% CI: 1.08-6.13, $P = 0.033$) was independently associated with recurrence.

DISCUSSION

We demonstrate that conservative treatment with watchful observation appears to be a safe therapeutic option in patients with chronic GV. None of the study subjects develop devastating complications during the follow-up period (up to 69 mo), and only one out of 44 patients turned to elective surgery. Nevertheless, symptom recurrence is a common feature in the natural history of chronic GV. This study revealed that 64% of the patients without anatomical correction experienced recurrent symptoms within a median follow-up period of 16 mo. We also identified peritoneal adhesion as an independent risk factor associated with recurrence.

Chronic GV is a disease of recurrent non-specific symptoms in the absence of immediate life-threatening complications. The potential risk of acute strangulation and its associated high mortality rate is regarded as the major indication for surgery[4,12]. Nevertheless, the natural history of chronic GV is poorly understood, and no study has addressed the incidence of chronic GV transforming into acute GV. Catastrophic complications of acute GV result from vascular compromise secondary to strangulation, therefore, transient or partial volvulus might not suffice to bring about severe vascular insufficiency that causes subsequent gastric infarction. With ligamentous attachment, a twisting stomach can spontaneously reduce after intermittent rotation[1]. Therefore, sustained and complete volvulus might not occur in the majority of chronic GV patients as frequently as it was often feared. Consistent with our finding, Al-Salem *et al*[20] have reported successful conservative treatment in 11 pediatric patients with chronic GV, and have concluded that those with mild to moderate symptoms should be treated conservatively. Similarly, in a large series conducted by Teague *et al*[4], clini-

Table 4 Risk factors for symptom recurrence in patients with chronic gastric volvulus, as determined by log rank test

	Dichotomous variable	Patient number	Median recurrence-free period, mo (95% CI)	*P* value
Age (yr)	< 70/≥ 70	22/22	4 (2.2-5.8)/4 (2.9-5.1)	0.798
Sex	M/F	37/7	4 (2.2-5.8)/3 (0.4-5.6)	0.158
Delayed diagnosis[1]	Yes/no	20/24	2 (0.2-3.8)/24 (NA)	0.065
Presenting symptoms				
Abdominal fullness	Yes/no	24/20	2 (0.6-3.4)/5 (3.0-7.0)	0.101
Abdominal pain	Yes/no	25/19	4 (2.5-5.5)/4 (0-27.0)	0.751
Acid regurgitation	Yes/no	9/35	5 (1.5-8.5)/4 (2.7-5.3)	0.680
Non-cardiac chest pain	Yes/no	18/26	2 (0.6-3.4)/5 (3.6-6.4)	0.096
Dysphagia	Yes/no	8/36	3 (0-7.2)/4 (2.9-5.1)	0.620
Dyspnea	Yes/no	9/35	4 (NA)/4 (2.6-5.4)	0.218
Heartburn	Yes/no	10/34	2 (0-4.1)/4 (2.3-5.7)	0.079
Nausea	Yes/no	17/27	5 (2.9-7.1)/4 (2.8-5.2)	0.810
Vomiting	Yes/no	5/39	4 (1.9-6.1)/4 (2.3-5.7)	0.580
Hematemesis	Yes/no	2/42	2 (NA)/4 (2.4-5.6)	0.332
Previous thoracic or abdominal surgery	Yes/no	10/34	3 (2.1-3.9)/5 (3.3-6.7)	0.061
Reflux esophagitis	Yes/no	14/30	4 (2.2-5.8)/4 (1.7-6.2)	0.466
Diaphragm eventration	Yes/no	13/31	3 (0.6-5.4)/5 (3.0-7.0)	0.126
Diaphragm defect	Yes/no	8/36	4(2.7-5.3)/4 (2.4-5.6)	0.755
Paraesophageal hernia	Yes/no	7/37	4 (1.4-6.6)/3 (1.4-4.6)	0.828
Sliding hernia	Yes/no	10/34	4 (2.5-5.5)/3 (0.4-5.3)	0.510
Peritoneal adhesion	Yes/no	11/33	1 (0-2.0)/5 (3.3-6.7)	0.015
Choledocholithiasis	Yes/no	9/35	2 (0.5-3.4)/4 (2.7-5.3)	0.101
Diabetes mellitus	Yes/no	5/39	1 (NA)/4 (1.9-6.1)	0.307
Gastric ulcer	Yes/no	9/35	4 (1.1-6.9)/4 (1.8-6.2)	0.926

[1]NA: Not applicable (95% CI could not be calculated as a result of small sample size).

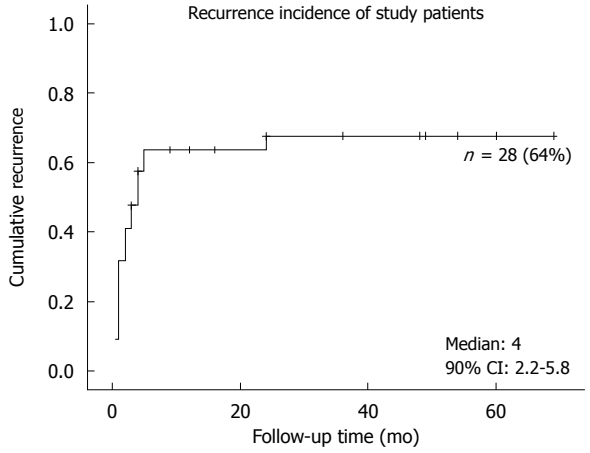

Figure 1 Cumulative incidence of recurrence in 44 chronic gastric volvulus patients who were treated conservatively.

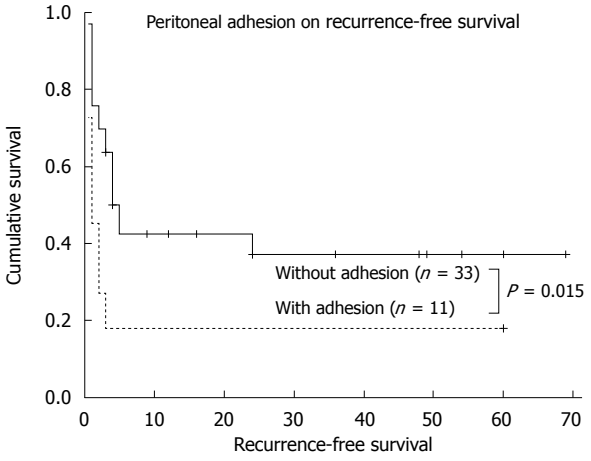

Figure 2 Peritoneal adhesion as a risk factor for symptom recurrence in patients with chronic gastric volvulus.

cal outcomes were uneventful in the two patients treated conservatively.

Diagnosis of chronic GV is difficult and requires a high index of suspicion and confirmatory upper GI barium study. In the present study, most patients presented with abdominal fullness, abdominal pain, chest pain, and nausea, all of which were non-specific and could be easily misdiagnosed as dyspepsia, peptic ulcer, gastroesophageal reflux disease, or other conditions. Furthermore, usual initial examinations for patients with similar symptoms were not diagnostic (Table 3). Plain radiography might be suggestive in a few patients[21,22], but generally it fails to uncover GV below the diaphragm. Abdominal sonography was not helpful in our study, and did not reveal GV in any of our patients. Even upper GI endoscopy cannot be regarded as an ideal diagnostic tool, because we demonstrated that, in the 25 patients who underwent endoscopy, the diagnosis of GV was missed in 17 (68%). Our results were consistent with previous studies, which reported upper GI barium examination as the most accurate diagnostic procedure, followed by endoscopy[4,6]. As a result of the poor performance of routine examinations, patients with chronic GV might remain undiagnosed unless a barium study is conducted, which is not usually part of routine work-up. As a result, many, and probably most of the chronic GV patients are unrecognized, and the prevalence of this disease is underestimated[23]. Our finding of the infrequent trans-

formation from chronic GV to an emergency situation lends additional support to this postulation, because misdiagnosis would be easy with only mild to moderate, non-specific symptoms.

Our study demonstrates that recurrence is a major feature in the natural history of chronic GV. The median recurrence-free period was only 3 mo. In fact, this was likely to have been an underestimate because mild or unreported symptoms were not categorized as recurrence for the purpose of this study. We further identified peritoneal adhesion as an independent risk factor associated with recurrence. Adhesion within the abdomen might predispose to GV by acting as an axis of rotation[7], and might also hamper spontaneous gastric reduction. Although it has long been reported that chronic GV recurs repeatedly if it is not anatomically corrected[8,9,24], this is believed to be the first study to explore specifically the incidence and risk factors of recurrence. Whether peritoneal adhesion heralds higher risk of gastric strangulation is a serious concern, but was not observed in this study. More studies are necessary to uncover the predictors of acute complications for chronic GV.

Laparoscopic surgery has recently become the trend, with encouraging results for anatomical correction of GV[4,5,25]. Accordingly, management of each chronic GV patient should be carefully individualized. It is important for clinicians to take patients' age, comorbidity, physical performance, life expectancy, and willingness into consideration. On one hand, chronic GV seems to result in acute complications uncommonly, but on the other, it recurs very frequently. Our results should not be misinterpreted to suggest that we undertook vigilant observation appropriate for every patient with chronic GV, because we did not correct the underlying pathology and recurrence was very common. With current evidence, we suggest that laparoscopic surgery can be regarded as the treatment of choice in good surgical candidates. However, conservative treatment with watchful follow-up appears as a safe alternative for those unwilling to accept or who are unsuitable for invasive procedures. Randomized trials might be necessary to elucidate the most appropriate management in chronic GV patients with different surgical risks, but they are very difficult to conduct.

Several limitations of this study should be noted. First of all, this was an observational study, rather than a comparative trial. We might have demonstrated that conservative management of chronic GV was not a risky alternative, but we did not claim conservative treatment was better than surgery. Second, the retrospective design precluded a standardized protocol of management. For example, not all patients underwent all kinds of diagnostic examinations, and their respective pharmacotherapy was different. Nevertheless, because the primary aim of this study was to explore outcome of conservative management, instead of the efficacy of any specific medication, our conclusion would not have been changed even if we had controlled the medical therapy. Third, the sample size of 44 patients might not permit detection of more predictors for adverse outcomes, although this study was actually a relatively large series of chronic GV patients, in view of the rarity of this disease. Furthermore, since there was no acute complication in our study, it might be controversial whether symptomatic recurrence should be considered as a poor clinical outcome or simply an inevitable part of the natural history. Finally, this was a hospital-based study that consisted of patients with established diagnosis. For reasons of diagnostic difficulty, we assumed there were many undiagnosed chronic GV patients whom we did not have chance to investigate.

In conclusion, clinical presentation of chronic GV is not specific and correct diagnosis remains a challenge. With poor performance of other modalities, upper GI barium study remains the diagnostic tool of choice and should be performed in appropriate settings. Our study reveals that symptomatic recurrence is very common and is independently associated with peritoneal adhesion in chronic GV patients, without anatomical correction. Nevertheless, conservative treatment might be regarded as a safe alternative to surgery, because acute complications infrequently occur in these patients.

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COMMENTS

Background

Chronic gastric volvulus (GV) usually presents with recurrent non-specific symptoms, but can potentially lead to acute strangulation of the stomach. The natural history of chronic GV remains unknown, and the clinical outcomes of patients managed conservatively without anatomical correction have not been investigated.

Research frontiers

It is debatable whether anatomical correction is necessary for all patients with chronic GV, who are usually at an advanced age, with comorbidity.

Innovations and breakthroughs

Symptomatic recurrence is common and independently associated with peritoneal adhesion for chronic GV without anatomical correction, although devastating complications infrequently occur.

Applications

Conservative treatment with vigilant follow-up can be regarded as a safe alternative in those patients unwilling to accept or who are unsuitable for invasive procedures.

Peer review

The aim of the present study was to investigate the natural history of chronic GV in elderly patients. The most significant finding that the authors reported was that peritoneal adhesion was an important risk factor associated with symptomatic recurrence of GV. In addition, the authors have provided some new important data with regard to symptom recurrence in the natural history of GV.

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BRIEF ARTICLE

A newly designed big cup nitinol stent for gastric outlet obstruction

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Abstract

AIM: To find out whether a newly designed big cup nitinol stent is suitable for treatment of patients with gastric outlet obstruction resulting from gastric cancer.

METHODS: The new stent is composed of a proximal big cup segment (20 mm in length and 48-55 mm in diameter), a middle part (60 mm in length and 20 mm in diameter) covered by a polyethylene membrane and a distal sphericity (20 mm in length and 28 mm in diameter). Half of the proximal big cup segment is also covered by a polyethlene membrane, which is adjacent to the middle part of the stent. The stent is preloaded in a 6.0-mm-diameter introducer system. Thirteen patients with gastric outlet obstruction resulting from gastric cancer received the new stents under endoscopic and fluoroscopic guidance.

RESULTS: Technical success was achieved in 12 of 13 (92.3%) patients. Among the 12 patients in whom endoscopic stent was placed successfully, the clinical success rate was 91.7% during a follow-up of average 6.5 mo. During the first month follow-up, the migration rate was 0%, recurrent obstruction 0% and gastric bleeding 8.3%. During the follow-up between 2-12 mo, no migration, recurrent obstruction and gastric bleeding occurred.

CONCLUSION: The proximal big cup segment seems to be effective and promising for technical efficacy, clinical outcome, and preventing migration and tumor ingrowth and increasing the emptying rate of sinus ventriculi.

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Key words: Endoscopic; Gastric outlet; Stenosis; Obstruction; Stents

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INTRODUCTION

Endoscopic stent placement has been increasingly used as a nonsurgical palliative treatment option for patients with gastric outlet obstruction caused by unresectable stomach cancer. However, conventional stents result in many complications including migration, restenosis and so on. Moreover, their proximal parts are not fit for roomy gastral cavity. To overcome the migration and restenosis, and to increase the emptying rate of sinus ventriculi, we designed a new big cup nitinol stent. This report is to evalu-

ate its clinical efficacy for gastric outlet obstruction caused by stomach cancer.

MATERIALS AND METHODS

Subjects

From April 2005 to May 2009, the newly designed big cup nitinol stent was placed in 13 patients (7 men, 6 women; mean age 77.6 years, range 71-93 yeas) with gastric outlet obstruction resulting from gastric cancer. The tumors were considered unresectable in all patients because of advanced, metastatic disease (10/13) and old age with medical comorbidity (3/13). All patients exhibited signs of gastric outlet obstruction, such as intractable vomiting and inability to eat. Endoscopic biopsy established the diagnosis of malignancy. Before endoscopy, the site and length of the stenosis were evaluated by radiography taken after oral contrast opacification. Patients were included following the criteria: (1) gastric outlet obstruction defined by symptoms resulting in decreased oral intake (nausea, vomiting, and inability to eat); and (2) the site of stenosis was between gastric body and duodenum bulb (Figure 1A). The study was approved by our hospital's ethics committee and was performed in compliance with our hospital's policies related to the use of animal and/or human subjects and human-derived material.

Stent

The proximal part of the newly designed stent (Figure 1B) contains a big cup with a length of 20 mm and a diameter 48-55 mm. The middle part is composed of a 60-mm-long segment with a diameter of 20 mm and the distal part presents a sphericity with a length of 20 mm and a diameter of 28 mm. Both the middle part and a half of proximal big cup segment are covered by a polyethylene membrane, but the rest part of the stent is not covered. The stent is mounted on a delivery system with an outer diameter of 6 mm and an overall length of 180 cm. The end of delivery system was adhered with 3 pieces of adhesive tape. The distance between two adhesive tapes in the neighborhood was 5 cm.

Procedure

The procedure was performed under endoscopic and fluoroscopic guidance as follows. The pharynx was sprayed with a topical anesthetic, lidocaine; meperidine was administered intravenously for sedation. The stomach was intubated with a endoscope (Fujinon EG 450HR, Japan) and the proximal and distal margins of the stricture were defined fluoroscopically by injection of nonionic contrast using a catheter passing through the accessory channel. Under endoscopic and fluoroscopic guidance, the locations of the proximal and the distal ends of the strictured segment were marked on the skin with metallic markers. Before implantation, an atraumatic, stiff guide wire was introduced via the endoscope through the stricture. If mild pressure was sufficient to pass the endoscope through the stricture, dilation was not performed. If there is complete obstruction, an atraumatic guide wire was placed endoscopically, and the stricture was dilated with a 15-mm diameter balloon catheter. When dilation was complete, the guide wire was exchanged for a super stiff metalic guide wire and then endoscope was removed with the guide wire left in place. Under fluoroscopic guidance, the delivery system was passed over the guide wire to a point slightly beyond the distal end of the stricture. Abdominal compression was used to aid advancement of the delivery system if necessary. This maneuver was helpful in preventing bending of the delivery device at particular locations such as the greater curvature of the stomach. If further assistance was needed to introduce the stent in patients with gastric outlet obstruction, the endoscope was reinserted to the stomach. The adhesive tape of delivery system was snatched up with a grasping forceps inserted through the endoscope accessory channel and the endoscope together with the delivery system was advanced across the stenotic region. When the stent within the delivery system was positioned between metallic markers on skin to indicate proximal and distal stricture margins, the outer sheath of the delivery system was slowly withdrawn to gradually release the stent and allowed it to lie within the stricture. With the guide wire left in place, the delivery system was removed. The endoscope is passed again to check the position of the proximal big cup of the stent (Figure 1C). If the stent was found in the correct position, the guide wire is removed. The fluoroscopic views were obtained immediately after stent placement (Figure 1D).

Follow-up study

After stent placement, the patients were arranged to resume oral intake of liquids within 24 h. The patients were not allowed to take a soft or solid diet until the follow-up showed full stent expansion. On the day after stent placement, barium contrast radiography was performed to document the position and the functionality of the stent. After this, the patients were called every month and inquired about what they ate and if there were any symptoms of obstruction such as pain and discomfort or vomiting after food intake. Follow-up barium study or endoscopy was carried out only in the patients with recurrent symptoms. The end point of this study was cessation of stent patency or patient death.

RESULTS

Stent implantation was successful in 12 patients with a technical success rate of 92.3%. All stents were transpyloric. However, one patient failed when we experienced the buckling of the delivery system in the proximal stomach and pushed over the super stiff guide wire through a narrow stenosis. All stents were 100 mm in length. The proximal port of placed stents ranged in diameter from 48 to 55 mm (mean 51.5 mm). Opacification showed that nitinol stents were integrated with sinus ventriculi residuary cavity in 11 of the 12 patients and barium could be emptied completely in all successful cases. No procedure-

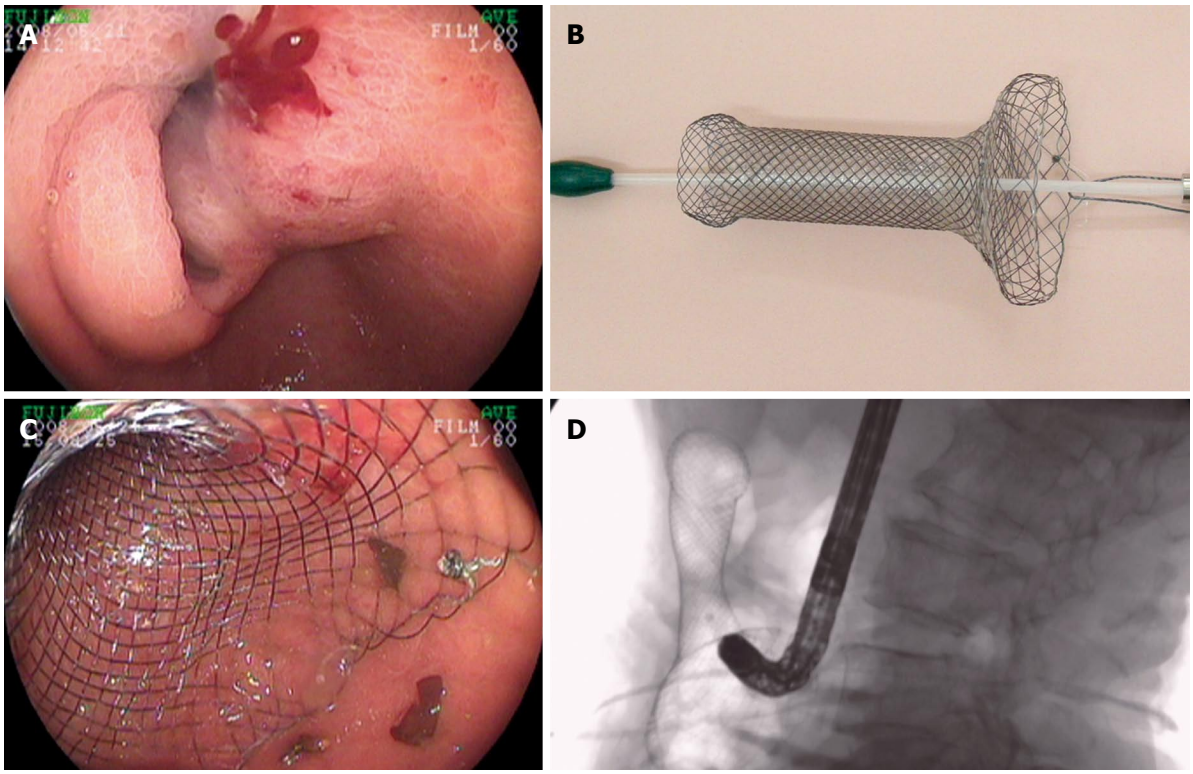

Figure 1 Endoscopic photography taken immediately before (A) and after (C) deployment of stent, the corresponding newly designed big cup nitinol stent (B), X-ray photography (D) taken immediately after deployment of stent.

related complications occurred, such as perforation. Eleven of the 12 patients had relief of obstructive symptoms, allowing oral intake of liquids. One patient who was already in a pre-terminal stage (death occurred 4 wk after stent implantation), had episodes of vomiting, gastric distention, and tolerated just liquid intermittently, although radiological examination showed free passage of barium. The mean survival of all patients who eventually died was 6.5 mo (range 4 wk-12 mo).

Nine patients were able to eat soft or semi-solid diet and two patients were able to take liquid, and no migration and recurrent obstruction occurred during the follow-up period. However, one patient had hematemesis and melena 3 wk after stent placement. After proper treatment, the bleeding stopped.

DISCUSSION

Expandable metal stents have been used generally to treat malignant gastric strictures in recent years[1,2]. Lowe *et al*[3] thought that the primary treatment in all patients with inoperable gastric outlet obstruction should be gastroduodenal stenting. However, a major problem of the stents was that the conventional proximal ends were not designed according to the shape and diameter of sinus ventriculi residuary cavity, which was not suitable for roomy gastral cavity[3-6]. Stent occlusion in uncovered stents and migration in covered stents were also problematic[2,7]. Therefore, the newly designed stent was developed to solve the problem of the disadvantages of conventional metal stents. The proximal big cup of stent can prevent stent migration and contribute to passage of food, and the middle part of the stent can inhibit ingrowth. Compared with other studies[8-10], recurrent symptoms did not occur in our patients during a mean follow-up of 6.5 mo. Stent migration was not found. Because the shape and diameter of proximal big cup are similar to the ones of the proximol end of obstruction, the newly designed stent seems to be more appropriate for treating gastric outlet obstruction caused by stomach cancer than conventional one. In our study, opacification showed that the big cup nitinol stents were integrated with sinus ventriculi residuary cavity in 11 of the 12 patients and barium could be emptied completely in all successful cases. Moreover, the mean survival of the 12 patients who eventually died was 6.5 mo.

The placement of the stent was technically successful in 12 patients and failed in one patient, with a technical success of 92.3%. After stent placement, 11 patients were able to ingest liquids, with a clinical success of 91.7%. The results were comparable to those of other studies (90%-100% and 75%-94%, respectively)[1,3,10-15]. Although the newly designed stent has a proximal big cup, it did not increase the difficulty of placement. Nevertheless, we found that one patient with gastric bleeding 3 wk after stent placement might have been relative to the proximal big cup, for the bleeding site was confirmed to be located on the gastric mucosa close to the proximal end of the stent by endoscope.

In conclusion, the placement of the newly designed big cup nitinol stent offers good palliation for inoperable

malignant gastric outlet obstruction resulting from stomach cancer. It suggests that it seems to be a reliable and safe therapeutic tool for gastric outlet obstruction for unresectable stomach cancers which are difficult to treat with conventional ones, although more cases are needed to be studied before any valid conclusions can be made. Proper techniques and settings should be followed to avoid any procedure-related complications.

COMMENTS

Background

Expandable metal stents have been used generally to treat malignant gastric strictures in recent years. However, a major problem of the stents was that the conventional proximal ends were not designed according to the shape and diameter of sinus ventriculi residuary cavity, which was not suitable for roomy gastral cavity. The authors designed a new big cup nitinol stent. It can not only overcome the migration and restenosis, but increase the emptying rate of sinus ventriculi.

Research frontiers

The newly designed stent was developed to solve the problems of the conventional metal stents. It can prevent the migration and restenosis for gastric outlet obstruction caused by stomach cancer.

Innovations and breakthroughs

A new big cup nitinol stent is designed on the basis of shape and dimension of remnant gastral cavity. The stent is obviously superior to the double-layered and conventional stents, for it is more appropriate for remnant gastric cavity.

Applications

The newly designed big cup nitinol stent can be used more appropriately for gastric outlet obstruction caused by stomach cancer compared with the double-layered and conventional stents.

Terminology

Big cup nitinol stent: The proximal part of the newly designed stent contains a big cup with a length of 20 mm and a diameter 48-55 mm. The middle part is composed of a 60-mm-long segment with a diameter of 20 mm and the distal part presents a sphericity with a length of 20 mm and a diameter of 28 mm.

Peer review

This article is interesting and can be better if a few corrections are made.

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BRIEF ARTICLE

Relationship between genetic polymorphisms of ALDH2 and ADH1B and esophageal cancer risk: A meta-analysis

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Abstract

AIM: To evaluate the contribution of alcohol dehydrogenase-1B (ADH1B) and aldehyde dehydrogenase-2 (ALDH2) polymorphisms to the risk of esophageal cancer.

METHODS: Nineteen articles were included by searching MEDLINE, EMBASE and the Chinese Biomedical Database, 13 on ADH1B and 18 on ALDH2. We performed a meta-analysis of case-control studies including 13 studies on ADH1B (cases/controls: 2390/7100) and 18 studies on ALDH2 (2631/6030).

RESULTS: The crude odds ratio [OR (95% confidence interval)] was 2.91 (2.04-4.14) for ADH1B*1/*1 (*vs* ADH1B*2/*2) and 1.32 (1.17-1.49) for ADH1B*1/*2. The crude OR for ALDH2*1/*2 (*vs* ALDH2*1/*1) was 2.52 (1.76-3.61). ADH1B*1/*1 increased the risk of esophageal cancer among never/rare [1.56 (0.93-2.61)], moderate [2.71 (1.37-5.35)], and heavy drinkers [3.22 (2.27-4.57)]. ADH1B*1/*2 was associated with a modest risk among moderate drinkers [1.43 (1.09-1.87)]. ALDH2*1/*2 increased the risk among never/rare [1.28 (0.91-1.80)], moderate [3.12 (1.95-5.01)], and heavy [7.12 (4.67-10.86)] drinkers, and among ex-drinkers [5.64 (1.57-20.25)]. ALDH2*2/*2 increased the risk among drinkers [4.42 (1.72-11.36)]. ADH1B*1/*1 plus ALDH2*1/*2 was associated with the highest risk for heavy drinkers [12.45 (2.9-53.46)]. The results of the meta-regression analysis showed that the effects of ADH1B*1/*1 and ALDH2*1/*2 increased with the level of alcohol consumption. ALDH2*1/*2 was associated with a high risk among Taiwan Chinese and Japanese drinkers, as opposed to a moderate risk among drinkers in high-incidence regions of Mainland China. ADH1B*1/*1 in heavy drinkers and ALDH2*1/*2 in moderate-to-heavy drinkers was associated with similarly high risk among both men and women.

CONCLUSION: ADH1B/ALDH2 genotypes affect the risk of esophageal cancer, and the risk is modified by alcohol consumption, ethnicity, and gender.

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Key words: Alcohol dehydrogenase-1B; Aldehyde dehydrogenase-2; Esophageal cancer; Meta-analysis

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INTRODUCTION

Esophageal cancer is a global health problem, and ranked the eighth in incidence and sixth in mortality in 2002[1]. Its incidence and mortality rates show a wide geographic variation at an international level, and there are marked differences between high-risk and low-risk areas[1-3]. Most esophageal cancer patients live in the "esophageal cancer belt", which stretches from Northern-Central China westward through Central Asia to Northern Iran, suggesting that genetic factors and environmental factors play a role in the development of esophageal cancer[2].

The ethanol in alcohol beverages[3] and the acetaldehyde associated with alcohol consumption[4] have recently been classified as Group 1 human carcinogens by the World Health Organization (WHO) and International Agency For Research On Cancer (IARC). The ethanol consumed in alcohol beverages is primarily metabolized by alcohol dehydrogenases (ADHs), including ADH1B (previously called ADH2), to acetaldehyde, and then to acetic acid, mainly by low-Km aldehyde dehydrogenase-2 (ALDH2)[5].

The mutant ADH1B*2 allele is highly prevalent among east Asians[6]. The homodimer of ADH1B encoded by ADH1B*1/*1 has only 1/100 and 1/200 of the ethanol oxidizing capacity of the isozymes encoded by ADH1B*1/*2 and ADH1B*2/*2[7], respectively, and ADH1B1*/1* genotype carriers experience prolonged exposure to ethanol after heavy drinking[8]. The mutant ALDH2*2 allele is also prevalent in east Asians[9] and encodes a catalytically inactive subunit. The ALDH2*2 allele acts in a semidominant manner[10]. ALDH2*2 allele carriers experience unpleasant flushing responses, including facial flushing, nausea, drowsiness, and a headache, after drinking a small amount of ethanol because of severe acetaldehydemia[11]. East Asian studies have identified that ADH1B*1 allele and ALDH2*2 allele as a strong positive factor and a strong negative risk factor, respectively, for heavy drinking[12,13], and both have been found to be a strong positive risk factor for esophageal cancer[5].

An earlier meta-analysis of ALDH2 genotype and risk of esophageal cancer included 7 case-control studies[14], and revealed only that cancer risk was reduced among ALDH2*2/*2 homozygotes because they are generally non-drinkers as a result of experiencing intense alcohol flushing responses, and that the cancer risk was higher among ALDH2*1/*2 heterozygotes. Only one case-control study has focused on the effect of the two genes in females, which showed that ALDH2*1/*2 alone is associated with a high risk of esophageal cancer among female heavy drinkers[15]. The influence of alcohol drinking on the risk of esophageal cancer varies with ethnicity, and gene polymorphisms may have different effects on cancer risk. In the present study, we conducted a comprehensive meta-analysis to clarify the effects of ADH1B and ALDH2 genotypes alone and in combination on the risk of esophageal cancer, and evaluate how the gene effects are influenced by drinking habits, gender, and ethnicity.

MATERIALS AND METHODS

Selection criteria

Only case-control studies that investigated the relationship between ADH1B and/or ALDH2 polymorphisms and esophageal cancer were included in the meta-analysis. Trials had to be original and report ADH1B and/or ALDH2 genotype frequencies in cases and controls or estimate the odds ratios (ORs). When the results of a study were published more than once, only the study that contained the most complete data was included in the analysis. Moreover, participants from high-risk populations and general populations were eligible for inclusion, and the controls were non-cancer or disease-free subjects.

Searching strategy for identification of studies

We searched MEDLINE (from January 1966 to April 2009), EMBASE (from January 1988 to April 2009), and the Chinese Biomedical Database (CBM; from January 1980 to April 2009). A comprehensive and exhaustive search strategy was formulated in an attempt to identify all relevant studies regardless of language or publication status using the following terms: "esophagus", "oesophagus", "carcinoma or cancer or neoplasm or tumour or tumor", "ADH2 or ADH1B or alcohol dehydrogenase", or "ALDH2 or aldehyde dehydrogenase", without any restriction on language.

Two reviewers (Yang SJ and Huang YC) independently examined abstracts of all candidate articles to decide whether to include or exclude them in the subsequent detailed review. We also attempted to identify additional studies by searching the reference lists of relevant trials, and scrutinized author names, location, setting, number of participants, and study data to ensure that each trial would be included only once. Of the 126 articles identified, 107 were excluded because 91 were studies regarded as animal models or studies conducted at a cellular level, 6 were reviews, 5 were not case-control studies, and 5 were duplicate publications. In addition, two articles only included part of data since the overlapped data were excluded. Ultimately, 19 articles were included (Tables 1 and 2), 13 on ADH1B and 18 on ALDH2. The overlapping data in these studies were excluded. ADH1B and ALDH2 genotype frequencies were not reported in 8 studies. Through contacting the authors, the data missing in four of the

Table 1 Characteristics of studies of alcohol dehydrogenase-1B polymorphism and risk of esophageal cancer[15-27]

Study ID	Country, subjects	Cases	Controls	ADH1B			
				Adjusted OR (95% CI)		Crude OR (95% CI)	
				*1/*2 *vs* *2/*2	*1/*1 *vs* *2/*2	*1/*2 *vs* *2/*2	*1/*1 *vs* *2/*2
Yokoyama *et al*[15], 2006[a]	Japan, female	52	412	1.56 (0.56-4.35)	2.08 (0.76-5.56)	1.29 (0.7-2.4)	2.15 (0.81-5.7)
Boonyaphiphat *et al*[16], 2002[a]	Thailand	202	261	NA[b]	NA	1.15 (0.56-2.47)	2.01 (0.96-4.3)
Ding *et al*[17], 2009	China	221	191	1.21 (0.79-1.86)	2.78 (1.06-7.29)	1.30 (0.85-1.99)	2.42 (0.96-6.66)
Chao *et al*[18], 2000	China (Taiwan), alcoholics	88	327	NA	NA	1.61 (0.84-3.16)	4.85 (2.32-10.18)
Guo *et al*[19], 2008	China, male	80	480	2.89 (1.11-5.64)	NA	1.13 (0.66-1.93)	5.37 (2.45-11.46)
Li *et al*[20], 2008	Africa	220	241	-	-	-	-
Yang *et al*[21], 2005	Japan	165	494	1.57 (1.04-2.36)	0.62 (0.22-1.72)	2.08 (1.44-2.99)	1.12 (0.44-2.86)
Yang *et al*[22], 2007	China	191	198	1.89 (1.1-3.22)	1.91 (0.92-3.95)	1.35 (0.88-2.08)	1.92 (1.04-3.56)
Yokoyama *et al*[23], 2001[a]	Japan, alcoholic male	112	526	NA	NA	0.81 (0.46-1.43)	2.4 (1.48-3.88)
Yokoyama *et al*[24], 2002	Japan, male	234	634	NA	NA	1.16 (0.82-1.65)	5.73 (3.4-9.71)
Hori *et al*[25], 1997	Japan	94	130	1.7 (0.9-3.0)	6.2 (2.6-14.7)	1.33 (1.15-1.53)	6.2 (2.6-14.7)
Lee *et al*[26], 2008	China (Taiwan)	406	656	NA	NA	1.3 (0.97-1.73)	6.09 (4.07-9.23)
Hashibe *et al*[27], 2006	Europe	325	2550	NA	NA	0.64 (0.14-2.97)	0.85 (0.19-3.79)
Pooled results		2390	7100	1.60 (1.28-2.0)	2.17 (1.08-4.34)	1.32 (1.17-1.49)	2.91 (2.04-4.14)
P for heterogeneity				0.54	< 0.05	0.45	< 0.05

[a]The genotype frequencies among the controls differed significantly from the Hardy-Weinberg equilibrium ($P < 0.05$); [b]NA: Not available because not mentioned or calculated in the study. ADH1B: Alcohol dehydrogenase-1B; OR: Odds ratio; CI: Confidence interval.

Table 2 Characteristics of studies of aldehyde dehydrogenase-2 polymorphism and risk of esophageal cancer[15-26,28-33]

Study ID	Country, subjects	Cases	Controls	ALDH2			
				Adjusted OR (95% CI)		Crude OR (95% CI)	
				*1/*2 *vs* *1/*1	*2/*2 *vs* *1/*1	*1/*2 *vs* *1/*1	*2/*2 *vs* *1/*1
Yokoyama *et al*[15], 2006[a]	Japan, female	52	412	1.01 (0.54-1.87)	1.49 (0.47-4.78)	1.13 (0.62-2.06)	1.56 (0.5-4.88)
Boonyaphiphat *et al*[16], 2002[a]	Thailand	202	261	1.57 (0.89-2.76)	0.52 (0.05-5.33)	1.33 (0.82-2.17)	0.22 (0.03-1.87)
Ding *et al*[17], 2009	China, male	221	191	1.71 (1.1-2.66)	4.84 (2.25-10.61)	1.7 (1.08-2.68)	5.69 (2.51-12.18)
Chao *et al*[18], 2000	China (Taiwan), alcoholics	88	327	NA[b]	NA	3.54 (2.16-5.8)	1.33 (0.28-6.4)
Guo *et al*[19], 2008	China, male	80	480	NA	NA	1.5 (0.93-2.42)	0.1 (0.01-1.67)
Li *et al*[20], 2008[a]	Africa	237	268	NA	NA	1.24 (0.62-2.46)	2.73 (0.79-10.69)
Yang *et al*[21], 2005	Japan	165	494	6.43 (4.02-10.3)	1.92 (0.23-15.7)	2.08 (1.44-2.99)	1.12 (0.44-2.86)
Yang *et al*[22], 2007	China	191	198	1.67 (1.02-2.72)	0.26 (0.06-1.09)	1.35 (0.88-2.08)	1.92 (1.04-3.56)
Yokoyama *et al*[23], 2001	Japan, alcoholic male	112	526	13.50 (8.06-22.6)	-	11.8 (7.36-18.94)	Excluded[c]
Yokoyama *et al*[24], 2002	Japan, male	234	634	7.46 (4.71-11.81)	7.83 (1.33-46.08)	3.66 (2.62-5.1)	0.25 (0.06-1.07)
Hori *et al*[25], 1997	Japan	93	171	4.4 (2.5-7.7)	0.9 (0.2-3.6)	4.35 (2.42-7.83)	0.93 (0.24-3.55)
Lee *et al*[26], 2008	China (Taiwan)	406	656	NA	NA	2.88 (2.19-3.77)	0.91 (0.48-1.72)
Cai *et al*[28], 2006	China	205	394	0.76 (0.5-1.16)	1.72 (0.85-3.48)	0.69 (0.47-0.99)	2.25 (1.2-4.22)
Ding *et al*[29], 2002	China	98	235	NA	NA	0.91 (0.56-1.47)	0.24 (0.05-1.07)
Itoga *et al*[30], 2004	Japan, male	74	241	NA	NA	2.28 (2.19-3.77)	0.95 (0.2-4.57)
Matsuo *et al*[31], 2001	Japan	102	241	3.72 (1.88-7.36)	0.8 (0.09-6.88)	2.48 (1.52-4.03)	0.19 (0.025-3.52)
Yokoyama *et al*[32], 1996	Japan, male	29	28	NA	-	9.2 (4.22-20.07)	Excluded[c]
Yokoyama *et al*[33], 2006	Japan, alcoholic male	42	273	NA	NA	13.83 (6.16-31)	Excluded[c]
Pooled results		2631	6030	2.06 (1.09-3.89)	1.31 (0.52-3.34)	2.52 (1.76-3.61)	0.76 (0.42-1.40)
P for heterogeneity				< 0.001	< 0.001	< 0.001	< 0.001

[a]The genotype frequencies among the controls differed significantly from the Hardy-Weinberg equilibrium ($P < 0.05$); [b]NA: Not available; [c]No studies were included because there were no cases and controls with ALDH2*2/*2. ALDH2: Aldehyde dehydrogenase-2; OR: Odds ratio; CI: Confidence interval.

articles were obtained, but the missing data of the other four were not because of no response from the authors or failure to contact the authors. Finally, two independent reviewers (Yang SJ and Shao Y) independently extracted the data using a standard extraction form.

Stratification by alcohol drinking

Information on alcohol drinking is essential in risk analyses of ADH1B and ALDH2 genotypes for two reasons. One reason is that the alcohol drinking could be a strong confounding variable in comparing genotypes and the risk of esophageal cancer because the genotypes are also related to the amount of alcohol consumed (suppressive in ALDH2*2 and facilitating in ADH1B*1[12,13]), thus misleading to analyze the risk of esophageal cancer without taking the alcohol consumption level into account[14]. It

should be noted that such a confounding effect is a unique property of alcohol-related genes. The second reason is that from the stand point of mechanism, alcohol-related enzymes would not be expected to play a role in the development of esophageal cancer among non-drinkers, whereas the adverse roles of the inactive enzymes would be enhanced among moderate to heavy drinkers. Thus, the primary analysis was done by stratifying the data according to alcohol-drinking status (ex-drinkers, non-/rare drinkers, moderate drinkers, and heavy drinkers).

Statistical analysis

The STATA statistical package (version 9, STATA, College Station, TX) was used to perform the meta-analysis. The ADH1B*2/*2 group served as the control for the ADH1B*1/*2 group and the ADH1B*1/*1 group, and the ALDH2*1/*1 group served as the control for the ALDH2*1/*2 group and the ALDH2*2/*2 group. We used the ADH1B*2 and ALDH2*1/*1 group as the control for the analysis of the combined effect of ADH1B and ALDH2, because most studies have used it as the control group. The crude ORs of the studies included were calculated according to the available genotype frequencies in order to obtain pooled ORs. Overall effect was tested using Z scores with significance set at $P < 0.05$. Heterogeneity was tested using the χ^2 test for goodness of fit with significance set at $P < 0.05$, and its possible sources were assessed by subgroup analyses as described below. A random-effect model was applied to obtain summary ORs and their 95% confidence interval (CI). The Hardy-Weinberg equilibrium in the controls in each study was assessed using the χ^2 test, and publication bias was statistically assessed by Egger's test. In addition, a sensitivity analysis was performed to explore whether omitting large-sample studies would greatly change or reverse the results. The influence of alcohol drinking on the relationship between the ADH1B and ALDH2 genotypes and esophageal cancer was assessed by a meta-regression analysis[34]. A subgroup analysis in regard to ethnicity and sex to detect heterogeneity across the trials was performed. Since the number of drinkers with the ALDH2*2/*2 genotype was very small, we combined the category of moderate and heavy drinkers into a single category to analyze the effect of this genotype in drinkers.

RESULTS

A total of 19 case-control studies were identified, including 13 studies on the ADH1B genotype (2390 cases and 7100 controls) (Table 1) and 18 studies on the ALDH2 genotype (2631 cases and 6030 controls) (Table 2). All of the studies, except for three studies[21,23,35] that did not mention the pathologic types, were about esophageal squamous cell carcinoma. The majority of the studies were conducted in Asian populations, except one study conducted in Europe and one in Africa. Seven studies were conducted in male populations alone, and three were conducted in alcoholics alone.

In comparison with ADH1B*2/*2, the overall crude OR (95% CI) of ADH1B*1/*2 and ADH1B*1/*1 was 1.32 (1.17-1.49) and 2.91 (2.04-4.14), respectively, and it was similar to their overall adjusted OR of 1.60 (1.28-2.0) and 2.17 (1.08-4.34) (Table 1). Significant study-heterogeneity was observed in ADH1B*1/*1 ($P < 0.05$), but it disappeared after stratifying by drinking status (Figure 1). ADH1B*1/*1 increased the risk of esophageal cancer among never/rare drinkers [1.56 (0.93-2.61)], moderate drinkers [2.71 (1.37-5.35)] and heavy drinkers [3.22 (2.27-4.57)], especially among the moderate/heavy drinkers. The results of the meta-regression analysis showed that the larger the amount of alcohol consumed the greater the OR for ADH1B*1/*1 became ($P = 0.043$, Figure 1), indicating a so-called gene-environment interaction. ADH1B*1/*2 was associated with a modest but significantly increased risk of esophageal cancer among moderate drinkers [1.43 (1.09-1.87)] (Figure 2).

In comparison with ALDH2*1/*1, the overall crude OR (95% CI) of ALDH2*1/*2 was 2.52 (1.76-3.61), and it was similar to the overall adjusted OR of 2.06 (1.09-3.89) (Table 2). There was significant study-heterogeneity in ALDH2*1/*2 ($P < 0.001$) and ALDH2*2/*2 ($P < 0.001$). After stratified by drinking status, the significant study-heterogeneity in ALDH2*1/*2 persisted among the moderate and heavy drinkers, and in ALDH2*2/*2 it persisted among the never/rare-drinkers (Figures 3 and 4). ALDH2*1/*2 increased the risk of esophageal cancer among never/rare drinkers [1.28 (0.91-1.80)], moderate drinkers [3.12 (1.95-5.01)], heavy drinkers [7.12 (4.67-10.86)], and ex-drinkers [5.64 (1.57-20.25)], and especially among the heavy drinkers. The results of the meta-regression analysis showed that the larger the amount of alcohol consumed, the greater the OR for ALDH2*1/*2 was ($P < 0.001$, Figure 3), clearly demonstrating a very strong gene-environment interaction. Although the proportion of drinkers among the ALDH2*2/*2 homozygotes was very small, ALDH2*2/*2 also greatly increased the cancer risk among drinkers [4.42 (1.72-11.36)].

The analysis of combined effects of ADH1B and ALDH2 genotypes showed that ADH1B*1/*1 plus ALDH2*1/*2 was associated with the highest risk of esophageal cancer among heavy drinkers [12.45 (2.9-53.46)] (Table 3), but no significant increase in risk was seen among never/rare drinkers.

A subgroup analysis was performed in regard to ethnicity and drinking status (Table 4). The ADH1B*1/*1 genotype was associated with a high risk of esophageal cancer in heavy drinkers among all three ethnic groups: Mainland Chinese, Taiwan Chinese, and Japanese [ORs = 3.55 (1.01-11.83), 3.36 (1.73-6.53), and 4.02 (1.83-8.80), respectively]. ALDH2*1/*2 was associated with a very high risk among Taiwan Chinese and Japanese moderate-to-heavy drinkers [ORs = 6.21 (3.78-10.22) and 4.74 (3.29-6.83) in moderate drinkers; OR = 9.21 (5.54-15.31) and 9.75 (7.34-12.97) in heavy drinkers], but only a moderate risk among moderate drinkers in the high-incidence regions of Mainland China [OR = 1.98 (1.20-3.27)].

The analysis of the effect of ADH1B and ALDH2 genotypes on esophageal cancer risk was stratified by both

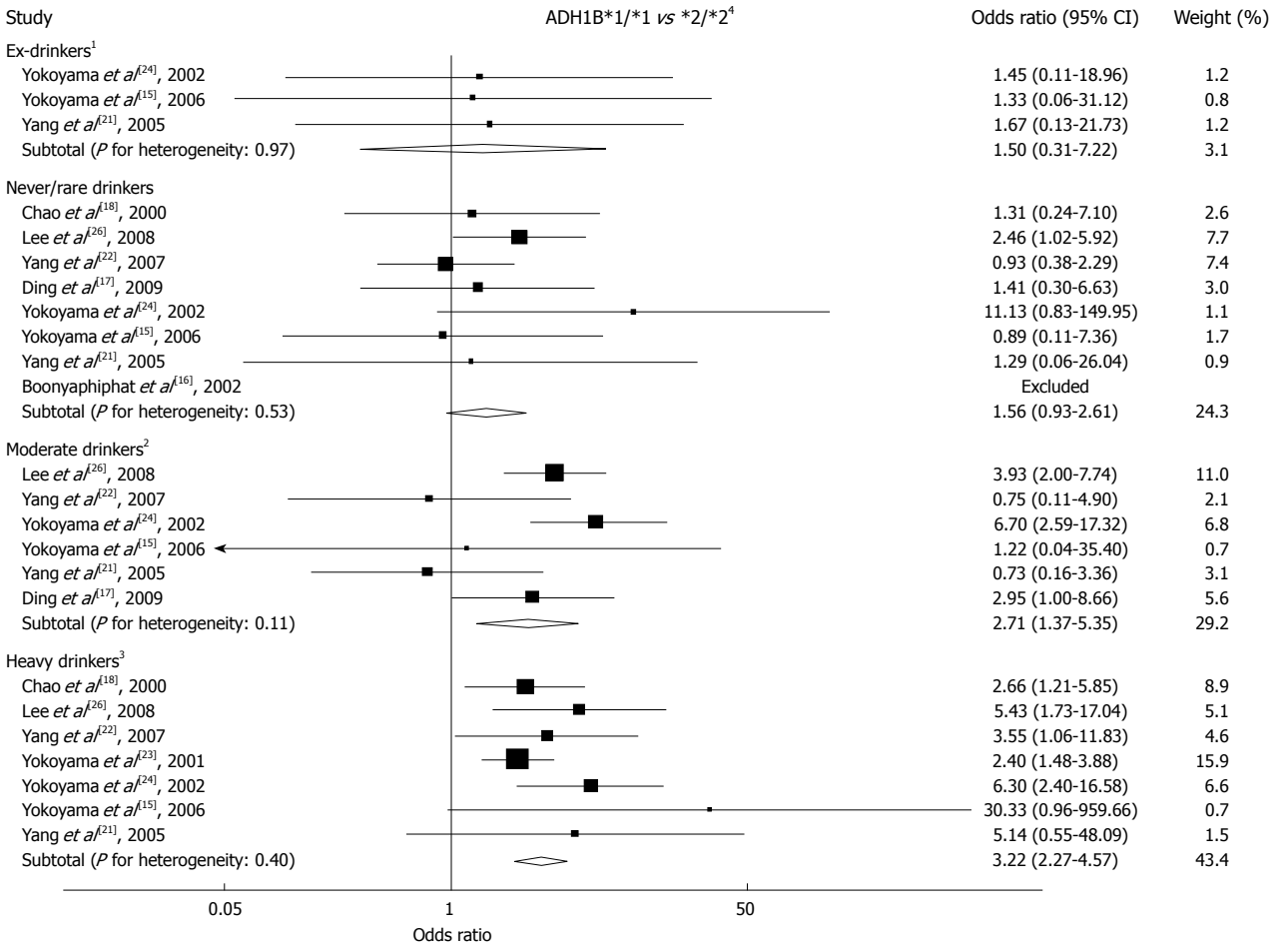

Figure 1 Relationship between alcohol dehydrogenase-1B*1/*1 and esophageal cancer stratified by alcohol consumption status. Weights are from random effects analysis. [1]Ex-drinkers mean alcohol quit for more than one year; [2]Moderate drinkers mean consumers of 1-40 g/d alcohol (Lee 2008), 1-50 g ethanol < 5 d/wk (Yang 2005, Yang 2007), 1-60 g/d ethanol (Boonyaphiphat 2002), 1-17.9 units/wk and 1 unit = 22 g ethanol (Yokoyama 2002, Yokoyama 2006), and > 40 g alcohol/wk (Ding 2009); [3]Heavy drinkers mean consumers of > 40 g/d alcohol (Lee 2008), > 50 g ethanol ⩾ 5 d/wk (Yang 2005, Yang 2007), > 60 g/d ethanol (Chao 2000, Yokoyama 1996), > 18 units/wk (Yokoyama 2002, Yokoyama 2006[15]), and alcoholics (Yokoyama 2001, Yokoyama 2006[33]); [4]Significant trend for meta-regression from never/rare drinkers to heavy drinkers is observed (P = 0.043). ADH1B: Alcohol dehydrogenase-1B.

Table 3 Risk of esophageal cancer associated with combinations of alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 genotypes

Genotype	ALDH2 *1/*1 pooled OR (95% CI)	*P* for heterogeneity	ALDH2 *1/*2 pooled OR (95% CI)	*P* for heterogeneity
ADH1B *2/*2 or *1/*2				
Never/rare drinkers[21,22]	1.00 (reference)	-	1.99 (0.53-7.5)	0.28
Moderate drinkers[21,22,26]	1.00 (reference)	-	5.64 (3.74-8.5)	0.68
Heavy drinkers[21-23,26]	1.00 (reference)	-	7.56 (3.4-16.78)	0.86
ADH1B *1/*1				
Never/rare drinkers[21,22]	1.13 (0.22-5.7)	< 0.05	1.36 (0.41-4.51)	< 0.05
Moderate drinkers[21,22,26]	2.37 (1.13-4.97)	< 0.05	9.17 (0.6-140.38)	0.06
Heavy drinkers[21-23,26]	2.43 (1.48-3.99)	0.08	12.45 (2.9-53.46)	0.08

ALDH2: Aldehyde dehydrogenase-2; ADH1B: Alcohol dehydrogenase-1B; OR: Odds ratio; CI: Confidence interval.

sex and drinking status (Table 4). ADH1B*1/*1 was associated with a high risk among men irrespective of drinking status [2.71 (1.13-6.51) among never/rare drinkers, 3.93 (2.43-6.36) among moderate drinkers, and 3.22 (2.16-4.83) among heavy drinkers]. ADH1B*1/*1 was associated with a high risk among heavy drinking women as well [3.55 (1.06-11.84)].

Men with ALDH2*1/*2 were highly susceptible to esophageal cancer, even among never/rare drinkers [1.92 (1.28-2.88)]. The ORs of ALDH2*1/*2 in moderate-to-heavy drinkers were similarly high in both men and women [3.53 (1.98-6.30) and 2.64 (1.24-5.68), respectively, in moderate drinkers; 7.04 (4.18-11.85) and 6.85 (1.04-44.95) in heavy drinkers]. The results of the meta-regression

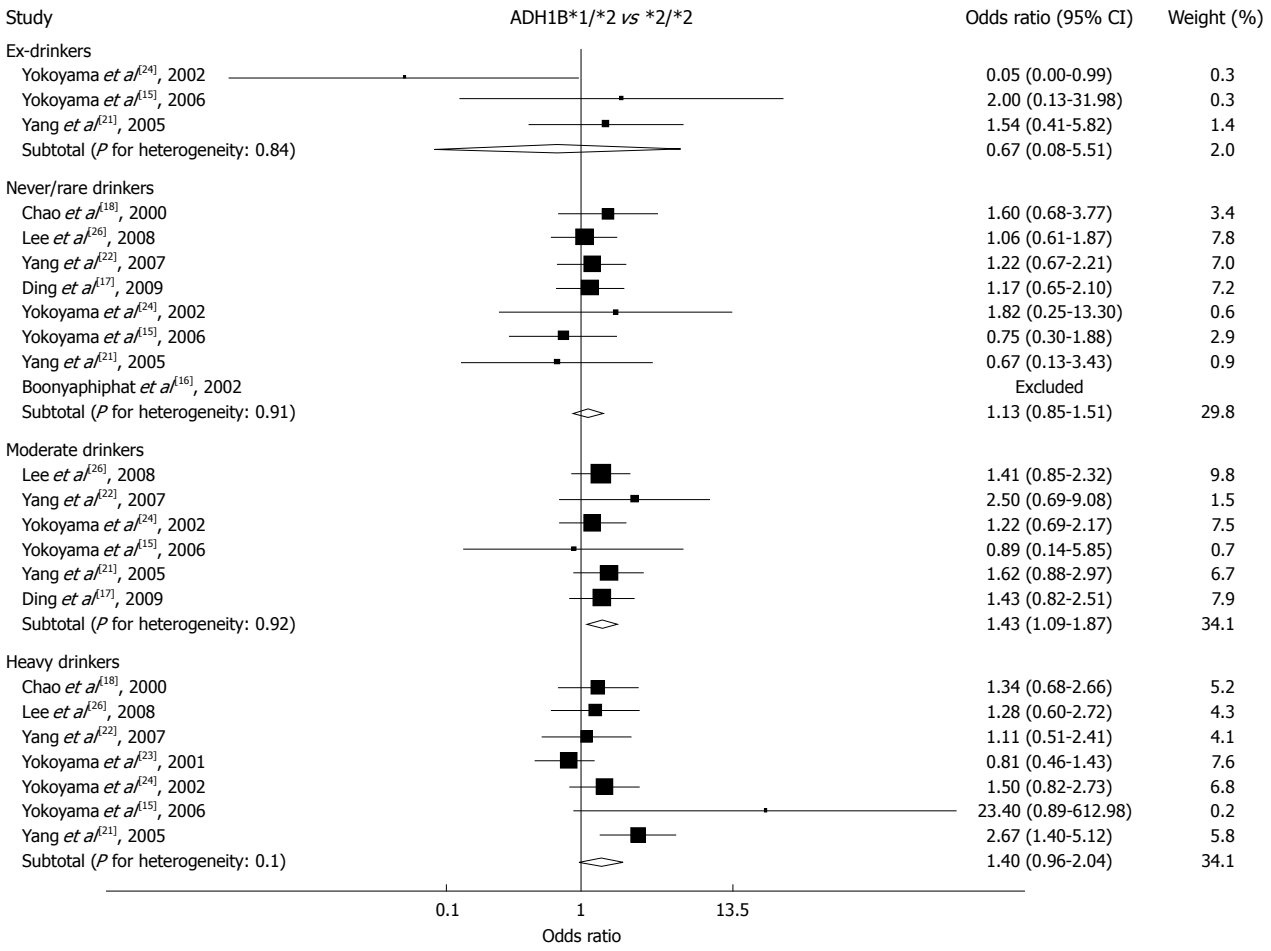

Figure 2 Relationship between alcohol dehydrogenase-1B*1/*2 and esophageal cancer stratified by alcohol consumption status. Weights are from random effects analysis. ADH1B: Alcohol dehydrogenase-1B.

analysis showed that the larger the amount of alcohol consumed by men, the greater the OR for ALDH2*1/*2 was (P = 0.003). Men with ALDH2*2/*2 also were at high risk of esophageal cancer among never/rare drinkers and drinkers [2.96 (1.61-5.44) and 3.47 (1.09-11.03), respectively].

Sensitivity analysis and publication bias

There was no evidence of publication bias in the ADH1B and ALDH2 polymorphism studies according to Egger's test, and the P values for ADH1B*1/*2, ADH1B*2/*2, ALDH2*1/*2 and ALDH2*2/*2 were 0.82, 0.21, 0.68 and 0.51, respectively. The sensitivity analysis showed that the overall ORs of the ADH1B*1/*2 genotype *vs* the ADH1B*2/*2 genotype did not appear to have changed greatly after excluding two large-sample studies [ORs = 1.36 (1.14-1.63)][24,26], and that the overall ORs of the ADH1B*1/*1 genotype *vs* the ADH1B*2/*2 genotype did not change either after removing two large-sample studies [ORs= 2.69 (1.81-3.98)][23,26]. The effect of ALDH2*1/*2 and ALDH2*2/*2 *vs* ALDH2*1/*1 on risk of esophageal cancer was not reversed and did not change greatly after removing one large-sample study [ORs = 2.51 (1.69-3.73) and 0.72 (0.36-1.45), respectively][26], indicating that the finding was robust. After dropping one large-sample study[21], ADH1B*1/*1 combined with ALDH2*1/*2 was associated with a higher risk with an OR (95% CI) of 17.02 (2.99-96.71) in heavy drinkers than ADH1B*2 combined with ALDH2*1/*1.

DISCUSSION

The results of the meta-analysis in this study shed light on some aspects of the association between ALDH2 and ADH1B genotypes and the risk of esophageal cancer that had not been clarified earlier because of the smaller numbers of subjects. After drinking alcohol, people with inactive ALDH2 experience unpleasant responses, including flushing responses[11] and hangovers[28]. The presence of inactive ALDH2 generally prevents East Asians from drinking heavily. Homozygotes for inactive ALDH2 are usually nondrinkers because of the very unpleasant responses they experience, and the previous meta-analysis showed that a protective effect of this genotype against esophageal carcinogenesis only resulted from non- or rare drinking. However, the inhibitory effect of ALDH2*2/*2 is not complete. In this meta-analysis, we found 24 cases and six controls who were drinkers and inactive ALDH2*2/*2 homozygotes, and confirmed that ALDH2*2/*2 greatly increased the risk of esophageal

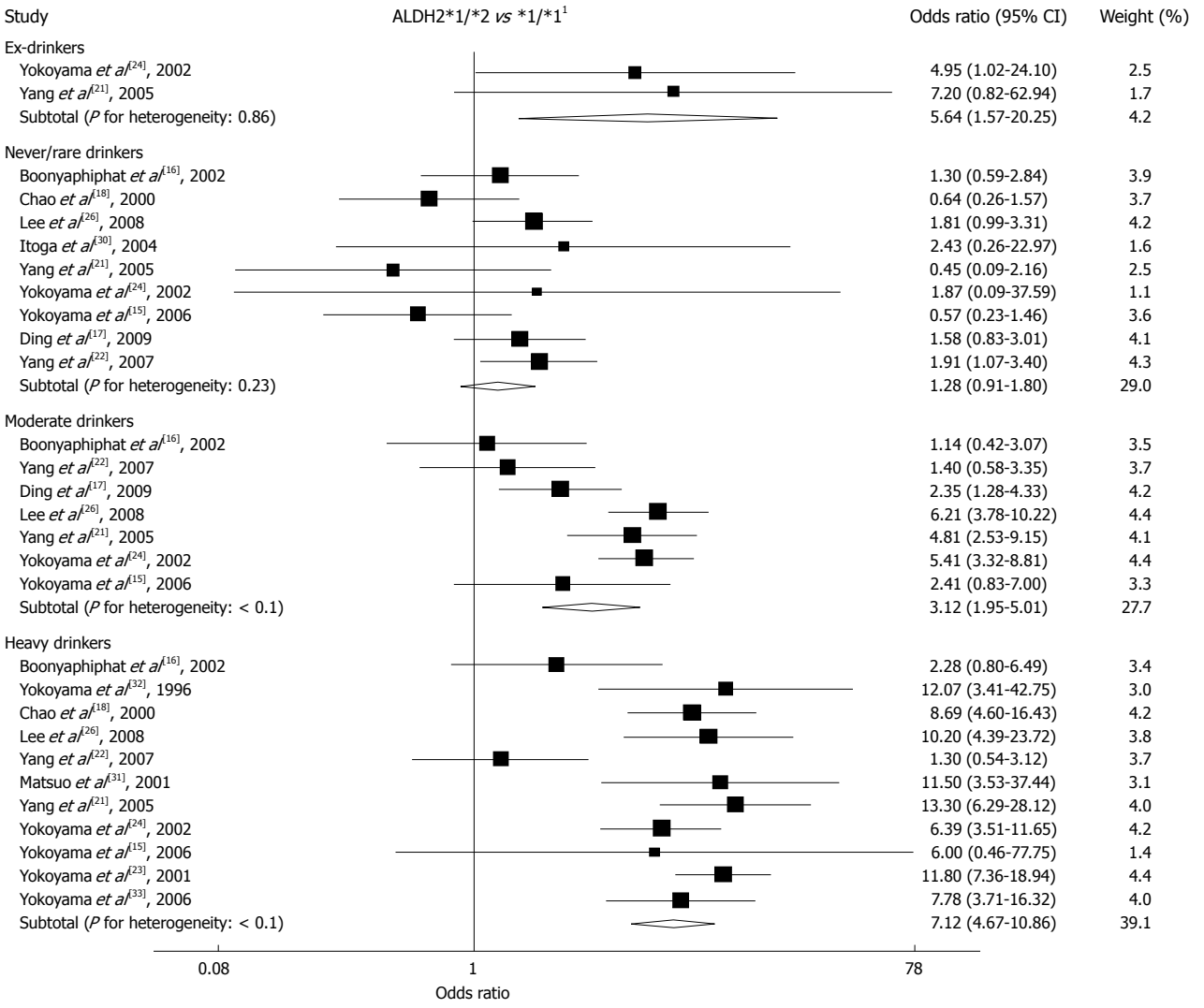

Figure 3 Relationship between aldehyde dehydrogenase-2*1/*2 and esophageal cancer stratified by alcohol consumption status. Weights are from random effects analysis. [1]Significant trend for meta-regression from never/rare drinkers to heavy drinkers is observed ($P < 0.001$). ALDH2: Aldehyde dehydrogenase-2.

cancer when the genotype carriers were drinkers (OR = 4.42, Figure 4). This finding is consistent with an earlier study that included only six cases and one control who were ALDH2*2/*2 drinkers[26], and it supports the hypothesis that acetaldehyde play a critical role in the development of esophageal cancer. Also, it suggested the genetic risk of esophageal cancer may be due to alcohol and genetic associations.

Although data regarding the duration of abstinence was unavailable, the results of the present study also confirmed that the strong effect of heterozygous ALDH2*1/*2 persisted in ex-drinkers (OR = 5.64, Figure 3). That may be explained by the "sick-quitter effect", meaning that the molecular changes that predisposed the ex-drinkers to esophageal cancer may not be able to return to the healthy status. A recent pooled analysis showed that the risk of esophagus cancer and the risk of head and neck cancer only decreased by five and 10 years, respectively, after the cessation of drinking[35]. Further study is needed to evaluate the long-term effect of drinking cessation on ALDH2-associated esophageal carcinogenesis.

Another intriguing finding was the ADH1B*1-allele effect on cancer risk. The ADH1B*2 allele acts in a semidominant manner, and the homozygous ADH1B*1/*1 enzyme exhibited 100 and 200 times lower *in vitro* activity[7] and was associated with an 11% and 18% slower ethanol elimination rate than the heterozygous ADH1B*1/*2 enzyme and homozygous ADH1B*2/*2 enzyme, respectively[36]. Most of the case-control studies have consistently shown an increased ADH1B*1/*1-associated risk of upper aerodigestive tract cancer, but there is controversy as to whether the risk appears in a dose-dependent manner as the number of ADH1B*1 alleles increases. The meta-analysis in this study showed that ADH1B*1/*1 greatly increased the risk of esophageal cancer in moderate drinkers and heavy drinkers (ORs = 2.71 and 3.22, respectively, Figure 1) and that ADH1B*1/*2 modestly but significantly increased the risk in moderate drinkers (OR = 1.43, Figure 2). These findings that the genetic risks are seen at all levels of alcohol exposure, may reveal that there is genetic risk for esophageal cancer, also show a dose-dependent relationship between the number of ADH1B*1 alleles and the risk of esophageal cancer as well. Such dose dependency has been clearly demon-

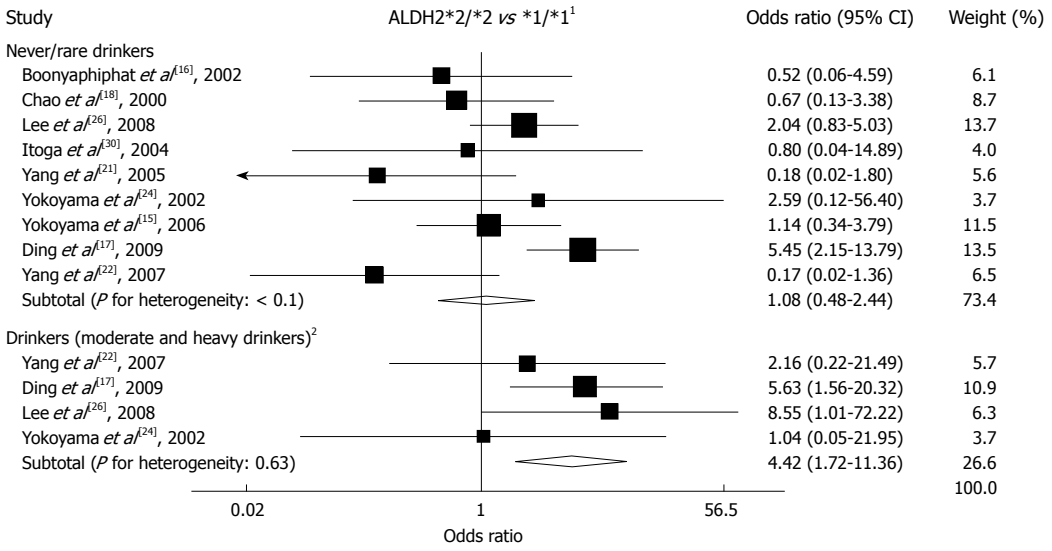

Figure 4 Relationship between aldehyde dehydrogenase-2*2/*2 and esophageal cancer stratified by alcohol consumption status. Weights are from random effects analysis. [1]Ex-drinkers were excluded because there were no cases and controls with the ALDH2*2/*2 for this drinking status; [2]Moderate and heavy drinkers were combined because of the small number of ALDH2*2/*2 drinkers. ALDH2: Aldehyde dehydrogenase-2.

Table 4 Risk of esophageal cancer associated with alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 polymorphisms according to ethnicity or sex

ADH1B	ADH1B*1/*1 *vs* *2/*2 pooled OR (95% CI)	*P* for heterogeneity	ALDH2	ALDH2*1/*2 *vs* *1/*1 pooled OR (95% CI)	*P* for heterogeneity
Ethnicity					
Chinese (Mainland)					
Never/rare drinkers[17,22]	1.20 (0.54-2.66)	0.81	Never/rare drinkers[17,22]	1.75 (1.14-2.70)	0.06
Moderate drinkers[17,22]	1.86 (0.52-6.60)	0.22	Moderate drinkers[17,22]	1.98 (1.20-3.27)	0.34
Heavy drinkers[22]	3.55 (1.01-11.83)	-	Heavy drinkers[22]	1.31 (0.54-3.12)	-
Chinese (Taiwan)					
Never/rare drinkers[18,26]	2.15 (0.99-4.69)	0.51	Never/rare drinkers[18,26]	1.14 (0.42-3.07)	-
Moderate drinkers[26]	3.93 (2.00-7.74)	-	Moderate drinkers[26]	6.21 (3.78-10.22)	0.67
Heavy drinkers[18,26]	3.36 (1.73-6.53)	0.3	Heavy drinkers[18,26]	9.21 (5.54-15.31)	0.76
Japanese					
Never/rare drinkers[15,21,24]	2.41 (0.50-11.62)	0.31	Never/rare drinkers[15,21,24,30]	0.68 (0.33-1.42)	0.55
Moderate drinkers[15,21,24]	2.18 (0.38-12.23)	0.05	Moderate drinkers[15,21,24]	4.74 (3.29-6.83)	0.4
Heavy drinkers[15,21,23,24]	4.02 (1.83-8.80)	0.64	Heavy drinkers[15,21,23,24,32,33]	9.75 (7.34-12.97)	0.69
Sex					
Male					
Never/rare drinkers[17,21,22,24,26]	2.71 (1.13-6.51)	0.24	Never/rare drinkers[17,21,22,24,26]	1.92 (1.28-2.88)	0.55
Moderate drinkers[17,21,22,24,26]	3.93 (2.43-6.36)	0.39	Moderate drinkers[17,21,22,24,26]	3.53 (1.98-6.30)	< 0.05
Heavy drinkers[21-24,26]	3.22 (2.16-4.83)	0.39	Heavy drinkers[21-24,26,32,33]	7.04 (4.18-11.85)	< 0.05
Female					
Never/rare drinkers[21,22,33]	1.48 (0.56-3.49)	0.17	Never/rare drinkers[21,22,33]	1.55 (0.50-14.78)	0.03
Moderate drinkers[21,22,33]	0.81 (0.21-3.15)	0.41	Moderate drinkers[21,22,33]	2.64 (1.24-5.68)	0.72
Heavy drinkers[22]	3.55 (1.06-11.84)	0.35	Heavy drinkers[21,33]	6.85 (1.04-44.95)	0.88

ALDH2: Aldehyde dehydrogenase-2; ADH1B: Alcohol dehydrogenase-1B; OR: Odds ratio; CI: Confidence interval.

strated in the effect of the ADH1B*1 allele on the risk of alcoholism[12,13].

Stratification according to status of alcohol consumption is essential to accurate assessment of the effects of ALDH2 and ADH1B genotypes on cancer risk. The ALDH2*2 allele plays a suppressive role in alcohol drinking, whereas ADH1B*1 plays a facilitating role. Without stratification, the OR associated with the ALDH2*2 allele is underestimated, and the OR associated with the ADH1B*1 allele is overestimated[25]. That is why the crude pooled OR is larger for ADH1B*1/*1 (OR = 2.91) than for ALDH2*1/*2 (OR = 2.52), while the alcohol-stratified OR for ALDH2*1/*2 in the moderate-to-heavy drinkers (OR = 3.12-7.12, Figure 3) is larger than for ADH1B*1/*1 (OR = 2.71-3.22, Figure 1). Moreover, the significant study-heterogeneity in ADH1B*1/*2, ALDH2*1/*2 and ALDH2*2/*2 disappeared or greatly decreased after stratification by alcohol consumption, indicating that alcohol consumption plays an important role on the effects of the two genes on cancer risk. The meta-analysis in this study demonstrated that the combination of ALDH2*1/*2 and ADH1B*1/*1 was

associated with the highest risk of esophageal cancer in heavy drinkers (OR = 12.45, Table 3).

The mechanism underlying the ALDH2*1/*2-associated increase in risk of esophageal cancer is straightforward. The WHO and IARC have concluded that the acetaldehyde associated with alcohol beverages is carcinogenic for humans (Group 1 carcinogen) and causes cancer of the esophagus and head and neck[4]. The extended tissue exposure to ethanol and acetaldehyde in persons with the ADH1B*1/*1 genotype is a plausible explanation for the high risk of esophageal cancer[8]. The distinctly high tissue exposure to, and accumulation of acetaldehyde in ALDH2*1/*2 carriers may increase the risk of esophageal cancer more greatly than, and synergistically with the prolonged tissue exposure to ethanol and acetaldehyde in the ADH1B*1 allele carriers.

Only one case-control study has focused on the cancer risk in women, and it showed a weaker increasing effect of ALDH2*1/*2 on the risk of esophageal cancer in female moderate drinkers than in male moderate drinkers and a markedly increasing effect in female heavy drinkers[15]. The small sample size in that study hampered the assessment of the effect of the ADH1B genotype in women. The meta-analysis in our study demonstrated that ALDH2*1/*2 affected the risk of esophageal cancer in a similar manner in both women and men who were moderate drinkers (ORs = 2.64 and 3.53, respectively, Table 4) or heavy drinkers (ORs = 6.85 and 7.04, respectively, Table 4). Two earlier meta-analyses showed that the magnitude of the ALDH2-effect on the risk of esophageal cancer and head and neck cancer was greater in heavy drinkers than in moderate drinkers[16,34], and the results of the present study demonstrated this phenomenon in both women and men. We also observed a significant increase in cancer risk by ALDH2*1/*2 and ALDH2*2/*2 in male never/rare drinkers alone (ORs = 1.92 and 2.96, respectively, Table 4), suggesting that the ALDH2*2-related susceptibility to esophageal cancer extends to very low dose or frequency levels of alcohol consumption in men. Similar effects of ADH1B*1/*1 on cancer risk were observed in both female and male heavy drinkers (ORs = 3.55 and 3.22, respectively, Table 4). These results are consistent with the results of a meta-analysis demonstrating a similarly high risk of esophageal cancer in women who consume alcohol at the same dose levels as men[38].

The magnitude of the ALDH2-associated risk depends on the strength of the association between the esophageal cancer and alcohol consumption evaluated. The incidence rate of esophageal cancer in the high-incidence regions in Mainland China is extremely high, e.g. 65/100 000 population in Taixing City[28], and alcohol drinking plays a less important role in esophageal carcinogenesis there than in Taiwan or Japan. The incidence rates in Taiwan and Japan are approximately 9/100 000 men and extremely low among women[37]. The four case-control studies conducted in the high-incidence regions in Mainland China showed a moderate-to-modest positive association[19,20,23] or no association[28] between heterozygous ALDH2 and esophageal cancer risk. The ALDH2*1/*2-associated risks in these Chinese studies were higher where the impact of alcohol consumption on esophageal cancer was greater and/or when only male populations were evaluated. The subgroup analysis in regard to ethnicity demonstrated that the ALDH2*1/*2 genotype was associated with a similarly very high risk of esophageal cancer among Taiwan Chinese and Japanese drinkers (OR = 4.74-6.21 in moderate drinkers, 9.21-9.75 in heavy drinkers, Table 4), but the OR of ALDH2*1/*2 in the high incidence regions of Mainland China was not so high in moderate-to-heavy drinkers (OR = 1.98 in moderate drinkers, OR = 1.31 in heavy drinkers, Table 4). However, it is possible to prevent a large number of esophageal cancer deaths by preventive strategies that include public education about high-risk drinking in Mainland China, because the incidence rates of esophageal cancer there are extremely high. Over the past 40 years, there has been a 9-fold increase in per capita alcohol consumption in Mainland China and a 2-fold increase in Japan[39]. It will be probably continue to increase in China, because current Chinese consumption is still comparable to Japanese consumption in the 1970s. The inhibitory effect of inactive heterozygous ALDH2 on heavy drinking is influenced by socio-cultural factors. Among Japanese alcoholics, the proportion of inactive ALDH2 heterozygotes has dramatically increased from 2.5% to 13% among Japanese alcoholics since the 1970s[40], and this change partially explains the alarming rate of increase in the prevalence of multiple upper aerodigestive tract cancers among Japanese esophageal cancer patients during the same period[41]. The proportion among Han Chinese alcoholics in Taiwan also increased from 10% to 18% during the 1990s[12]. Alcohol-related esophageal cancer will be an important problem in Mainland Chinese in the future.

Several limitations of this study should be considered. There was heterogeneity between the studies of ALDH2 polymorphisms, and some heterogeneity still existed after performing the subgroup analysis, suggesting that other factors should have been taken into account in the analysis. Smoking presents a critical risk factor for esophageal cancer, and drinkers also tends to smoke, and may influence the risk of drinking for esophageal cancer, but the further subgroup analysis on smoking would have been limited by the missing information in most of these studies. Five control samples were not in Hardy-Weinberg equilibrium. The disequilibrium in the controls suggests that the samples were not representative of the expected distribution of the genotypes and thus may have distorted the findings. Alcohol consumption measurement was defined by different criteria across the studies, which would have resulted in overestimation or underestimation of the ORs, but the ORs did not change greatly or reverse according to the sensitivity analysis, indicating that the results in the drinking categories are robust. Finally, some subgroups were included in only one study, and that may have lowered the power to identify the true effect. However, we did attempt to contact the authors, but some data were still unavailable because the data were missing or there was no response from the authors.

Based on the results of our meta-analysis, we concluded that the ADH1B*1-allele and ALDH2*2 allele increase the risk of esophageal cancer at all levels of exposure to ethanol and acetaldehyde after drinking and that these effects are modified by alcohol consumption, ethnicity, and gender. These findings broaden the concept of high-risk drinking and may provide a new strategic approach to the prevention of esophageal cancer.

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COMMENTS

Background

The ethanol in alcohol beverages and the acetaldehyde associated with alcohol consumption have recently been classified as Group 1 human carcinogens by the World Health Organization and International Agency For Research On Cancer. The ethanol in alcohol beverages is primarily metabolized by alcohol dehydrogenases (ADHs), including ADH1B (previously called ADH2), to acetaldehyde, and then to acetic acid, mainly by low-Km aldehyde dehydrogenase-2 (ALDH2) ADH1B*1 allele and ALDH2*2 allele have been found to be a strong positive risk factor for esophageal cancer, especially for heavy drinkers. Present study clarified the effects of ADH1B and ALDH2 genotypes alone and in combination on the risk of esophageal cancer, and evaluated how the gene effects are influenced by drinking habits, gender, and ethnicity.

Research frontiers

The effect of gene polymorphism and gene-environment interaction on esophageal cancer risk has become a hotspot in recent researches. There is no study comprehensively clarifying the effect of the two genes on the risk of esophageal cancer. Therefore, this study focused on the effect of ADH1B and ALDH2 on the risk of esophageal cancer, and its effect modification by drinking habits, gender and ethnicity.

Innovations and breakthroughs

The study comprehensively searched for all case-control studies about the effects of ADH1B and ALDH2 on the risk of esophageal cancer, and used meta-analysis to analyze the effects of ADH1B and ALDH2 genotypes alone and in combination on the risk of esophageal cancer, and evaluated how the gene effects are influenced by drinking habits, gender, and ethnicity.

Applications

The study concludes that the ADH1B*1-allele and ALDH2*2 allele increase the risk of esophageal cancer at all levels of exposure to ethanol and acetaldehyde after drinking and that these effects are modified by alcohol consumption, ethnicity, and gender. These findings broaden the concept of high-risk drinking and provide a new strategic approach to the prevention of esophageal cancer.

Terminology

ADH1B: A zinc-containing enzyme which oxidizes primary and secondary alcohols or hemiacetals in the presence of NAD. In alcoholic fermentation, it catalyzes the final step of reducing an aldehyde to an alcohol in the presence of NADH and hydrogen. ALDH2: An enzyme that oxidizes an aldehyde in the presence of NAD+ and water to an acid and NADH.

Peer review

This is a nicely done study with few limitations. The study clarifies the effects of ADH1B and ALDH2 genotypes alone and in combination on the risk of esophageal cancer by systematic review, and its effect modification by drinking habits, gender and ethnicity.

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BRIEF ARTICLE

Prevalence of irritable bowel syndrome in Chinese college and university students assessed using Rome Ⅲ criteria

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Abstract

AIM: To estimate the prevalence of irritable bowel syndrome (IBS) in college and university students of North China and certain related factors for IBS.

METHODS: A total of 2500 students from Shandong University in North China were asked in February-March 2009 to complete questionnaires, including the Rome Ⅲ questionnaire, hospital anxiety and depression scale, and IBS-quality of life questionnaire (IBS-QOL).

RESULTS: Among the 2126 students with complete data, the prevalence of IBS was 7.85% according to the Rome Ⅲ criteria, with a female/male ratio of 1.78:1. Most students had the IBS-constipation subtype (36.5%), followed by IBS-diarrhea subtype (31.1%) and IBS-mixed subtype (23.9%). The students with IBS had a higher anxiety and depression score than those without IBS. Low exercise level and anxiety indicated a high risk for IBS. The mean score of IBS patients was 74.2 ± 4.242 on the IBS-QOL.

CONCLUSION: The prevalence of IBS is 7.85% in Chinese college and university students according to the Rome Ⅲ criteria. Low exercise level and anxiety may be the risk factors for IBS.

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Key words: Irritable bowel syndrome; Prevalence; College and university students

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common, costly and potentially functional gastrointestinal disorder characterized by abdominal pain or discomfort with altered bowel habits, but without any organic damages to the intestine such as tumor or inflammation[1]. Men and women at all ages may have this disease.

The prevalence of IBS is 15%-24% in the general population of Western countries, regardless of sex, age or ethnicity, with a male/female ratio of 1:1.5[2,3]. The prevalence of IBS in most Asian communities is 5%-10%[4], which is lower than that in Western countries. The prevalence of IBS is 5.7% in Korean college and university

students[5], and 10.4% in undergraduates of Southeast China[6,7]. The prevalence of IBS has not been investigated in students of North China.

It has been shown that some factors, especially psychological factors[8], dietary habits[9], and frequency of exercise, are associated with the onset and course of IBS[5,10]. Many college and university students have psychological problems such as anxiety and depression[11,12], which can be predictive of IBS. Research is needed to compare IBS patients and healthy controls and the 3 IBS subtypes classified by bowel movement disturbance. Furthermore, many patients with IBS have a history of chronic recurrent abdominal pain from childhood[13].

The symptoms of IBS greatly affect the quality of life of patients. It was reported that the score of mental health and social function domains in patients with IBS is very low[14]. A questionnaire on health-related quality of life is an important measure to assess the impact of this chronic disease on the quality of life of IBS patients[15].

The Rome Ⅲ criteria were established in 2006 by the Rome Ⅲ Committee[1]. The Committee simplified the IBS subtype classification using the criteria for stool consistency, which is closest to the clinical criteria and increases the accurate diagnosis of IBS[1].

The prevalence of IBS has been reported most frequently in general populations, but not often in Chinese college and university students, especially in those of North China. Our study was to investigate the prevalence of IBS in college and university students of North China according to Rome Ⅲ criteria and the factors associated with IBS.

MATERIALS AND METHODS

Study setting

This investigation was carried out from February to March 2009 in Shandong University, located in North China.

Sample size

It has been reported that the prevalence of IBS in Chinese college and university students is 10%-25%. According to the established formula for sample size, we used a minimum sample size of 1800 to achieve a precision of ± 2% with a 95% confidence interval (CI)[16]. Therefore, 2500 participants were enrolled in this study.

Participants

A total of 2500 college and university students, randomly recruited from 3 areas of study (liberal arts, science, medicine), were asked to voluntarily complete questionnaires during their regularly scheduled class time. Trained staff answered any questions about the questionnaire. The study was approved by the Ethics Committee of Qilu Hospital of Shandong University, China.

Measures

Chinese version of Rome Ⅲ questionnaire: The Rome Ⅲ criteria have been widely used in the diagnosis of functional gastrointestinal disorders. We used the Chinese version of the previously validated Rome Ⅲ diagnostic questionnaire. The diagnosis of IBS was based on the presence of abdominal pain or discomfort for at least 3 mo in the previous 6 mo, with 2 or more of the following symptoms: pain improved after defecation, symptoms associated with a change in frequency of stool, and symptoms associated with a change in the form (appearance) of stool. Subjects with IBS were divided into constipation-predominant type (IBS-C), diarrhea-predominant type (IBS-D), and mixed type with IBS (IBS-M) according to the proportion of lumpy and hard stools. Other students who could not be classified into the 3 subtypes were considered a subtype without IBS (non-IBS).

Hospital anxiety and depression scale: The hospital anxiety and depression scale (HADS), a specifically reliable scale developed by Zigmond and Snaith[17], was used in estimating the emotional disorder of anxiety and depression.

The HADS is a short, self-reporting questionnaire consisting of 14 questions with two 7-item subscales for anxiety and depression assessment. The items are coded on a 4-point scale from 0 to 3 scores. Moreover, anxiety and depression scores are summed separately. For each subscale, the scores can be divided into 0-7 (normal cases), 8-10 (borderline cases), and over 11 (cases).

Daily lifestyle factors: Some questions about the daily lifestyle of college and university students, such as dietary habits, exercise frequency, and alcohol consumption, were included in the questionnaire. For dietary habits, we obtained information on the intake of dairy products and cold dishes, which may be associated with the onset of IBS.

Red-flag items: Referring to the guidelines for IBS recommended by the American Gastroenterological Association, there are 7 red-flag items used to distinguish organic intestinal disease from IBS. Participants who chose one or more of the 7 items were included into a special "others" group: drastic weight loss, a history of organic bowel disease, a history of digestive surgery, blood in stool, awakening due to abdominal pain during night, anemia, fever or arthralgia.

Negative life events in childhood: A life event scale was used in this study[18]. Considering the specific situations of college and university students in China, we assessed 5 of the most stressful events in childhood (occurred under the age of 12 years) on the scale, including death of a close family member or divorced parents, extreme financial difficulty, major natural disaster, experiencing serious illness or major surgery, and other major setbacks. Whether students with IBS have recurrent abdominal pain in childhood was also assessed.

Quality of life of patients with IBS: The quality of life of patients with IBS (QOL-IBS patients), a self-reported

quality-of-life measure specific to people with IBS, was used to assess the impact of IBS on the quality of life of IBS patients. The questionnaire consists of 34 items, each with a 5-point response scale. The scores are summed and averaged for a total score and then transformed to a 0-100 scale for ease of interpretation. The score of the quality-of-life scale is a general parameter reflecting the healthy status of IBS patients, with some questions about health care-seeking behavior.

Baseline information: The remaining questions were about the sociodemographic characteristics, such as age, sex, schooling level, place of birth, health condition, and family information.

Statistical analysis

All eligible questionnaires were coded. Data analysis was performed using the SPSS 12.0 (SPSS Inc., Chicago, IL). Distributions of sex and lifestyle factors were analyzed by Pearson's χ^2 or Fisher's exact test. For each of the lifestyle factors and psychological measures, the choice or scores of participants were divided into 3 degrees. Analysis of variance was used to compare the anxiety and depression levels between groups. Possible risk factors were assessed by logistic regression analysis. Odds ratio (OR) with 95% confidence interval (CI) was calculated. Data are presented as mean ± SD. All calculated *P*-values were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Response rate and characteristics of subjects

Of the 2500 enrolled participants, 2376 completed the questionnaires. Valid responses were obtained from 2126 participants, with a response rate of 89.48%. Of the 2376 participants, 917 (43.1%) were males and 1209 (56.9%) were females, with a mean age of 20.79 ± 1.590 years and 20.53 ± 1.587 years, respectively. The average college grade of the students was 2.58 ± 0.368. Of the 2376 participants, 1768 (74.4%) were natives of Shandong Province. The characteristics of participants are listed in Table 1. The non-IBS group included healthy subjects and the "others" group (participants with other gastrointestinal disorders excluded).

Prevalence of IBS

Of the 2126 participants, 167 fulfilled the Rome Ⅲ criteria for IBS with a male/female ratio of 1.78:1. The prevalence of IBS was 7.85%, with no statistically significant difference among areas of study or schooling level. Sixty-one cases were IBS-C (36.5%), 51 were IBS-D (31.1%), 40 were IBS-M (23.9%), and 25 were non-IBS cases (8.5%).

Daily lifestyle factors

The daily lifestyle factors, including dietary habits and exercise frequency, were compared between IBS and non-IBS groups (Table 2). No difference was found in measures related to the intake of dairy products, cold dishes and alcohol between the two groups, but a difference was observed in exercise level, indicating that a low exercise level is a high risk factor for IBS.

Table 1 Characteristics of students with or without irritable bowel syndrome *n* (%)

	Total (*n* = 2126)	IBS (*n* = 167)	Non-IBS (*n* = 1959)	χ^2	*P* value
Sex					
Male	917 (43.1)	60 (6.5)	857 (93.5)	3.835	0.05
Female	1209 (56.9)	107 (8.9)	1102 (91.1)		
Branch of learning					
Liberal arts	701 (33.0)	43 (6.1)	658 (93.9)	5.119	0.077
Science	697 (32.8)	56 (8.0)	641 (92.0)		
Medicine	728 (34.2)	68 (9.3)	660 (90.7)		
Schooling level					
Lower	1425 (67.0)	117 (8.2)	1308 (91.8)	0.754	0.385
Upper	701 (33.0)	50 (7.1)	651 (92.9)		

IBS: Irritable bowel syndrome; Non-IBS: Subjects without IBS.

QOL-IBS patients and health care-seeking behavior

The mean score of students with IBS was 74.2 ± 4.242 on the health quality questionnaire, indicating that the occurrence of IBS can influence their quality of life and daily health.

Of the participants with IBS, 38.3% did not seek for medical advice on their abdominal discomfort, 1.2% received long-term treatment, and 3.0% worried about their health. No difference was found in seeking for help from physicians between medical students and others.

HAD scores and negative life events in childhood

The anxiety and depression scores were significantly higher for students with IBS than for those without IBS (Table 3), especially for those with normal scores (0-7) than for those with abnormal scores (> 11). Of the participants with IBS, 65.9% believed that their discomfort was related to the negative moods.

The mean anxiety score was 6.52 ± 4.036, 6.78 ± 4.912, and 5.82 ± 4.545, respectively, for IBS-C, IBS-D, and IBS-M ($F = 0.373$, $P = 0.773$). The mean depression score was 6.92 ± 3.822, 6.88 ± 4.097, and 6.90 ± 4.787, respectively, for IBS-C, IBS-D, and IBS-M ($F = 0.008$, $P = 0.999$). No difference was found in anxiety and depression scores between the 3 subtype groups.

Of the participants with IBS, 47.3% had a recurrent abdominal pain in childhood.

Risk factors for IBS

After univariate analysis, multivariable logistic regression analysis was adjusted by sex. Anxiety ($P = 0.031$) but not depression ($P = 0.329$) was independently associated with IBS (Table 4).

DISCUSSION

To our knowledge, this is the first school-based investigation on the epidemiology of IBS in college and university students of North China. The prevalence rate of IBS was

Table 2 Dietary and lifestyle factors in irritable bowel syndrome and non-irritable bowel syndrome groups *n* (%)

Variables	Categories	Total (*n* = 2126)	IBS (*n* = 167)	Non-IBS (*n* = 1959)	χ^2	*P* value
Dairy produce						
	Almost everyday	676 (31.8)	57 (8.4)	619 (91.6)	0.456	0.796
	Sometimes	1305 (61.4)	99 (7.6)	1206 (92.4)		
	Almost never	145 (6.8)	11 (7.6)	134 (92.4)		
Cold dish	Percentage per day					
	< 25%	1650 (77.6)	132 (8.0)	1518 (92.0)	0.512	0.774
	25%-75%	468 (22.0)	34 (7.3)	434 (92.7)		
	> 75%	8 (0.4)	1 (12.5)	7 (87.5)		
Alcohol						
	No	1912 (89.9)	149 (7.8)	1763 (92.2)	0.102	0.750
	Yes	214 (10.1)	18 (8.4)	196 (91.6)		
Exercise frequency					9.823	0.007
	Almost everyday	160 (7.5)	11 (6.9)	149 (93.1)	0.121	0.728
	Sometimes	1039 (48.9)	64 (6.2)	975 (93.8)	1.479	0.224
	Almost never	927 (43.6)	92 (9.9)	835 (90.1)	9.505	0.002

IBS: Irritable bowel syndrome; Non-IBS: Subjects without IBS.

Table 3 Univariate analysis of some factors in irritable bowel syndrome and non-irritable bowel syndrome groups *n* (%)

Factors	Categories	Total (*n* = 1902)	IBS (*n* = 167)	Non-IBS (*n* = 1735)	χ^2	*P* value
Anxiety					60.431	0.000
	0-7	1496 (78.7)	103 (6.9)	1393 (93.1)	2.864	0.091
	8-10	253 (13.3)	25 (9.9)	228 (90.1)	61.053	0.000
	⩾ 11	153 (8.0)	39 (25.5)	114 (74.5)	17.493	0.000
Depression					35.597	0.000
	0-7	1357 (71.3)	94 (6.9)	1263 (93.1)	2.549	0.110
	8-10	326 (17.2)	31 (9.5)	295 (90.5)	35.894	0.000
	⩾ 11	219 (11.5)	42 (19.2)	177 (80.8)	10.557	0.001
Negative life events in childhood	Normal	967 (50.8)	99 (10.2)	868 (89.8)	6.282	0.987
	Mild	626 (32.9)	48 (7.7)	578 (92.3)		
	Moderate	273 (14.4)	19 (7.0)	254 (93.0)		
	Severe	36 (1.9)	1 (2.8)	35 (97.2)		

IBS: Irritable bowel syndrome; Non-IBS: Subjects without IBS.

Table 4 Evaluation of risk factors for irritable bowel syndrome by multivariate logistic regression analysis

Variables	β	Standard error	Wald	Odds ratio	95% CI		*P* value
					Lower	Upper	
Anxiety	0.106	0.027	15.383	1.112	1.054	1.172	0.031
Depression	0.028	0.029	0.951	1.028	0.972	1.088	0.329

CI: Confidence interval.

7.85% and more female students suffered from IBS than male students, which is similar to that in previous studies[2,3].

The prevalence of IBS varied greatly among different investigations, which may be due to the differences such as varied study population from different countries and different diagnostic criteria used. Rome Ⅱ criteria have been used to examine a 12-wk period of time in the past 12 mo, which is less than a continuous 6-mo period, thus expanding the scope of diagnosis[19]. Furthermore, IBS is a functional disease with organic intestinal damage excluded. The usual method of using questionnaires for a large population without any further examination is too simple to judge the accuracy of diagnosis.

In the general population, IBS of the diarrhea-dominant type is more frequent than the constipation-dominant type[1]. However, the results of our study are opposite. Most college and university students have irregular eating habits and bedtimes and are also burdened with jobs or school work.

In the present study, the anxiety and depression score was high in IBS group, indicating that anxiety is a predictor of IBS diagnosis and psychological factors play an important role in development of IBS[8,11,12,19]. It has been shown that although IBS symptoms influence negative moods such as anxiety and depression, psychological factors themselves influence motor abdominal functions, sensory threshold and stress reactivity of the intestine[20].

It was reported that cold diets, such as ice water and cold dishes, can induce IBS onset by influencing visceral hypersensitivity[21]. However, we did not find any significant correlation between intake of cold dishes and IBS in college and university students which may be due to the change in dietary habits in young Chinese people who are accustomed to a cold diet in childhood. In this study, about 50% of the IBS patients complained of pediatric recurrent abdominal pain, which is similar to the reported findings[13].

No research prior to this study has directly assessed the important role of exercise frequency in development of IBS. In this study, the students with a low exercise level were more likely to have IBS symptoms. Nowadays, many college and university students are too busy to do physical exercises. Some students with IBS are restrained in their desire to do physical exercise, perhaps because of chronic abdominal pain or discomfort. A positive association between exercise level and course of IBS should be interpreted with caution.

Although many patients with IBS do not seek for medical advice on discomfort, they have significant demands. It was reported that medical students are more likely to see physicians for their discomfort, perhaps because they worry more about their health[22], which is not consistent with the findings in our study. The psychological factors we describe may be responsible for such a behavior.

IBS is not a life threatening disease, but as a functional intestinal disease, its chronic symptoms greatly influence their daily life[3,14,23], which is consistent with the finding in this study. Most students with IBS have more or less troubles with their health.

Although this investigation provided some new insights into IBS in Chinese college and university students, it was based on only the self-reporting questionnaires, without any further examinations to exclude organic intestinal diseases.

The public and university students in China are not informed about digestive health and IBS. Considering some predictive factors for the onset of IBS, more attention must be paid to the primary prevention of IBS in the public and university students.

In conclusion, the prevalence of IBS in college and university students of North China is 7.85%. A low exercise level and anxiety are the risk factors for IBS. IBS greatly influences the quality of life of students.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder characterized by abdominal pain or discomfort with altered bowel habits. Its prevalence is 15%-24% in the general population of Western countries.

Research frontiers

Many studies focused on the related factors for the prevalence of IBS, such as age, gender, family history, negative mood and daily lifestyle.

Innovations and breakthroughs

This is the first school-based investigation on the epidemiology of IBS in college and university students of North China. The prevalence rate of IBS was 7.85% using the Rome Ⅲ criteria, which is consistent the previous findings.

Applications

The questionnaire used in the program was a comprehensive Chinese version. It will be useful in the future.

Terminology

IBS is a common, costly and potentially functional gastrointestinal disorder characterized by abdominal pain or discomfort with altered bowel habits, but without any organic damages to the intestine such as tumor or inflammation.

Peer review

The authors investigated the prevalence of IBS in Chinese college and university students using the Rome Ⅲ criteria, and found that a low excise level and anxiety are the risk factors for IBS. This article is well written with excellent readability.

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BRIEF ARTICLE

Efficacy evaluation of imatinib treatment in patients with gastrointestinal stromal tumors: A meta-analysis

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Abstract

AIM: To perform a meta-analysis to derive a more precise estimation of imatinib treatment for different genotypes of gastrointestinal stromal tumors (GIST).

METHODS: Studies were identified by searching PubMed and Embase. Inclusive criteria were patients with exon 9-mutant, exon 11-mutant or wide type (WT) GIST, receiving chemotherapy of imatinib for clinical trial, and efficacy evaluation was cumulative response (CR) including complete response and partial response. The odds ratios (OR) for CR in stem cell factor receptor (KIT) mutation patients *vs* WT genotype patients, KIT exon 11-mutant genotype patients *vs* KIT exon 9-mutant genotype patients and KIT exon 9-mutant genotype patients *vs* WT genotype patients were calculated with 95% confidence interval (CI) for each study as an estimation of the efficacy of imatinib.

RESULTS: Five studies including 927 patients were involved in this meta-analysis. The overall OR (KIT group *vs* WT group) was 3.34 (95% CI: 2.30-4.86, $P < 0.00001$, $P_{heterogeneity} = 0.04$). The overall OR in KIT exon 11 group *vs* KIT exon 9 group was 3.29 (95% CI: 2.17-5.00, $P < 0.00001$, $P_{heterogeneity} = 0.33$). The overall OR in KIT exon 9 group *vs* WT group was 1.23 (95% CI: 0.73-2.10, $P = 0.44$, $P_{heterogeneity} = 0.42$).

CONCLUSION: Most patients with different genotypes of GIST and KIT exon 11-mutant will benefit from the individualized treatment of imatinib.

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Key words: Gastrointestinal stromal tumors; Gene; Imatinib; Efficacy; Meta-analysis

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Chen P, Zong L, Zhao W, Shi L. Efficacy evaluation of imatinib treatment in patients with gastrointestinal stromal tumors: A meta-analysis. *World J Gastroenterol* 2010; 16(33): 4227-4232 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i33/4227.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i33.4227

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is a rare tumor, but the most common mesenchymal malignancy of the gastrointestinal tract[1]. GIST expresses the tyrosine kinase receptor KIT, which is the protein product of the KIT proto-oncogene. GIST is generally characterized by gain-of-function mutations of KIT[2]. These mutations result in the constitutive activation of KIT signaling and are the likely causal molecular events of GIST[3,4]. No effective systemic treatment is available. Imatinib (STI571) inhibits a similar tyrosine kinase, BCR-ABL, leading to responses in chronic myeloid leukemia, and has also been shown to inhibit KIT.

Imatinib, an active tyrosine kinase inhibitor against

KIT and platelet-derived growth factor receptor, has been shown to be highly effective in the treatment of advanced GIST. Clinical benefit was demonstrated in more than 80% of patients, resulting in a substantial improvement in the 2-year survival rate from 26% to 76%[5,6]. Imatinib has, therefore, become the standard of care in patients with advanced GIST. However, secondary resistance to imatinib often occurs within the first or second year of treatment[7], which indicated the need for differential treatment of patients with GIST. According to the previous reports, laboratory studies revealed significant molecular heterogeneity among GIST. Notably, 75%-85% of GIST had an activating mutation of KIT, 5%-7% had an activating mutation of the homologous PDGFRA kinase, and approximately 12%-15% of GIST did not have a detectable mutation of either Kinase[8-10]. And several studies have been designed to test the sensitivity of imatinib to different genotypes of GIST. Therefore, we made a meta-analysis of response to different genotypes to identify which one is more sensitive to imatinib.

MATERIALS AND METHODS

Publication search

Two electronic databases (PubMed and Embase) were searched (the last search was done on January 1, 2010, using the terms: "gastrointestinal stromal tumor" and "imatinib"). All eligible studies were retrieved, and their bibliographies were checked for other relevant publications. Only published studies with full-text articles were included. When more than one of the same patient population was included in several publications, only the most recent or complete study was used in this meta-analysis.

Inclusion criteria

The inclusion criteria were as follows: (1) assessing the efficacy of imatinib in treatment of patients with different genotypes of GIST; (2) clinical trial studies; and (3) sufficient data for estimating an odds ratio (OR) with 95% confidence interval (CI).

Data extraction

Information was carefully extracted from all eligible studies. The following data were collected from each study: first author's surname, publication date, treatment protocols and total number of KIT mutation cases, KIT exon 11 cases, KIT exon 9 cases and WT cases, and numbers of KIT mutation cases, KIT exon 11 cases, KIT exon 9 cases and wild type (WT) cases, with the clinical CR after the treatment of imatinib, respectively. We did not define any minimum number limit of patients to include a study in our meta-analysis.

Statistical analysis

Odd ratios with 95% CI were used to assess the efficacy of imatinib in treatment of patients with different genotypes of GIST according to the method of Woolf. Heterogeneity assumption was checked by the χ^2-based Q test. $P > 0.10$ for the Q test indicates a lack of heterogeneity among studies, so the OR estimate of each study was calculated by the fixed-effects model (the Mantel-Haenszel method). Otherwise, the random-effects model (the DerSimonian and Laird method) was used. The significance of the pooled OR was determined by the Z test and $P > 0.05$ was considered as statistically significant. Sensitivity analyses were carried out to check if modification of the inclusion criteria of this meta-analysis affected the final results. Potential publication bias was estimated by the funnel plot, in which the OR of each study was plotted against its log. An asymmetric plot suggests a possible publication bias. Funnel plot asymmetry was assessed by the method of Egger's linear regression test, and funnel plot asymmetry on the natural logarithm scale of the OR was measured by a linear regression approach. The significance of the intercept was determined by t test ($P < 0.05$ was representative of statistically significant publication bias). All the statistical tests were performed with Review Manager Version 4.2 (The Cochrane Collaboration, Oxford, England) and STATA version 9.2 (Stata Corporation, College Station, TX, USA).

RESULTS

Study characteristics

Five publications met the inclusion criteria. The study by Blanke *et al*[11] was excluded due to the fact that it only revealed the prognostic factor and so did the study by Tzen *et al*[12]. Likewise, the study by Andersson *et al*[13] was excluded because the study was designed for a random, double-blind, 400 mg *vs* 600 mg imatinib controlled trial only used to prove the effective dosage to treat GIST. Hence, five groups including 927 patients were used in the pooled analyses. Table 1 lists the studies identified and their main characteristics. Of the five groups, sample sizes ranged from 32 to 392. Almost all of the patients with GIST were confirmed by histology and immunohistochemistry, and DNA sequence was identified by polymerase chain reaction technique. No significant differences were found in the age distributions and sex difference among all the studies.

Meta-analysis results

Overall meta-analysis indicated that the cumulative response of KIT mutation group to imatinib was significantly different compared with that of WT group (OR 3.34, 95% CI: 2.30-4.86; $P < 0.00001$, $P_{heterogeneity} = 0.04$) (Figure 1A). A significant heterogeneity was found by simply comparing those five combined samples ($P < 0.10$). The overall OR for KIT exon 11 group *vs* KIT exon 9 group and KIT exon 9 group *vs* WT group were 3.29 (95% CI: 2.17-5.00, $P < 0.00001$, $P_{heterogeneity} = 0.33$) and 1.23 (95% CI: 0.73-2.10, $P = 0.44$ $P_{heterogeneity} = 0.42$), respectively (Figure 1B and C). Although the CR in the study of Wardelmann *et al*[15] and Yeh *et al*[16] did not follow the tendency of other studies, the corresponding pooled OR was not materially altered with or without these two studies. No other single study influenced the pooled OR qualitatively as indicated by sensitivity analyses (data not shown).

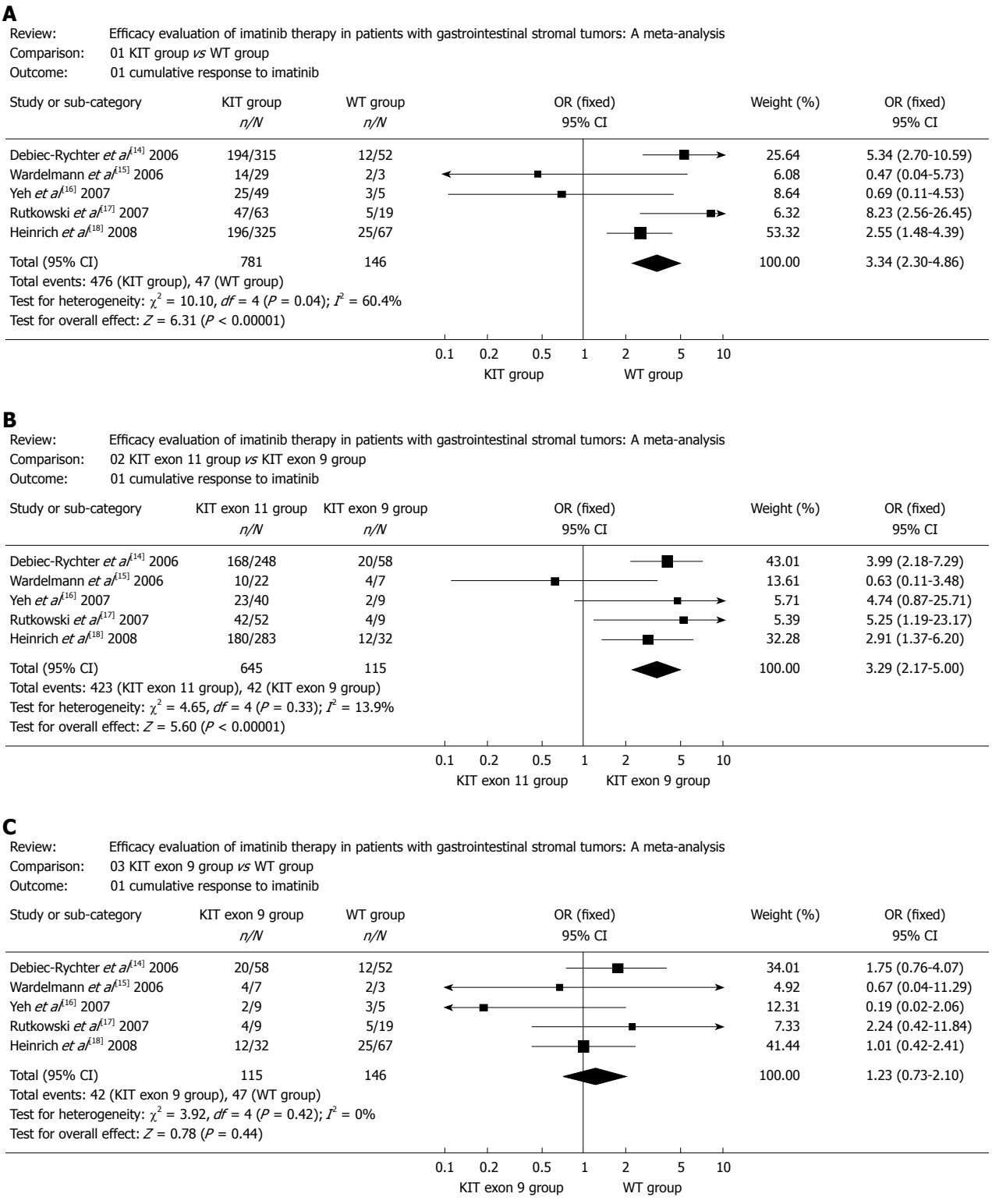

Figure 1 Meta-analysis. A: KIT group *vs* wide type (WT) group; B: KIT exon 11 group *vs* KIT exon 9 group; C: KIT exon 9 group *vs* WT group. OR: Odds ratios.

Publication bias

Begg's funnel plot was performed to access the publication bias of literatures. The shapes of the funnel plots did not reveal any evidence of obvious asymmetry (Figure 2A-C).

DISCUSSION

Before the introduction of imatinib mesylate (formerly known as STI571), poor responses to radiotherapy and chemotherapy made surgery the only realistic treatment to cure GIST[19-21].

Molecularly targeted therapy with imatinib can inhibit the etiologic aberrant cell signaling mechanisms in GIST, leading to major objective responses and prolonged disease control. Patients experienced a dramatic response, supporting the rational use of imatinib in this disease.

Table 1 Main characteristics of all studies included in the meta-analysis

Author	Dose distribution	Cumulative response (%)	Genotype	*n*			
				KIT	Exon 11	Exon 9	WT
Debiec-Rychter *et al*[14], 2006	400 mg/800 mg	56	Exon 11, 9, 13, 17 WT	315	248	58	52
Wardelmann *et al*[15], 2006	NA	50	Exon 11, 9 WT	29	22	7	3
Yeh *et al*[16], 2007	400 mg	52	Exon 11, 9 WT	49	40	9	5
Rutkowski *et al*[17], 2007	400 mg/800 mg	63	Exon 11, 9, 13, 17 WT	63	52	9	19
Heinrich *et al*[18], 2008	400 mg/800 mg	56	Exon 11, 9, 8, 13, 17 WT	325	283	32	67

WT: Wide type; NA: Not available.

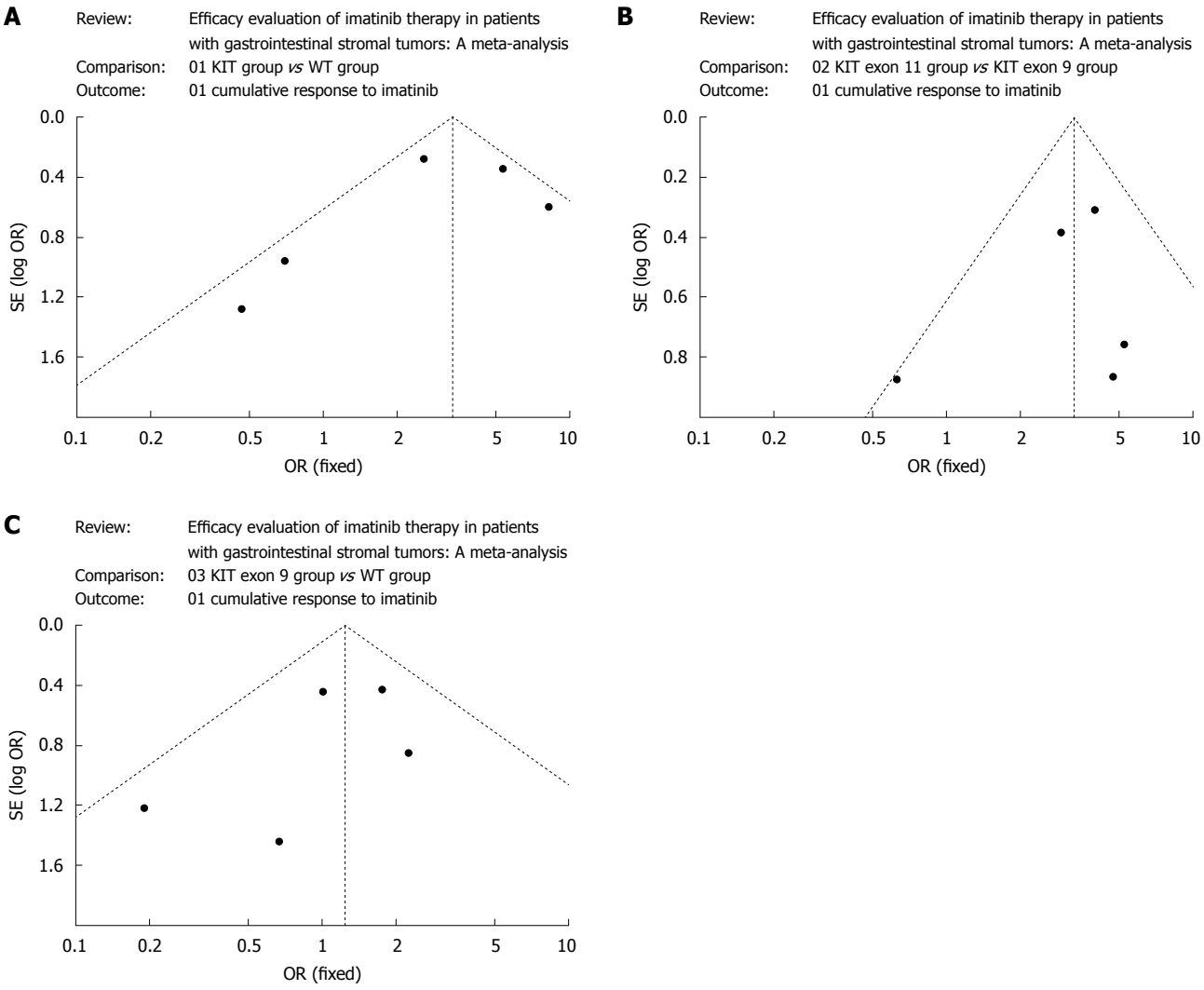

Figure 2 Begg's funnel plot for publication bias test. A: KIT group *vs* wide type (WT) group; B: KIT exon 11 group *vs* KIT exon 9 group; C: KIT exon 9 group *vs* WT group. OR: Odds ratios.

Prior studies have noted that imatinib can be effectively and safely administered. Imatinib has, therefore, become the standard of care in patients with advanced GIST. However, the secondary resistance to imatinib often occurs within the first or second year of treatment, which indicated the need for differential treatment protocol for patients with GIST. According to previous reports, laboratory studies revealed significant molecular heterogeneity among GIST. Notably, 75%-85% of GIST had an activating mutation of KIT, 5%-7% had an activating mutation of the homologous PDGFRA kinase, and approximately 12%-15% of GIST did not have a detectable

mutation of either kinase. Mutations in KIT exon 11 were the most common imatinib-target mutation found among the confirmed and unconfirmed CD117-positive GISTs (71.3%), followed by mutations in KIT exon 9 (8.2%), KIT exon 13 (1.2%), PDGFRA exon 18 (1.2%), and KIT exon 17 (approximately 1%)[18]. Several studies have been designed to test the sensitivity of different genotypes of GIST to imatinib. Therefore, we made a meta-analysis of response to imatinib at different genotypes to identify which one is more sensitive to imatinib.

Objective tumor response was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST)[22]. The best clinical response to imatinib was classified as cumulative response (CR) including complete response and partial response, stable disease, progressive disease, or not assessable. The conclusion shows that KIT mutation genotype correlates with improved treatment outcome when compared with WT genotype for cumulative response. Furthermore, patients whose tumor had a KIT exon 11 mutation were significantly more likely to achieve a CR than patients with tumors having a KIT exon 9 mutation, or WT genotype. There was no statistically significant difference in the likelihood of achieving a CR for patients with KIT exon 9-mutant GIST compared with WT GIST. Our findings confirmed that KIT exon 11 mutation is a positive predictive factor for cumulative response.

According to some phase Ⅲ trials that were designed to compare 400 mg and 800 mg daily doses of imatinib, 400 mg remains the standard starting dose[23,24]. The survival of the patients with exon 9-mutant, exon 11-mutant or WT GIST was not affected by imatinib dosage. However, there was evidence of improved response rates for patients with exon 9-mutant tumors treated with imatinib 800 mg *vs* 400 mg (complete response/partial response, 67% *vs* 17%, $P = 0.02$)[16]. Remarkably, patients with tumors expressing an exon 9 mutant KIT protein show significant imatinib dose dependency for CR as compared with patients with tumors harbouring mutant exon 11 or wild-type KIT isoforms. These results suggest that 400 mg imatinib should be administered twice a day to patients with tumors bearing KIT exon 9 mutations. Other patients could safely start at an initial imatinib dose of 400 mg once daily, and increase to 800 mg when there is evidence of disease progression.

It appears that the WT expression of KIT is not sufficient to confer the antitumor activity of imatinib mesylate. Thus, inhibiting a normal target may not have antitumor activity if the target does not provide an essential function to the tumor cell. Therefore, identification of molecular abnormalities that are essential for tumorigenesis will help develop new anticancer therapies.

COMMENTS

Background

Gastrointestinal stromal tumor (GIST) commonly shows oncogenic activating mutations of the KIT tyrosine kinase. Imatinib mesylate, a small-molecule inhibitor of BCR-ABL, KIT and PDGFR tyrosine kinases, targets the aberrant signaling pathways that are critical for tumor cell proliferation and survival. Recent advances in understanding the molecular pathogenesis of GIST has led to the remarkably successful use of imatinib in the treatment of advanced tumors, inducing high response rates resulting in unprecedented improvement in the overall survival of the patients. Although several studies reported clinical response to imatinib with the mutational status to explore if the response to imatinib is linked to tumor genotype, a small sample can not provide persuasive evidence.

Research frontiers

Several studies with limited samples have concluded that clinical response to imatinib may correlate with mutational status. It is essential to give a personalized treatment by genotypes so as to improve the effectiveness in clinical treatment of GIST.

Innovations and breakthroughs

In the previous studies, it was found that the reason why a small sample size could not supply remarkable evidence to prove the response to imatinib may be linked to tumor genotype. The statistical analysis of a large collection of analysis results from individual studies for the purpose of integrating the findings. Meta-analysis is a statistical technique for assembling the results of several studies in a review into a single numerical estimate so as to provide a best evidence in making decisions about the treatment of individual patients.

Applications

The results indicate that most patients with different genotypes of GIST and KIT exon 11 mutant will benefit from the personalized treatment of imatinib.

Peer review

In this report, Chen *et al* performed a meta-analysis to confirm the prognostic importance of KIT mutation exon location with respect to imatinib sensitivity. Although this work does not add new information beyond what is already known, it is confirmatory and potentially useful for other investigators in this field.

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CASE REPORT

Agressive inflammatory myofibroblastic tumor of the liver with underlying schistosomiasis: A case report

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Abstract

Inflammatory myofibroblastic tumor (IMT) occurs infrequently in the liver. It is controversial whether it represents a low grade mesenchymal neoplasm or a reactive inflammatory lesion. Local recurrence and metastasis are rare and some tumors are associated with infectious agents. We report on a case of a large and partially resected IMT with local recurrence and diaphragm and kidney infiltration detected on routine surveillance two years later. Histologically, the tumor showed spindle cells without atypia, mitosis or necrotic areas in a myxoid and collagenized background with inflammatory cells. In the liver portal tracts, granulomatous lesions with viable eggs of *Schistosoma mansoni* were identified. Immunohistochemistry demonstrated spindle cells which were smooth-muscle actin and vimentin positive. In conclusion, this case points out that these histological patterns do not predict the aggressive biological behavior of the lesion. A reason for the recurrence and the infiltration may be incomplete tumor resection. Further investigation is necessary in order to better clarify an infectious cause in some IMTs.

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Key words: Inflammatory myofibroblastic tumor; Liver; Recurrence; *Schistosoma mansoni*

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INTRODUCTION

Inflammatory myofibroblastic tumor (IMT) is a lesion composed of myofibroblastic spindle cells, plasma cells, lymphocytes, and eosinophils. It can occur in soft tissues and visceras[1]. It was previously called plasma cell granuloma, inflammatory myofibrohistiocytic proliferation and inflammatory pseudotumor, but IMT is the designation currently used[1]. IMT is more frequently described in the lung and abdomen of young patients, but it can also be found in the central nervous system, salivary glands, larynx, bladder, breast, spleen, skin and liver[1].

Pack and Backer published the first case occurring in the liver[2]. Since then, reports on IMT in the liver with different progression have been described[3]. Concerning pathogenesis, it is controversial whether IMT is a neo-

plasm or a reactive pseudotumoral lesion[4]. The inflammatory pseudotumor has been associated with trauma, auto-immune disease, and infectious disease[5-9]. Recently, anaplastic lymphoma kinase (ALK) gene translocations or ALK protein expression in IMT has been reported, mainly in young patients[10].

Differential diagnosis of malignant disease is sometimes difficult. The clinical presentation is frequently associated with local mass and upper abdominal pain, as well as jaundice, intermittent fever, and weight loss[1,11]. Surgical resection is the principal treatment, but corticosteroids and nonsteroidal anti-inflammatory therapy are sometimes used[12].

Here, we report on a case of a large incompletely resected IMT, with local recurrence and kidney and diaphragm infiltration on routine surveillance two years later.

CASE REPORT

A 40-year-old Brazilian woman born in northeast Brazil, presented with abdominal pain, fatigue and weight loss of 2 kg. She denied having fever or abdominal trauma, and she was receiving oxamniquine therapy for hepatic schistosomiasis. On physical exam, she presented with a hard mass that occupied the superior right quadrant of the abdomen and was painful at superficial and deep palpation. It was difficult to distinguish it from the liver that seemed enlarged primarily in its right lobe. Laboratory exams showed a cholestatic pattern, with elevated alkaline phosphatase (1176 U/L) and γ glutamyl transpeptidase (1341 U/L). The aminotransferases were also abnormal (aspartate transaminase = 108 mg/dL and alanine transaminase 92 mg/dL). Alpha-feto-protein, CEA, CA19.9 and CA125 levels were all normal.

At the ultrasound exam, a large isoechoic mass was identified in the 5th, 6th and 8th segments of the liver, and there was no mechanical obstruction of the biliary tract. Abdominal computer tomography (CT) confirmed its localization in the liver and described a close contact with the inferior caval vein. Laparotomy was indicated, and a right lobectomy of the liver was performed, but the tumor was incompletely resected because it was in contact with a large vessel. Two years later, routine CT scan detected local recurrence with diaphragm infiltration and a well defined mass in the right kidney. Again, the tumor was partially resected (including a right nephrectomy).

The gross appearance revealed a right hepatectomy specimen with a large mass measuring 11 cm (Figure 1) and other multinodular masses measuring 31 cm × 23 cm × 10 cm (Figure 2), which altogether weighed 3 kg. Those lesions were well circumscribed, and the cut surface was whorled, firm, and shining, with a myxoid aspect and a whitish or yellowish appearance (Figures 1 and 2). The adjacent liver parenchyma was unremarkable. Histologically, the tumor was characterized by spindle cell proliferation with plasmocytes, lymphocytes, histiocytes, and a few neutrophils, dispersed in a myxoid or dense collagen background (Figure 3). Cellular atypia was not identified, neither were mitotic or necrotic areas, and staining did not show the presence of microorganisms. By immunohistochemical analysis, the spindle cells were uniformly positive for vimentin and smooth muscle actin (SMA), supporting the myofibroblastic nature of these cells (Figure 4). There was no reactivity for CD34, CD23, CD117, EBV, P53 and ALK1. The CD68 was positive in histiocytes. In the liver parenchyma, granulomatous lesions with viable eggs of *Schistosoma mansoni* were identified in the portal tracts. The right kidney showed a large mass in its upper portion measuring 19 cm × 16 cm × 15 cm with the same macroscopic, microscopic and immunohistochemical findings as those described in the liver tumors.

Figure 1 Well circumscribed and nodular mass, tan-white.

Figure 2 Multinodular mass, tan-white and shining.

DISCUSSION

IMT is a lesion with intermediate biological behavior that may recur with local or surrounding infiltration or rarely metastasizes. Its usual radiological aspects are similar to those of a malignant neoplasm[13]. It is described in almost all solid organs, including the liver. Patients with hepatic IMT may complain of abdominal pain, jaundice and obliterative phlebitis, but, in this report, the clinical symptoms were nonspecific. As described in the laboratory setting, we observed a cholestatic laboratory pattern suggestive of an infiltrative liver disease. The aminotransferases were fairly abnormal and the radiological exams suggested a malignant tumor.

The pathogenesis of IMT is uncertain. Some cases

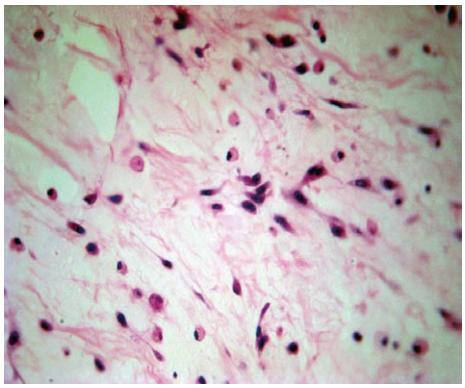

Figure 3 Spindle and inflammatory cells in a myxoid background (HE stain, × 100).

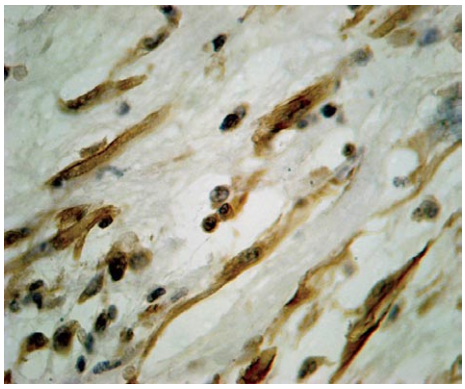

Figure 4 Smooth muscle actin positive in the spindle cell tumor (immunostaining, × 200).

are related to infectious or reparative processes, and the association of IMT with some infectious agents has been related to its pathogenesis, although it is rarely observed. Parasitic infections were also implicated, as *Schistosoma mansoni*[6] were identified in the midst of the tumor tissue. In this instance, we are not sure whether the hepatic schistosomiasis is an incidental diagnostic finding or if it is associated with the pathogenesis of the lesion, even though numerous slides demonstrated viable eggs of *Schistosoma mansoni* not in the tumor but, rather, in the adjacent liver tissue. Abdominal and intestinal pseudotumors have been described as a complication of this parasitosis[14]. In the liver, *Schistosoma mansoni* maturation is finished in the intra-hepatic portion of the portal vein, leading to a pyelophlebitis and to a granulomatous peripyelophlebitis together with a *de novo* formation of a portal conjunctive[15]. According to some authors[16], the IMT could develop secondary portal venous infection, and the inflammatory mass might have enlarged together with the obliterating phlebitis, which might possibly explain the tumoral development in this patient. However, there are tumors with evidence of being truly neoplastic, characterized by rearrangements involving chromosome 2q23 upon which the ALK gene is located[17].

As already described in the literature[1], this tumor was found in the right lobe of the liver, characterized by a well-circumscribed lesion that was firm, with myxoid areas, and a yellow or whitish color. The differential diagnosis depends upon the histological patterns observed, and, although we observed a hypocellular fibrous pattern with some inflammatory cells, the SMA immunoreactivity in the spindle cell was important in the diagnosis. CD34, CD23 and CD117 were non-immunoreactive in the spindle cells, which excluded the diagnosis of fibrous solitary tumor, follicular dendritic cell tumor and gastrointestinal stromal tumor, respectively. What draws our attention in this case report is the tumor progression showing right kidney and diaphragm infiltration (benign histological features were also observed in infiltrative lesions) in addition to the same immunohistochemical pattern observed in the primary tumor. Morphological and genetic features have been studied as prognostic factors, and this case report did not show such features as p53 immunoreactivity, ganglion-like cells, and mitotic figures, which are sometimes associated with aggressive tumors[4]. Although clinical, histopathological or molecular features do not predict biological behavior[4,11], recurrences are associated with abdominal site, large size of the tumor and older age. In general, histological features are not different between ALK positive and negative inflammatory pseudotumors[18]. Cases with local recurrence and an aggressive clinical course have also been described, especially in patients with incomplete resection[19]. This was probably the main reason for the aggressive clinical and biological course observed in this case because, aside from the large size, the proximity between the tumor and the vital structures such as the vena cava did not allow the complete resection of the tumor. Finally, the histological patterns did not predict the aggressive biological behavior, and further investigation is necessary in order to better clarify an infectious cause in some cases of IMT.

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CASE REPORT

Multi-site abdominal tuberculosis mimics malignancy on ^{18}F-FDG PET/CT: Report of three cases

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Abstract

^{18}F-fluorodeoxyglucose positron emission/computed tomography (^{18}F-FDG PET/CT) imaging, an established procedure for evaluation of malignancy, shows an increased ^{18}F-FDG uptake in inflammatory conditions. We present three patients with abdominal pain and weight loss. Conventional imaging studies indicated that abdominal neoplasm and ^{18}F-FDG PET/CT for assessment of malignancy showed multiple lesions with intense ^{18}F-FDG uptake in abdomen of the three cases. However, the three patients were finally diagnosed with multi-site abdominal tuberculosis (TB). Of them, two were diagnosed with TB by pathology, one was diagnosed with TB clinically. They recovered after anti-TB therapy. Few reports on accumulation of ^{18}F-FDG in abdominal TB are available in the literature. A high index of suspicion is necessary to achieve an early diagnosis and a better outcome of the disease.

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Key words: Abdominal tuberculosis; Differential diagnosis; ^{18}F-fluorodeoxyglucose; Positron emission tomography/computed tomography; Malignancy

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INTRODUCTION

Patients with abdominal mass caused by benign lesions such as infection and benign tumor are often encountered in daily clinical practice. Sometimes, it is difficult to differentiate them from malignancy with the conventional imaging techniques, such as ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI)[1-3]. In spite of the great success achieved by ^{18}F-fluorodeoxyglucose positron emission tomography/CT (^{18}F-FDG PET/CT) imaging in the evaluation of malignant disorders[4-6], ^{18}F-FDG is not specific for malignant lesions, and can accumulate in inflammatory lesions with an increased glucose metabolism[7,8].

Abdominal tuberculosis (TB) may involve the gastrointestinal tract, peritoneum, mesenteric lymph nodes, liver, spleen, or genitourinary tract. Its presentations are non-

specific and its diagnosis is frequently elusive[9,10]. Since the presentations of multi-site abdominal TB are even more atypical and insidious, its definitive diagnosis requires a high index of suspicion, various laboratory findings, imaging modalities, and histologic exclusion of malignancy[9,10].

We present three patients with abdominal mass diagnosed as malignancy by ultrasonography and/or CT. ^{18}F-FDG PET/CT also showed an increased activity. However, these three patients were finally diagnosed with multi-site abdominal TB.

CASE REPORT

Case 1

A 50-year-old male presented with a 4-mo history of left abdominal pain and body weight loss of about 6 kg in the past 2 mo. He had not any past clinical or radiological evidence of TB. Ultrasound examination imaging showed a mass behind the pancreatic body, multiple enlarged and confluent lymph nodes in retroperitoneal space and on the left side of abdominal aorta. Physical examination of the abdomen showed a slight tenderness in upper left abdominal area. Liver function tests, electrolytes, and complete blood count were normal. Whole-body ^{18}F-FDG PET/CT scan demonstrated a mass-like area with an intense ^{18}F-FDG uptake extending up behind the pancreatic body and tail, a focal area with an intense ^{18}F-FDG uptake in spleen, multiple focal areas with an intense ^{18}F-FDG uptake along the arcus minor ventriculi, around the abdominal aorta, and post crura diaphragm (Figure 1). Delayed ^{18}F-FDG PET/CT scan was performed 120 min after ^{18}F-FDG was injected into the patient. The maximal standardized uptake value (SUVmax) measurements of each tuberculous lesion were obtained both on early- (SUV1) and on delayed-scans (SUV2). Furthermore, we calculated the retention index (RI) of SUVmax (RI-SUVmax) from SUVmax according to the following formula: RI-SUVmax (%) = [SUV2 (delayed-phase) - SUV1 (early-phase)] × 100/SUV1 (early-phase). The images of delayed-scan demonstrated almost the same multiple areas with an intense ^{18}F-FDG uptake. The SUVmax was increased in some lesions and decreased in others (Table 1), indicating that the patient has lymphoma or retroperitoneal tumor with multiple metastases. The subsequent laparoscopic biopsy of the mass extending up behind the pancreas and histopathological examination revealed TB in the patient (Figure 2).

The patient was diagnosed with multi-site abdominal TB. Six months after anti-TB therapy, a second ^{18}F-FDG PET/CT scan showed remission of all his prior lesions. Three weeks after anti-TB therapy, his abdominal pain disappeared and 10 mo after anti-TB therapy, his general condition including body weight loss was much improved.

Case 2

A 61-year-old female was admitted to our hospital due to right abdominal pain for 1 wk and a body weight loss of about 5 kg in the past 2 mo. The patient had a 3-year history of hypertension but no past clinical or radiological evidence of TB. Liver function tests, electrolytes, and complete blood count were normal. The serum levels of α-fetoprotein, carcinoembryonic antigen, carbohydrate antigen 50, carbohydrate antigen 125, carbohydrate antigen 153, and carbohydrate antigen 199 were normal. Ultrasound and abdominal CT showed a mass in the hepatic hilar region, and multiple enlarged lymph nodes around the abdominal aorta. Whole-body ^{18}F-FDG PET/CT scan demonstrated multiple lesions with an intense ^{18}F-FDG uptake in the hepatic hilar region, para-abdominal aorta, right iliac arterial region, thoracic and lumbar vertebrae (Figure 3), suggesting malignancy with multiple metastases. A biopsy of the 1st lumbar vertebra showed osteomyelitis (Figure 4A). Subsequently laparoscopic biopsy of the mass in hepatic hilar region and histopathological examination revealed TB (Figure 4B).

The patient was diagnosed with multi-site abdominal and bone TB. Ten months after anti-TB therapy, a second ^{18}F-FDG PET/CT scan showed remission of all her prior lesions. Two weeks after anti-TB therapy, her abdominal pain disappeared. Twelve months after anti-TB therapy, her general condition including body weight loss was much improved.

Table 1 Maximum standardized uptake values and retention index of maximal standardized uptake value

Lesion location	SUV1	SUV2	RI-SUVmax (%)
Patient 1			
Behind pancreas	17.7	16.5	-6.78
In retroperitoneal space	14.4	12.1	-15.97
In spleen	6.7	9.2	37.31
Patient 3			
In liver hilum	13.2	15.3	15.91
Near the pancreatic head	13.7	12.8	-8.03
Peritoneum lesion	13.9	14.3	2.88
In retroperitoneal space	12.4	11.8	-4.84

SUV1: Maximal standardized uptake value obtained on early-scan; SUV2: Maximal standardized uptake value obtained on delayed-scan; RI-SUVmax: Retention index of maximal standardized uptake value [RI-SUVmax (%)] = (SUV2 - SUV1) × 100/SUV1.

Case 3

A 37-year-old male presented with malaise and abdominal pain in the past 6 mo, as well a body weight loss of about 3 kg in the past 3 mo. He had not any past clinical or radiological evidence of TB. Ultrasound and abdominal CT showed multiple enlarged nodes in hepatic hilar region, pancreatic head, retroperitoneal space, and disorganization of the pancreatic head. Laboratory test showed an elevated white-cell count (15 250 per cubic millimeter) with an increased neutrophil differential count (12 060 per cubic millimeter). His serum biochemistry values, including liver-function test, were normal. Whole-body ^{18}F-FDG PET/CT scan demonstrated multiple focal or lamellar lesions with an intense ^{18}F-FDG uptake in hepatic hilar region, curvatura ventriculi minor, radix mesenterii region,

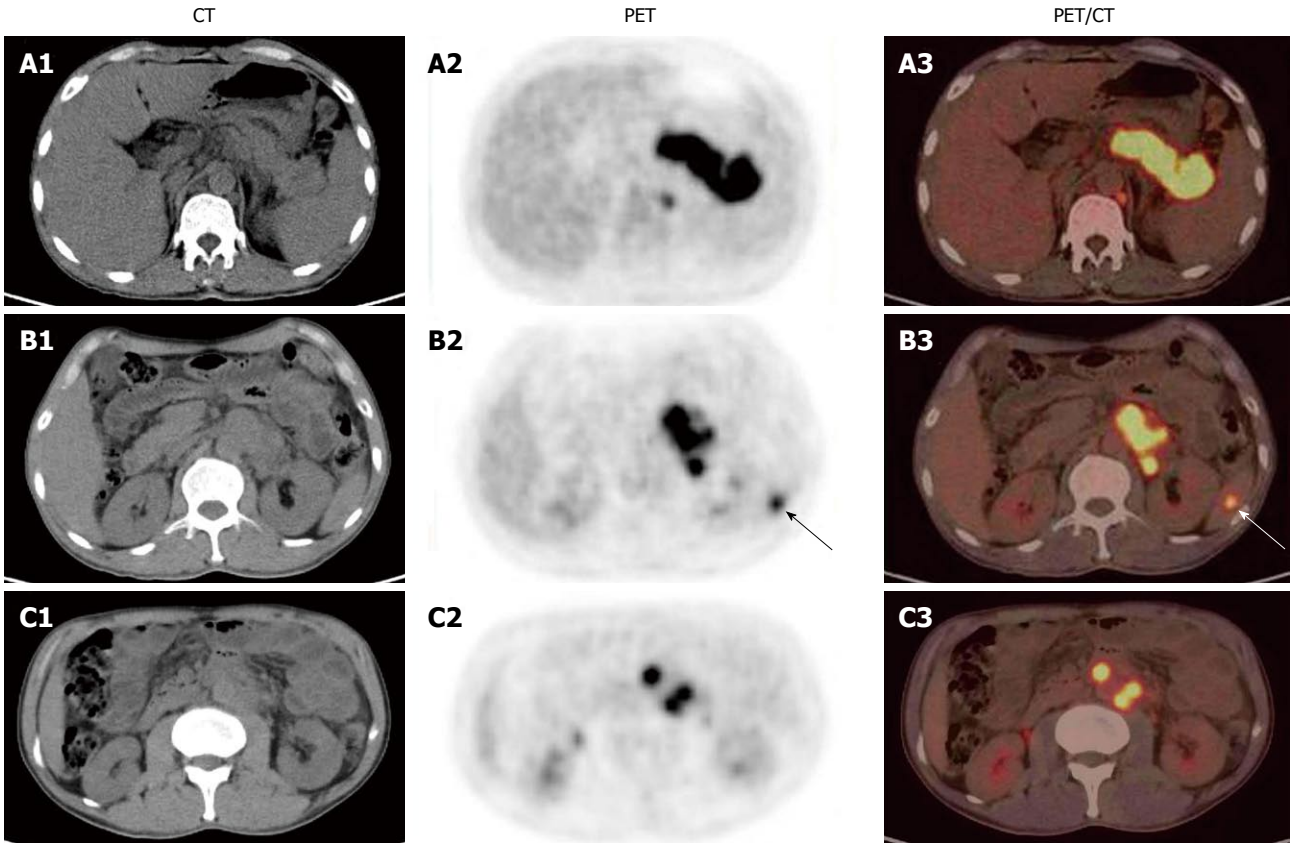

Figure 1 **^{18}F-fluorodeoxyglucose positron emission/computed tomography images of patient 1 showing a mass-like area with an intense ^{18}F-fluorodeoxyglucose uptake extending up behind the pancreatic body and tail (A1-A3), a focal area with an intense ^{18}F-fluorodeoxyglucose uptake in spleen (arrows) (B1-B3), and multiple focal areas with an intense ^{18}F-fluorodeoxyglucose uptake around the abdominal aorta (C1-C3).** CT: Computed tomography; PET: Positron emission tomography.

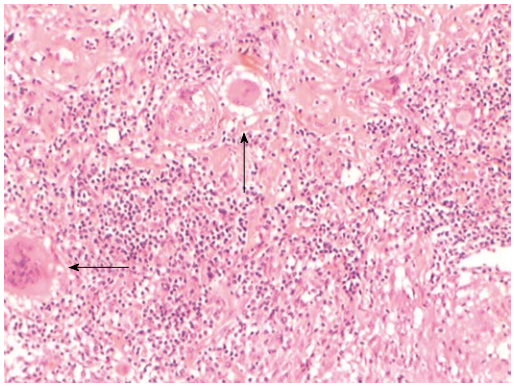

Figure 2 **Hematoxylin and eosin staining of laparoscopic biopsy specimen (× 200) from mass extending to behind the pancreas showing amorphous, eosinophilic and granular debris, as well as Langhans giant cells (arrows) in granulomas.**

retroperitoneal space, pancreatic body and tail, peritoneum, and around the pancreatic head (Figure 5), suggesting TB or cancer. Delayed ^{18}F-FDG PET/CT scan, 112 min after ^{18}F-FDG injection, demonstrated almost the same multiple areas with an intense ^{18}F-FDG uptake. The SUVmax was increased in some lesions and decreased in others (Table 1). Blood tests showed an accelerated erythrocyte sedimentation rate of 96 mm/h. Based on the experience with the reported two patients, this patient was suspected to have TB, a Mantoux test was thus done. The size of induration on Mantoux test was 35 mm (strongly positive). The patient was diagnosed with multi-site abdominal TB.

Ten days after anti-TB therapy, his abdominal pain disappeared and his white-cell count returned to normal. A second ^{18}F-FDG PET/CT scan 5 mo after anti-TB therapy showed remission of all his prior lesions. His treatment was terminated 4 mo after the second PET/CT. Nine months after anti-TB therapy, his general condition including body weight loss and malaise was much improved. Five months after anti-TB therapy, the patient was in good health.

DISCUSSION

Differentiation of TB from malignancy is very important, because the treatment modalities for them are different. Abdominal TB and malignancy often have common clinical features, such as weight loss, abdominal mass, and malaise. Overlap of symptoms of multi-site abdominal TB and metastatic carcinoma makes their differential diagnosis rather difficult and sometimes confusing. Histology is the only way of making its diagnosis as all available imaging methods are not sufficiently specific[1-3]. It has been shown that laparoscopic biopsy is a good diagnostic tool

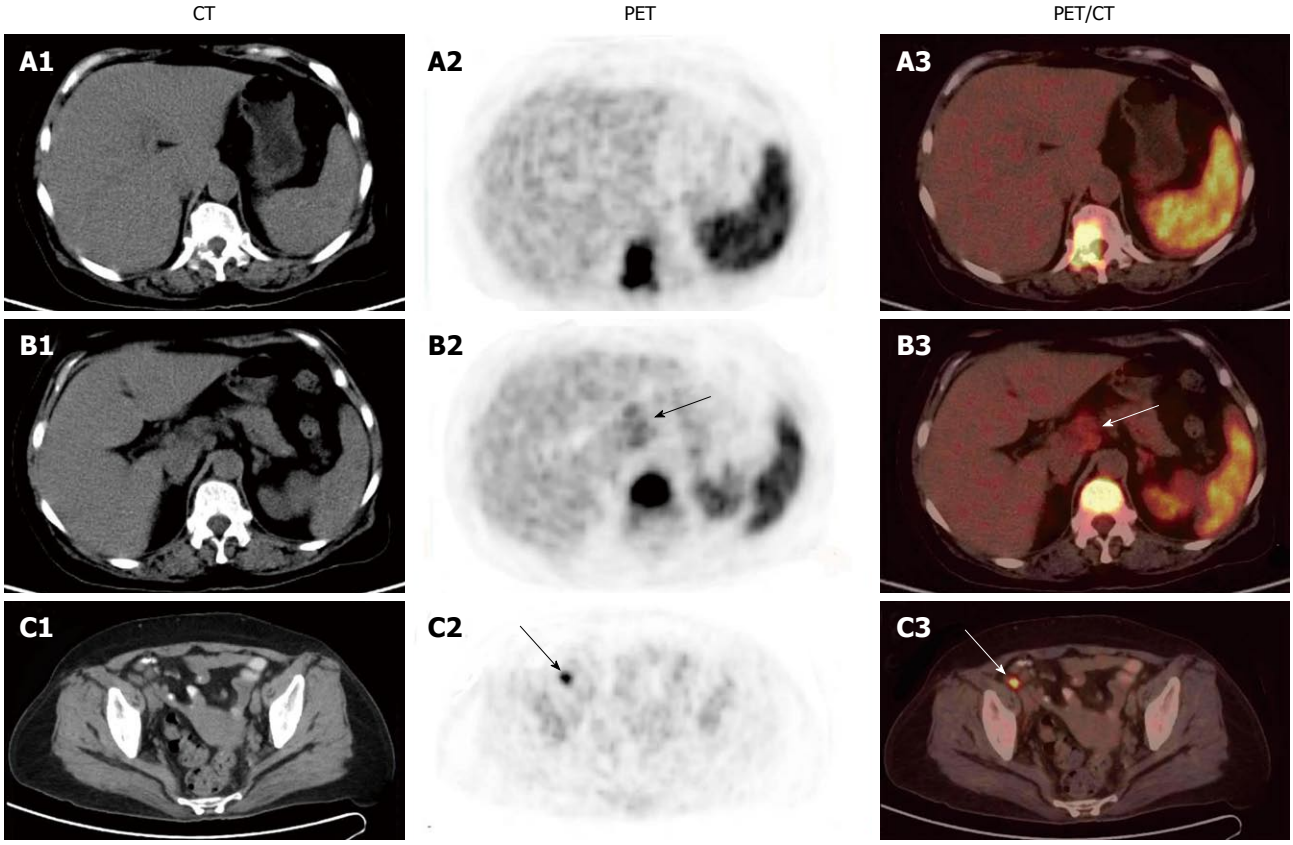

Figure 3 **^{18}F-fluorodeoxyglucose positron emission/computed tomography images of patient 2 showing diffuse intense uptake of ^{18}F-fluorodeoxyglucose in spleen and intense ^{18}F-fluorodeoxyglucose uptake in thoracic vertebrae (A1-A3), a mass-like area with an intense ^{18}F-fluorodeoxyglucose uptake in hepatic hilar region (arrows) and thoracic vertebrae (B1-B3), and a focal intense uptake of ^{18}F-fluorodeoxyglucose in right iliac arterial region (arrows) (C1-C3).** CT: Computed tomography; PET: Positron emission tomography.

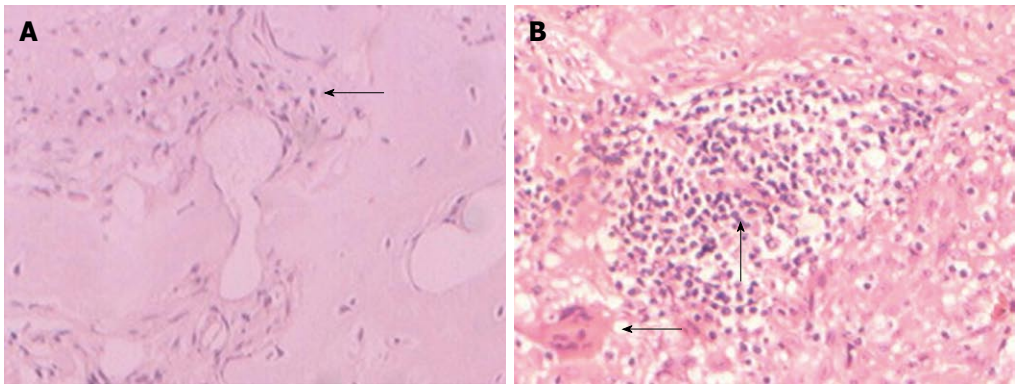

Figure 4 **Hematoxylin and eosin staining of the biopsy specimen from the 1st lumbar vertebra and the mass in hepatic hilar region showing thickened irregular bone trabeculae with a slight infiltration of lymphocytes and plasmocytes (arrow) (A), granulomatous lymphadenitis and amorphous, eosinophilic and granular debris, and Langhans giant cells in granulomas (arrows) (B).**

for doubtful abdominal TB[11]. Laparoscopic biopsy confirmed the diagnosis of abdominal TB in our two patients.

Few reports are available on ^{18}F-FDG accumulation in abdominal TB lesions[12,13]. The abdominal appearance on TB ^{18}F-FDG PET/CT image is nonspecific and various[12,13]. These ^{18}F-FDG positive lesions are often proliferative lesions composed of epithelioid cells, Langhans giant cells, and lymphocytes. These cells highly metabolize glucose and show a high uptake of ^{18}F-FDG. In this study, a focal accumulation pattern (Figure 1) and a diffuse accumulation pattern (Figure 3) of ^{18}F-FDG were observed in spleen TB. Although a SUVmax, greater than 2.5, is attributed to malignant lesions[14], in this study, the peak SUVmax value was 17.7 for abdominal TB (include TB of the spleen, lymph nodes, and peritoneum) (Figures 1, 3 and 5). It has been shown that inflammatory lesions show an increased ^{18}F-FDG washout on delayed images obtained 90-120 min after ^{18}F-FDG injection, whereas cancerous

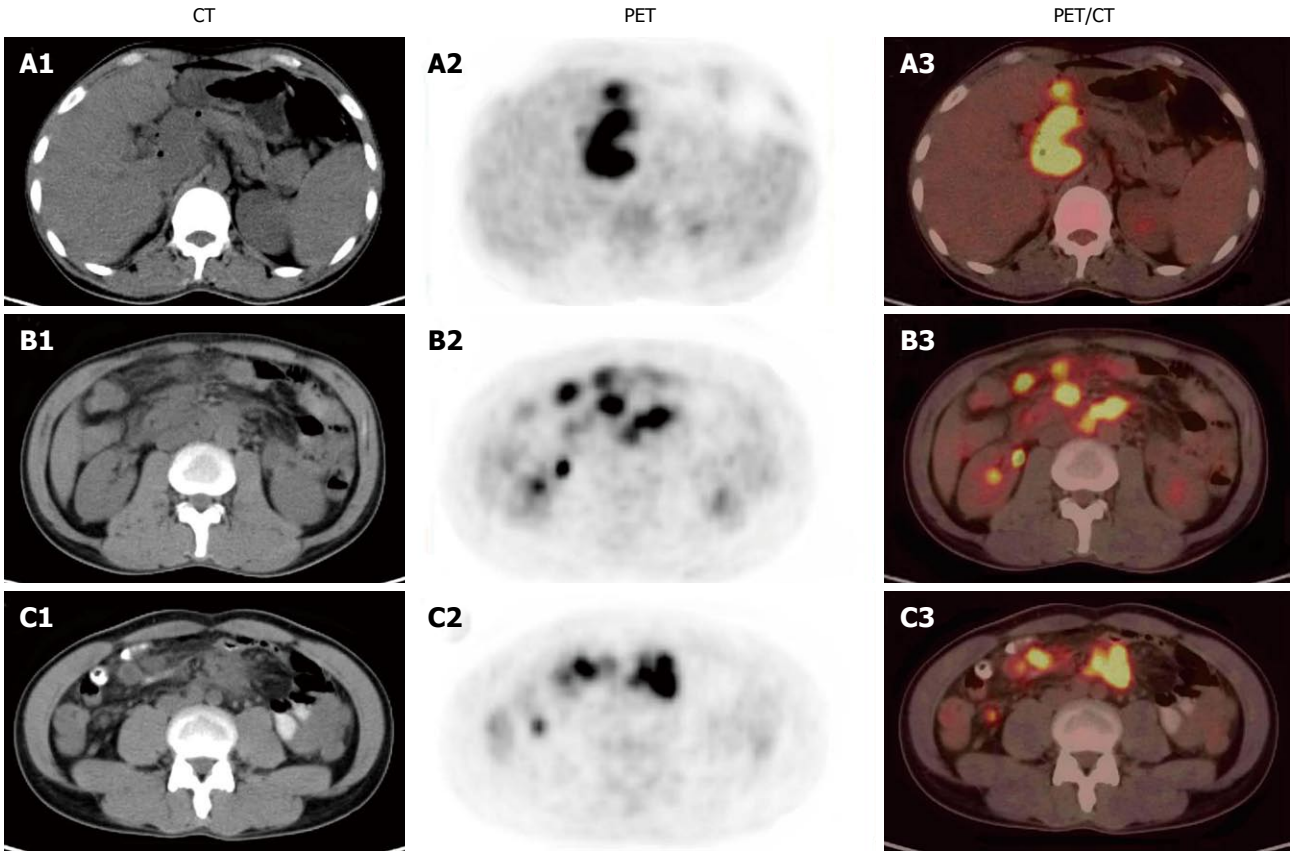

Figure 5 ^{18}F-fluorodeoxyglucose positron emission/computed tomography images of patient 3 A showing a mass-like area with an intense ^{18}F-fluorodeoxyglucose uptake in hepatic hilar region (A1-A3), multiple focal lesions (B1-B3) or multiple lamellar lesions (C1-C3) with an intense ^{18}F-fluorodeoxyglucose uptake in peritoneal cavity, retroperitoneal space, and peritoneum. CT: Computed tomography; PET: Positron emission tomography.

lesions usually exhibit a further accumulation of tracer[15,16]. However, it was reported that TB exhibits a further accumulation of tracer, mimicking malignant lesions[17,18]. In our study, the SUVmax was reduced in some TB lesions and increased in others.

In conclusion, it is difficult to distinguish multi-site abdominal TB from malignancy even with ^{18}F-FDG PET/CT. A high index of suspicion is necessary to achieve an early diagnosis and a better outcome of the disease.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Contents

Weekly Volume 16 Number 34 September 14, 2010

EDITORIAL

4243 Obstructive sleep apnea syndrome and fatty liver: Association or causal link?

Ahmed MH, Byrne CD

4253 Endoscopic ultrasound in chronic pancreatitis: Where are we now?

Seicean A

REVIEW

4264 Intestinal epithelial cells in inflammatory bowel diseases

Roda G, Sartini A, Zambon E, Calafiore A, Marocchi M, Caponi A, Belluzzi A, Roda E

ORIGINAL ARTICLE

4272 α,β-amyrin, a natural triterpenoid ameliorates L-arginine-induced acute pancreatitis in rats

Melo CM, Carvalho KMMB, Neves JCS, Morais TC, Rao VS, Santos FA, Brito GAC, Chaves MH

4281 Antitumor effect of matrine in human hepatoma G2 cells by inducing apoptosis and autophagy

Zhang JQ, Li YM, Liu T, He WT, Chen YT, Chen XH, Li X, Zhou WC, Yi JF, Ren ZJ

BRIEF ARTICLE

4291 Peripancreatic collections in acute pancreatitis: Correlation between computerized tomography and operative findings

Vege SS, Fletcher JG, Talukdar R, Sarr MG

4297 Pre-illness changes in dietary habits and diet as a risk factor for inflammatory bowel disease: A case-control study

Maconi G, Ardizzone S, Cucino C, Bezzio C, Russo AG, Bianchi Porro G

4305 Exertional esophageal pH-metry and manometry in recurrent chest pain

Budzyński J

4313 Effects of glutamine and curcumin on bacterial translocation in jaundiced rats
Karatepe O, Acet E, Battal M, Adas G, Kemik A, Altiok M, Kamali G, Koculu S, Cagatay A, Kamali S, Karahan S

4321 HCV genotype distribution and possible transmission risks in Lahore, Pakistan
Ahmad W, Ijaz B, Javed FT, Jahan S, Shahid I, Khan FM, Hassan S

4329 Associated factors for a hyperechogenic pancreas on endoscopic ultrasound
Choi CW, Kim GH, Kang DH, Kim HW, Kim DU, Heo J, Song GA, Park DY, Kim S

4335 Limited endoscopic sphincterotomy plus large balloon dilation for choledocholithiasis with periampullary diverticula
Kim HW, Kang DH, Choi CW, Park JH, Lee JH, Kim MD, Kim ID, Yoon KT, Cho M, Jeon UB, Kim S, Kim CW, Lee JW

4341 Pathophysiological significance of gallbladder volume changes in gallstone diseases
Huang SM, Yao CC, Pan H, Hsiao KM, Yu JK, Lai TJ, Huang SD

4348 Association of NOD1 (CARD4) insertion/deletion polymorphism with susceptibility to IBD: A meta-analysis
Lu WG, Zou YF, Feng XL, Yuan FL, Gu YL, Li X, Li CW, Jin C, Li JP

4357 Standard triple, bismuth pectin quadruple and sequential therapies for *Helicobacter pylori* eradication
Gao XZ, Qiao XL, Song WC, Wang XF, Liu F

CASE REPORT

4363 Therapy-refractory gastrointestinal motility disorder in a child with c-kit mutations
Breuer C, Oh J, Molderings GJ, Schemann M, Kuch B, Mayatepek E, Adam R

4367 Gastrojejunostomy followed by induction chemotherapy for incurable gastric cancer with outlet obstruction
Okumura Y, Ohashi M, Nunobe S, Iwanaga T, Kanda T, Iwasaki Y

World Journal of Gastroenterology
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Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
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EDITORIAL

Obstructive sleep apnea syndrome and fatty liver: Association or causal link?

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Abstract

Obstructive sleep apnea (OSA) is a complex disorder that consists of upper airway obstruction, chronic intermittent hypoxia and sleep fragmentation. OSA is well known to be associated with hypoxia, insulin resistance and glucose intolerance, and these factors can occur in the presence or absence of obesity and metabolic syndrome. Although it is well established that insulin resistance, glucose intolerance and obesity occur frequently with non-alcoholic fatty liver disease (NAFLD), it is now becoming apparent that hypoxia might also be important in the development of NAFLD, and it is recognized that there is increased risk of NAFLD with OSA. This review discusses the association between OSA, NAFLD and cardiovascular disease, and describes the potential role of hypoxia in the development of NAFLD with OSA.

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Key words: Sleep apnea syndrome; Hyperlipidemia; Non-alcoholic fatty liver disease; Insulin resistance

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INTRODUCTION

Obstructive sleep apnea (OSA) is a condition that affects 1%-4% of the general population and 25%-35% of obese individuals. OSA is more common in men than women and is characterized by loud and frequent snoring, periods of apnea during sleep and excessive day somnolence[1]. Initially, OSA was thought to be due to failure to maintain small upper airway tone, which causes airway collapse and apnea, but recently, unstable ventilatory control and changes in lung volume have been implicated. An OSA disorder is generally defined as five or more apnea-hypopnea episodes per hour of sleep [i.e. the apnea-hypopnea index (AHI)][1,2]. OSA is associated with insulin resistance and hyperlipidemia and both conditions are associated with non-alcoholic fatty liver disease (NAFLD)[3,4]. Importantly, and potentially relevant to OSA, hypoxia is now considered as one of the aggravating factors for development of NAFLD[4], and interestingly, OSA is also regarded as one of the factors that accelerate the progression of NAFLD to non-alcoholic steatohepatitis (NASH)[5].

NAFLD is emerging as an important public health problem across the globe[6]. NAFLD refers to a wide spectrum of liver damage, which ranges from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. NAFLD is strongly associated with insulin resistance and is defined by accumulation of liver fat > 5% per liver

weight, in the presence of < 10 g daily alcohol consumption[7]. The diagnosis of NAFLD can be established by ultrasound and can be confirmed by liver biopsy. The characteristic histology of NAFLD resembles that of alcohol-induced liver injury, but occurs in people who consume minimal or no alcohol. NAFLD is regarded as the most common cause of increased liver enzyme concentrations and is associated with type 2 diabetes, obesity and hyperlipidemia[8]. The reported prevalence of obesity with NAFLD varies between 30% and 100%, whereas the prevalence of NAFLD with type 2 diabetes varies between 10% and 75%[7]. In routine clinical practice, most cases of fatty liver disease are attributable to alcohol excess; however, fatty liver disease can also occur in association with a wide range of toxins, drugs, and diseases, such as morbid obesity, cachexia, type 2 diabetes, hyperlipidemia, and after jejuno-ileal bypass surgery. As important risk factors for NAFLD such as obesity and type 2 diabetes are increasing in prevalence this could explain the marked increase in numbers of individuals with NAFLD[9].

NAFLD can progress silently to cirrhosis, portal hypertension, and liver-related death in early adulthood. Importantly, NAFLD is also associated with an increased risk of all-cause death and predicts future cardiovascular disease (CVD) events, independently of age, sex, low-density lipoprotein (LDL)-cholesterol, smoking and the cluster of features of the metabolic syndrome[9]. Currently, there are no sensitive and specific biochemical markers for NAFLD. An increase (or decrease) in alanine aminotransferase (ALT) is often used as a biochemical marker to monitor progression (or amelioration) of NAFLD, despite the fact that ALT concentrations can be misleading and do not reflect the severity or outcome. Mass screening for significant liver injury in patients with NAFLD will be an important medical challenge in the years to come because of the epidemics of obesity and diabetes[10].

We have previously summarized the studies that have shown that NAFLD is associated with an increase in incidence of CVD[7]. Importantly, considerable numbers of studies have shown an increase in incidence of CVD with OSA[11-13]. The subsequent discussion focuses on the association of OSA with hypoxia, insulin resistance and hyperlipidemia, and how ultimately this can lead to NAFLD.

OSA AND FATTY LIVER DISEASE

Experimental studies have shown that OSA can lead to an increase in insulin resistance and an alteration in lipid metabolism and can precipitate NAFLD[14-16]. Savransky *et al*[14] have exposed lean C57BL/6J mice (n = 15) on a regular chow diet to chronic intermittent hypoxia (CIH) for 12 wk and compared these mice with pair-fed mice, exposed to intermittent air (IA, n = 15). CIH caused liver injury with an increase in serum ALT (224 ± 39 U/ L *vs* 118 ± 22 U/L in the IA group, $P < 0.05$). CIH also induced hyperglycemia, lipid peroxidation of liver tissue, and increased activity of nuclear factor (NF)-κB but not an inflammatory response [as tumor necrosis factor (TNF)-α was not detectable], which suggests that CIH induces oxidative stress in the liver. Liver histology shows swelling of hepatocytes, with marked accumulation of glycogen in hepatocytes, but no evidence of hepatic steatosis. CIH greatly exacerbates acetaminophen-induced liver toxicity, which causes fulminant hepatocellular injury[14]. It is therefore likely that in the absence of factors that induce obesity as a primary stressor on the liver, IH *per se* leads to mild liver injury. The same authors have repeated the same experiment in C57BL/6J mice on a high-fat, high-cholesterol diet, exposed to CIH for 6 mo. CIH caused liver injury with an increase in serum ALT (461 ± 58 U/L *vs* 103 ± 16 U/L in the control group, $P < 0.01$) and aspartate aminotransferase (AST) (637 ± 37 U/L *vs* 175 ± 13 U/L in the control group, $P < 0.001$). Histology revealed evidence of inflammation and fibrosis in the liver, which was not evident in the control mice. CIH caused marked increases in lipid peroxidation in serum and liver tissue; marked increases in hepatic levels of myeloperoxidase, pro-inflammatory cytokines interleukin (IL)-1β, IL-6, the chemokine macrophage inflammatory protein-2; a trend towards an increase in TNF-α; and an increase in α1 (I)-collagen mRNA[15]. Thus, it is plausible that a high-fat diet that occurs in the presence of hypoxia with OSA promotes NAFLD. Furthermore, in a rat model of NAFLD (a choline-deficient high-fat diet) IH has been shown to induce NASH[16]. The metabolic disorders that predispose patients to NASH include insulin resistance and obesity but the mechanism by which repeated hypoxic events, such as occur in OSA, can lead to the progression of liver disease is unclear. It has been shown that hypoxia decreases insulin sensitivity in mice and might ultimately increase expression of the lipogenic genes sterol-regulatory-element-binding protein-1c (SREBP-1c), peroxisome-proliferator-activated receptor-γ (PPAR-γ), acetyl-CoA carboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2). Furthermore, hypoxia also decreases expression of genes that regulate mitochondrial β oxidation [e.g. PPAR-α and carnitine palmitoyltransferase-1 (CPT-1)][17], which suggests that fat oxidation is also inhibited. Therefore, hypoxia can increase lipogenesis and inhibit fat oxidation; both factors that promote fat accumulation and development of NAFLD.

Human studies have shown that OSA is associated with an increase in liver enzymes, and treatment of OSA has been shown to decrease liver enzymes. For example, Chin *et al*[18] have shown that OSA is associated with an increase in liver enzyme concentrations in 14 of 44 (35%) obese individuals. Furthermore, continuous positive airway pressure (CPAP) therapy decreases concentrations of liver enzymes (ALT and AST). In contrast, in a randomized controlled trial, administration of CPAP for 4 wk had no effect on liver enzymes[19]. In a cohort of morbidly obese patients who required bariatric surgery, OSA was found to be a risk factor for increased liver enzyme concentrations but not for NASH[20]. However, Kallwitz *et al*[21] have shown that, in obese patients with NAFLD, OSA is associated with elevated ALT levels and a trend toward histological evidence of progressive

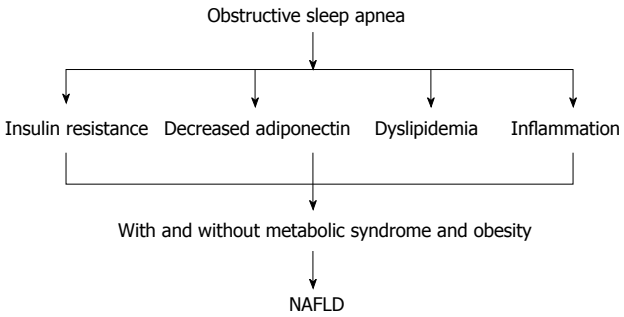

Figure 1 Obstructive sleep apnea can induce non-alcoholic fatty liver disease through increasing insulin resistance, dyslipidemia and inflammation. The presence of metabolic syndrome and obesity with obstructive sleep apnea (OSA) can aggravate non-alcoholic fatty liver disease (NAFLD). OSA might aggravate NAFLD in the absence of obesity and metabolic syndrome.

liver disease. This finding has been endorsed by Mishara *et al*[22], who have shown that, in 101 patients awaiting bariatric surgery, OSA was a risk factor for progression of NAFLD to NASH. Histopathological evidence from 20 obese individuals has shown that OSA is associated with NASH and insulin resistance[23]. Importantly, in 109 patients with OSA, serum aminotransferase levels were better predicted by markers of oxygen desaturation than by factors traditionally associated with the metabolic syndrome[24]. Markers of hypoxia were correlated significantly with AST and ALT levels, whereas the AHI, body mass index (BMI), blood pressure, fasting glucose, triglyceride, and cholesterol levels did not[24]. Importantly, in obese children, OSA has also been shown to be associated with hepatic steatosis and insulin resistance[25], which suggests that early exposure to relative hypoxia also has a deleterious impact on the liver (Figure 1).

OSA AND INSULIN RESISTANCE

There is a strong link between insulin resistance and excessive deposition of triglyceride in hepatocytes, which is the hallmark of NAFLD[26]. Although clinical and experimental studies have shown an association between OSA and insulin resistance, whether CPAP therapy improves insulin resistance remains a controversial issue[27]. In 14 obese individuals with OSA, there were marked increases in leptin, insulin resistance, TNF-α and IL-6, compared with non-apneic obese men. The sleep apnea patients had a significantly greater amount of visceral fat compared to obese controls ($P < 0.05$) and indexes of sleep disordered breathing were positively correlated with visceral fat, but not with BMI, or total or subcutaneous fat. Furthermore, a greater degree of insulin resistance was observed in the group of apnea patients than in BMI-matched non-apneic controls[28]. This finding suggests that OSA is not only associated with insulin resistance but also with inflammation.

Punjabi *et al*[29] have shown that the prevalence of sleep-disordered breathing in 150 healthy mildly obese men, without diabetes, ranged from 40% to 60%, and impairment in glucose tolerance was related to severity of oxygen desaturation. For a 4% decrease in oxygen saturation, the associated OR for worsening glucose tolerance was 1.99 (95% CI: 1.11-3.56) after adjusting for percent body fat, BMI, and AHI. Multivariate linear regression analyses revealed that increasing OSA was associated with worsening insulin resistance independent of obesity[29]. Ip *et al*[30] also have shown that OSA is independently associated with insulin resistance. Furthermore, Meslier *et al*[31] have carried out a cross-sectional study in 494 patients with OSA and have found that the prevalence of type 2 diabetes was 30% and impaired glucose tolerance was 20%, and importantly, diabetes and BMI were independent predictors of OSA[32]. Mallon *et al*[33] have shown in a 12-year follow-up study that difficulty maintaining sleep, or short sleep duration ($\leqslant$ 5 h), was associated with an increased incidence of diabetes in men; whereas in contrast, the Finnish type 2 diabetes survey (FIN-D2D) (a large population study in Finland) has shown that sleep duration of $\leqslant$ 6 h, or $\geqslant$ 8 h, was independently associated with type 2 diabetes in middle-aged women, but not in men[34]. Taken together, these data suggest that an alteration in the normal sleep pattern increases risk of diabetes in men and women.

Data from experimental animal models have shown that IH is also associated with insulin resistance. In obese mice, short-term IH led to a decrease in blood glucose levels accompanied by a marked increase in serum insulin levels, and intriguingly, this effect was completely abolished by prior leptin infusion. Obese mice exposed to IH for 12 wk developed a time-dependent increase in fasting serum insulin levels (from 3.6 $\pm$ 1.1 ng/mL at baseline to 9.8 $\pm$ 1.8 ng/mL at wk 12, $P < 0.001$) and worsening glucose tolerance, consistent with an increase in insulin resistance[35]. However, in lean C57BL/6J mice, exposure to IH for 5 d did not induce the same metabolic changes seen in obese mice[35]. Furthermore, in lean C57BL/6J mice IH induced insulin resistance. This effect was seen during the time of exposure to IH[36]. These data suggest that the presence of obesity or metabolic syndrome (as a first insult) in association with OSA (a second insult) might lead to NAFLD and ultimately NASH. Therefore, it is plausible to suggest that OSA in association with insulin resistance increases risk of type 2 diabetes. Several mechanisms are thought to contribute to the development of insulin resistance with OSA (Figure 2).

Death of adipose tissue and associated excess release of free fatty acid

Adipose tissue hypoxia (ATH) is a new concept in understanding the pathogenesis of insulin resistance and inflammation in OSA. The concept suggests that inhibition of adipogenesis and triglyceride synthesis by hypoxia might be a mechanism for the increased free fatty acid (FFA) concentrations in obesity that occurs with insulin resistance. Gross obesity might be associated with ATH and adipocyte death. However, the exact cause of adipocyte death in obesity is not known. It has been suggested that gross obesity *per se* is associated with a reduction in

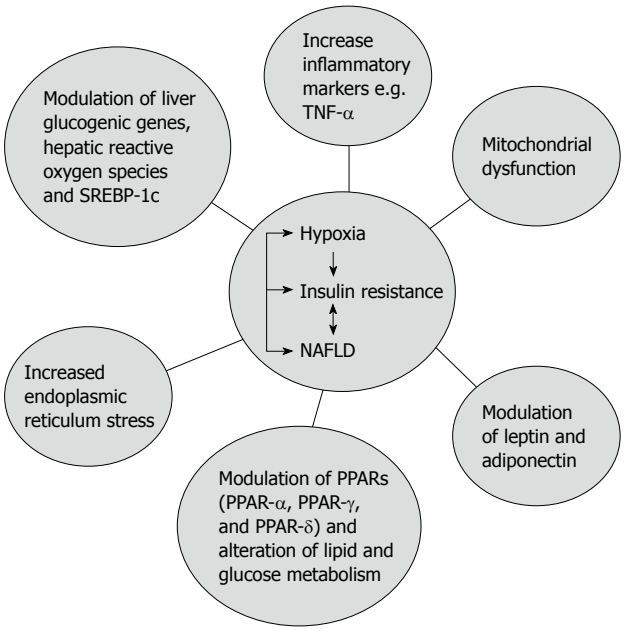

Figure 2 The complex relationship between non-alcoholic fatty liver disease, obstructive sleep apnea and insulin resistance. TNF: Tumor necrosis factor; NAFLD: Non-alcoholic fatty liver disease; PPAR: Peroxisome-proliferator-activated receptor; SREBP-1c: Sterol-regulatory-element-binding protein-1c.

blood flow to adipocytes due to diminished angiogenesis or vasoconstriction[37]. Yin *et al*[38] have shown that hypoxia might inhibit insulin-induced glucose uptake by reducing concentrations of the insulin-signaling molecules insulin receptor β and insulin receptor substrate-1 in mice. Hypoxia might also stimulate lipolysis and inhibit uptake of FFA in adipocytes, which leads to FFA elevation in the plasma of obese subjects, because the increase in FFA might occur as a result of the inhibitory effect of hypoxia on the fatty acid transporters (FATP1, CD36) and the transcription factor (PPAR-γ)[38]. It is tempting to speculate that OSA might act in synergism with gross obesity to accelerate the process of adipocyte death that could ultimately aggravate the course of insulin resistance.

Inflammation

Numerous studies have shown that OSA is a trigger for inflammation. This might explain the associated increase in insulin resistance, dyslipidemia and hypertension in OSA[36]. NF-κB is the transcription factor that is involved in inflammatory pathways and might be involved in modulating insulin sensitivity[39]. Importantly, NF-κB is also increased not only with OSA but also with obesity and metabolic syndrome. NF-κB is the master regulator of inflammatory process and its activation with hypoxia also leads to activation of TNF-α, IL-1, IL-6, monocyte chemoattractant protein-1, macrophage migration-inhibition factor, inducible nitric oxide synthase and matrix metalloproteinase 9. Some of these mediators are also activated by the hypoxia inducible factor (HIF)[40-43]. TNF-α and IL-1 are known to be increased not only with OSA, but also with obesity and metabolic syndrome[40], and the increase in TNF-α and IL-1 is known to be associated with an increase in insulin resistance[44].

Modulation of transcription factors

The modulation of transcription factors has a crucial role in the development of insulin resistance. Evidence is now emerging that hypoxia stimulates SREBP-1c, which is a positive transcription factor for activity of ACC and fatty acid synthase genes, both genes whose activity can promote development of fatty liver[45]. Hypoxia-induced fatty liver has been shown to be associated with an increase in the expression of SREBP-1c[45]. Insulin-resistant ob/ob mice have increased concentrations of SREBP-1c and also develop spontaneous fatty liver[45]. We have presented evidence that suggests strongly that abnormalities of SREBP-1c function play an important pathogenetic role in contributing to the NAFLD phenotype[46]. PPAR-γ is required for maintenance of insulin sensitivity and lipid metabolism[47,48]. Importantly, hypoxia and the associated increase of NF-κB, TNF-α, IL-1 and IL-6 are all known to inhibit PPAR-γ[49]. Overexpression of hepatic PPAR-γ leads to lipid accumulation and it is suggested as a mechanism for hypoxia-induced fatty liver. Furthermore, PPAR-α is also reduced by hypoxia. PPAR-α is highly expressed in the liver, and animal models deficient in PPAR-α develop NAFLD and insulin resistance[49]. In addition, hypoxia also decreases the expression of mitochondrial fatty acid transporter CPT-1[17], which might decrease fat oxidation and promote lipid accumulation. Hypoxia might also modulate AMP-activated protein kinase through mitochondrial respiration or oxidative stress, and ultimately, this might enhance insulin resistance[50].

Adiponectin

Adiponectin is a cytokine that is produced by adipocytes. Serum levels of adiponectin correlate with systemic insulin sensitivity[51]. A reduction in adiponectin contributes to insulin resistance in obesity. However, it is still not clear why adiponectin concentrations are decreased in obesity[52]. Decreased adiponectin is known to be associated with NAFLD[53] and studies have now shown that hypoxia reduces adiponectin expression in adipocytes[54,55]. In adipose tissue, the inhibitory effect of hypoxia on adiponectin might result in increased expression of inflammatory cytokines[56]. Furthermore, TNF-α has been shown to inhibit adiponectin in adipocytes[56]. Thus, these data suggest that hypoxia might directly inhibit adiponectin expression, directly or indirectly, through TNF-α, although whether the decrease in adiponectin causes NAFLD is still uncertain.

Leptin

Leptin is a hormone that is secreted by adipose tissue and increases with obesity. The main role of leptin is to reduce appetite[57]. OSA is known to be associated with an increase in leptin plasma levels, and the increase in leptin occurs in proportion to the severity of OSA[57]. Therefore, it is likely that adipose tissue hypoxia might in part

contribute to the increase in plasma leptin level. HIF-1α is associated with increased leptin level[58]. Despite the increase in plasma leptin in the majority of obese individuals with OSA, there is no improvement in appetite due to leptin resistance associated with excess fats[59-61]. CPAP has been shown to be associated with a decrease in leptin[59-61], which suggests that hypoxia might modulate insulin sensitivity at least in part *via* changes in leptin concentrations. In contrast, other studies have suggested that hypoxia is associated with a decrease in leptin level[62-64]. Yasumasu *et al*[62] have shown that hypoxia is associated with a decrease in leptin secretion in cultured rat adipocytes[62]. Furthermore, short-term hypoxia does not affect leptin in humans. Hypoxia for 8 wk in a neonatal animal model was not associated with marked changes in plasma leptin levels[64]. Therefore, further research is needed to establish the impact of hypoxia on leptin.

Mitochondrial dysfunction and endoplasmic reticulum stress

Hypoxia is known to inhibit biogenesis and respiration of the mitochondria[65]. Furthermore, hypoxia might also gradually decrease the number and function of the mitochondria and this could lead to insulin resistance[65]. We have shown that alteration in mitochondrial function is associated with NAFLD[66]. Hypoxia is known to induce endoplasmic reticulum stress and inhibition of this has been found to protect mice against insulin resistance. OSA is thought to induce endoplasmic reticulum stress in obesity[67-69]. An increase in endoplasmic reticulum stress is also associated with NAFLD[70].

OSA AND METABOLIC SYNDROME

NAFLD is regarded as the hepatic component of the metabolic syndrome[71]. In October 2009, a joint interim statement from the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and the International Association for the Study of Obesity was published that defined diagnostic criteria for identifying the presence of the metabolic syndrome, without having to resort to measurements that require sophisticated equipment[72]. Metabolic syndrome was defined by the presence of three of five criteria, including: increased waist circumference, elevated triglycerides, reduced high-density lipoprotein (HDL)-cholesterol levels, elevated blood pressure, and elevated fasting-glucose levels. In this new definition, waist circumference is not an obligate requirement for defining the syndrome and is one of five criteria that physicians can use when diagnosing the metabolic syndrome[72]. Furthermore, Vgontzas *et al*[73] have shown that sleep apnea patients have a significantly greater amount of visceral fat and insulin resistance compared to obese controls ($P < 0.05$), and indexes of sleep disordered breathing are positively correlated with visceral fat, but not with BMI or total or subcutaneous fat. This finding has led to the suggestion that OSA should be considered as part of the metabolic syndrome[73]. Coughlin *et al*[74] have shown that subjects with OSA are more obese, have higher blood pressure and fasting insulin concentrations, are more insulin resistant, and have lower HDL-cholesterol concentrations, which provides evidence that there is increased prevalence of metabolic syndrome (87% *vs* 35%, $P < 0.0001$). A regression analysis adjusted for age, BMI, smoking and alcohol consumption, has demonstrated that OSA is independently associated with increased systolic and diastolic blood pressure, higher fasting insulin and triglyceride concentrations, decreased HDL-cholesterol, increased cholesterol:HDL ratios, and a trend towards higher homeostasis model assessment values. Importantly, the authors have concluded that, in individuals with OSA, the prevalence of metabolic syndrome was 9.1 times higher (95% CI: 2.6-31.2, $P < 0.0001$) than in the general population. These data suggest that OSA is independently associated with an increase in the cardiovascular risk factors, and are supported by the work of Tkacova *et al*[75] who have shown that severe OSA is associated with an increased incidence of CVD independent of insulin resistance and obesity.

There could be a differing contribution of risk factors for OSA between ethnic groups. In 819 Japanese patients with OSA (719 men and 100 women) and 89 control subjects without OSA, metabolic syndrome was significantly more common in patients with OSA than in the controls (49.5% *vs* 22.0% for men, $P < 0.01$; 32.0% *vs* 6.7% for women, $P < 0.01$)[70]. Men and women with moderate and severe OSA have a higher risk of metabolic syndrome compared with controls. In men, age, BMI and OSA are significantly associated with metabolic syndrome, whereas, in women, BMI is the only risk factor for metabolic syndrome[76].

In a small study in China, the independent determinants of OSA in men and women were age, sex, BMI and the metabolic syndrome[77]. In another study in the Japanese population with OSA, the concurrent presence of metabolic syndrome constituted an additional cardiovascular risk factor[78]. From the evidence mentioned above, it is possible to conclude that OSA is associated with an increase in risk of CVD in the presence, (or absence), of metabolic syndrome. In addition, when the metabolic syndrome (including NAFLD) occurs in association with OSA, there might be a further increase in risk of CVD. Therefore, the complex relationship between OSA, metabolic syndrome and NAFLD to increase risk of CVD suggests the importance of identifying and treating NAFLD in individuals with metabolic syndrome and OSA.

OSA, NAFLD AND HYPERLIPIDEMIA

NAFLD is not only associated with insulin resistance but also with dyslipidemia[79]. Importantly, in numerous studies, NAFLD has been shown to be associated with an increase in risk of CVD. NAFLD is associated with increased incidence of CVD in type 2 diabetes[80-87]. Furthermore, OSA is associated with significant cardio-

vascular morbidity and mortality[88]. There is increasing evidence that OSA is associated with dyslipidemia in animal models as well as human studies. Acute exposure to hypoxia increases LDL-cholesterol concentrations but does not influence the concentration of cholesterol and fatty acids in rats[89]. Repeated exposure to hypobaric hypoxia causes a significant increase in the concentration of cholesterol, fatty acids, chylomicron, LDL-cholesterol and very-low-density beta-lipoproteins (VLDL) in rats, whereas the level of HDL-cholesterol decreases[89]. Furthermore, Li *et al*[90] have shown that, in leptin-deficient obese C57BL/6J-Lep(ob) mice, exposure to IH increases fasting serum levels of total cholesterol, HDL-cholesterol, and triglycerides, as well as liver triglyceride content. These changes are not observed in obese mice, which have hyperlipidemia and fatty liver at baseline. In lean mice, IH increases SREBP-1c levels in the liver, increases mRNA and protein levels of stearoyl-coenzyme A desaturase 1 (SCD-1), an important enzyme that is involved in desaturation of fatty acids, controlled by SREBP-1, and increases monounsaturated fatty acid content in serum, which indicates augmented SCD-1 activity. In addition, in lean mice, IH decreases protein levels of scavenger receptor B1, which regulates uptake of cholesterol esters and HDL by the liver[91]. In mice with a conditional knockout of SREBP cleavage-activating protein (SCAP) in the liver, which exhibits low levels of an active nuclear isoform of SREBP-1c (nSREBP-1c), IH does not have any effect on serum and liver lipids, and expression of lipid metabolic genes is not altered[89]. In wild-type mice, IH increases fasting levels of serum total and HDL-cholesterol, serum triglycerides, serum and liver phospholipids, mRNA levels of SREBP-1c and mitochondrial glycerol-3-phosphate acyltransferase (mtGPAT), and protein levels of SCAP, nSREBP-1, and mtGPAT in the liver. These data suggest that hyperlipidemia in response to IH is mediated in part *via* the SREBP-1c pathway[91], and we have previously suggested that modulation of SREBP provides a potential treatment of NAFLD[46].

In contrast, Savransky *et al*[92] have shown that C57BL/6J mice exposed to CIH and a high-cholesterol diet develop dyslipidemia, aortic atherosclerosis, and upregulation of SCD-1. Therefore, inhibition of SCD-1 might have the potential to prevent dyslipidemia and atherosclerosis during OSA. In another study by Savransky *et al*[93] in mice and in obese humans, C57BL/6J mice were exposed to CIH or normoxia for 10 wk while being treated with SCD-1 or control antisense oligonucleotides. In mice, hypoxia increased hepatic SCD-1 and plasma VLDL levels and induced atherosclerotic lesions in the ascending aorta (the cross-section area of 156 514 ± 57 408 μm^2, and descending aorta (7.0% ± 1.2% of the total aortic surface). In mice exposed to CIH and treated with SCD-1 antisense oligonucleotides, dyslipidemia and atherosclerosis in the ascending aorta were abolished, whereas there was a 56% decrease in lesions in the descending aorta. None of the mice exposed to normoxia developed atherosclerosis. Furthermore, Savransky *et al*[93] have studied obese human subjects who have undergone an intraoperative liver biopsy at the time of bariatric surgery for treatment of sleep apnea and obesity. In these patients, hepatic SCD mRNA levels correlated with the degree of nocturnal hypoxemia ($r = 0.68$, $P = 0.001$) and patients who showed oxyhemoglobin desaturation at night showed higher plasma triglyceride and LDL-cholesterol levels, compared to subjects without hypoxemia[93].

Modulation of HIF-1 activity could also be a precipitating factor for dyslipidemia with OSA. HIF-1 is a master transcriptional regulator of genes that are involved in physiological responses to hypoxia, including erythropoiesis, angiogenesis, and glucose metabolism. Li *et al*[94] have hypothesized that HIF-1 might be involved in dyslipidemia associated with OSA. They have performed a 5-d IH experiment using C57BL/6J (wild-type) or heterozygous *Hif1*$\alpha^{+/-}$ mice (with partial HIF-1α deficiency). During IH, *Hif1*$\alpha^{+/-}$ mice experienced blunted rises in serum triglycerides, liver triglycerides, light-phase fasting insulin, and glucose level, and attenuated transcription or translation of several liver lipid biosynthesis enzymes. HIF-1α deficiency diminished the rise of SREBP-1 and SCD-1 protein levels during IH without affecting serum cholesterol[94]. This suggests that, besides obesity, insulin resistance and a high intake of dietary cholesterol, modulation of HIF-1α could represent another factor that mediates hypoxia-induced dyslipidemia. In summary, hypoxia might lead to an increase in plasma and hepatic lipid profile through different factors and this could precipitate fatty liver.

Clinical studies have shown that CPAP is associated with a reduction in cholesterol and C-reactive protein. In two studies, CPAP was associated with a reduction in cholesterol, LDL-cholesterol, C-reactive protein and homocysteine[95,96]. In children with OSA, tonsillectomy improves parameters of the lipid profile such as LDL-cholesterol, apolipoprotein B and HDL-cholesterol[97].

OSA AND NAFLD AND MODULATION OF CVD RISK

Recently, Floras and Bradley have reviewed the association between OSA and CVD[98]. Their conclusion was that OSA is associated with an increased risk of CVD and this has been demonstrated in epidemiological, clinical and physiological studies. Epidemiological studies have shown a significant independent association between OSA and hypertension, coronary artery disease, arrhythmias, heart failure and stroke[99-105]. Although the association between NAFLD and OSA and CVD is not yet fully elucidated, from the evidence presented, it is tempting to postulate that the association between OSA and NAFLD accelerates atherosclerosis development. The complex interaction between OSA and NAFLD, and the fact that they share similar metabolic pathways that are well known to be associated with an increase in the incidence of CVD, suggest the need for clinical trials in this field (Figure 3).

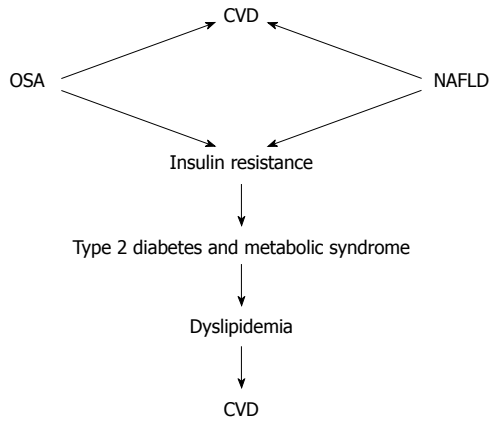

Figure 3 Association of non-alcoholic fatty liver disease and obstructive sleep apnea with cardiovascular disease. Whether the combination of non-alcoholic fatty liver disease (NAFLD) and obstructive sleep apnea (OSA) has a synergistic effect in the incidence of cardiovascular disease (CVD) needs to be demonstrated.

CONCLUSION

OSA is associated with NAFLD in experimental animals and in humans. Importantly, OSA can aggravate the development of NAFLD to NASH in obese individuals or those with metabolic syndrome. OSA might induce NAFLD in the absence of obesity and metabolic syndrome, and the link with hypoxia might be instrumental in precipitating fatty liver development. We suggest that the relationship between CVD, OSA and NAFLD requires further study to elucidate the precise nature of these relationships. Importantly, individuals with OSA require a full evaluation of their CVD risk, and clinicians should be aware that these individuals are also at increased risk of NAFLD.

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EDITORIAL

Endoscopic ultrasound in chronic pancreatitis: Where are we now?

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Abstract

Endoscopic ultrasonography (EUS) is well suited for assessment of the pancreas due to its high resolution and the proximity of the transducer to the pancreas, avoiding air in the gut. Evaluation of chronic pancreatitis (CP) was an early target for EUS, initially just for diagnosis but later for therapeutic purposes. The diagnosis of CP is still accomplished using the standard scoring based on nine criteria, all considered to be of equal value. For diagnosis of any CP, at least three or four criteria must be fulfilled, but for diagnosis of severe CP at least six criteria are necessary. The Rosemont classification, more restrictive, aims to standardize the criteria and assigns different values to different features, but requires further validation. EUS-fine needle aspiration (EUS-FNA) is less advisable for diagnosis of diffuse CP due to its potential side effects. Elastography and contrast-enhanced EUS are orientation in differentiating a focal pancreatic mass in a parenchyma with features of CP, but they cannot replace EUS-FNA. The usefulness of EUS-guided celiac block for painful CP is still being debated with regard to the best technique and the indications. EUS-guided drainage of pseudocysts is preferred in non-bulging pseudocysts or in the presence of portal hypertension. EUS-guided drainage of the main pancreatic duct should be reserved for cases in which endoscopic retrograde cholangiopancreatography has failed owing to difficult cannulation of the papilla or difficult endotherapy. It should be performed only by highly skilled endoscopists, due to the high rate of complications.

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Key words: Endoscopic ultrasonography; Pancreatic neoplasms; Chronic pancreatitis; Contrast agents; Nerve block; Pancreatic pseudocyst; Drainage; Elastography; Main pancreatic duct

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INTRODUCTION

Chronic pancreatitis (CP) is an irreversible and progressive inflammatory process featuring pathological modifications of fibrosis, inflammatory infiltration, and destruction of exocrine and endocrine tissue, resulting in characteristic morphological changes in the parenchyma and pancreatic ducts. These modifications vary in intensity and distribution (diffuse or patchy). This has several consequences: (1) Biopsy specimens are difficult to obtain and not always

relevant, because they do not fully display the signs of CP; moreover, duct biopsy is usually avoided due to the risk of acute pancreatitis; (2) Most imaging methods reflects only partially the CP modifications, especially those typical for late stages of the disease; some methods, such as endoscopic retrograde cholangiopancreatography (ERCP) and magnetic retrograde cholangiopancreatography (MRCP) detect only the ductal features of CP; and (3) The findings of pancreatic function tests are not modified until a late stage in the natural history of the disease. Endoscopic ultrasonography (EUS) accomplishes the quality of being an imaging method able to detect both early and late changes in the parenchyma and pancreatic ducts.

The pancreas is well assessed by EUS due to the method's high resolution and the proximity of the transducer to the pancreas with the possibility of avoiding air in the gut. In patients with CP, EUS was performed initially for diagnosis, then for differential diagnosis, and later for therapeutic purposes (Figure 1).

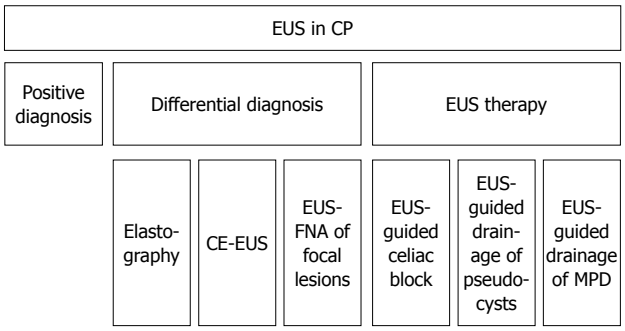

Figure 1 Flowchart of the endoscopic ultrasonography utility in chronic pancreatitis. EUS: Endoscopic ultrasonography; CP: Chronic pancreatitis; CE-EUS: Contrast enhanced endoscopic ultrasonography; MPD: Main pancreatic duct.

POSITIVE DIAGNOSIS

Despite its advantage of assessing the pancreas at very close range, EUS, being operator dependent, is still imperfect in establishing the diagnosis of chronic pancreatitis. The various pathological aspects of the disease are shown as different EUS features, and the same importance for diagnosis has been attributed to all of them. There have been several attempts to define the disease on ductal and parenchymal criteria, initially embracing 11 criteria[1,2], then focusing on nine factors corresponding to histopathological changes[3]: five parenchymal criteria (hyperechoic foci, hyperechoic strands, parenchymal lobularity, cysts, calcifications) and four ductal criteria (pancreatic duct dilatation, pancreatic duct irregularity, hyperechoic pancreatic duct walls, visible pancreatic side branches) (Figure 2). Very rarely are all these manifestations present simultaneously. Some of these features have been found also in elderly people[4], males (OR = 1.8, 95% CI: 1.3-2.55), persons with a history of alcohol consumption abuse (OR = 5.1, 95% CI: 3.1-8.5), smokers (OR = 1.7, 95% CI: 1.2-2.4), and those with history of acute pancreatitis[5-9]. Some features, like gland atrophy or lobularity aspect, can impede the complete assessment of all features (e.g. visualization of side branches of pancreatic ducts).

The interobserver agreement in one study using these criteria was moderate (k = 0.45), with good agreement only for duct dilatation and lobularity; the main drawback of the study was the limited experience of some examiners with pancreatic EUS. The most important criterion for the diagnosis was considered by all experts to be pancreatic stones, followed by visible side branches and lobularity, and the least significant was main pancreatic duct (MPD) dilatation[9]. In an EUS study in which both digital linear and radial echo endoscopes were employed, the interobserver variability also moderate (k = 0.50 and 0.61 respectively); the best concordance between the two methods was found for detection of cysts, calcifications, and visible side branches[10].

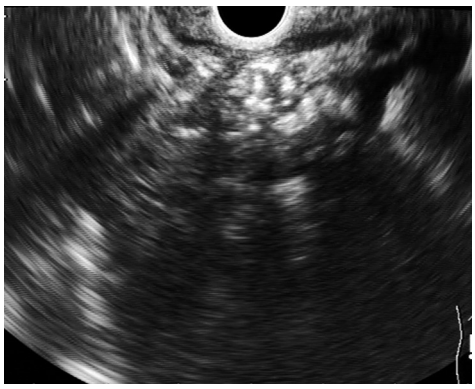

Figure 2 Chronic pancreatitis. Parenchymal and ductal pancreatic stones as hyperechoic structures with shadowing and stenosis of the main pancreatic duct.

Because histological evaluation of the pancreas is usually difficult, different gold standards have been used to establish the optimum number of EUS criteria for diagnosis of CP. The secretin direct pancreatic test has 85% sensitivity and 85% specificity for CP diagnosis, and the false-negative results are due to preserved pancreatic exocrine function[11]. Using one or two criteria for mild pancreatitis, three to five for moderate pancreatitis, and more than five for severe forms, the agreement with the secretin test as gold standard was 100% for normal parenchyma and severe disease, 50% for moderate forms, and 13% for mild disease[2]. On comparison of both EUS radial and linear assessment with the endoscopic secretin test during the same procedure, the best EUS accuracy was obtained for a cut-off point of more than four criteria (accuracy of 84% and 74%, respectively)[10]. The same group obtained lower sensitivity and specificity for diagnosis using four EUS criteria when cholecystokinin was used instead of secretin to test pancreatic function[12]. Comparison of assessment by non-blinded EUS (three to five criteria for diagnosis) and endoscopic retrograde cholangiopancreatography (ERCP; Cambridge classification) showed quite similar sensitivity (72% *vs* 68%) and specificity (76% *vs* 79%) for either mild or severe chronic pancreatitis, with the secretin endoscopic direct pancreatic test as the reference. However, the odds ratio for exocrine insufficiency was higher for EUS assessment than for ERCP[13]. To obtain the best specificity and

Table 1 Diagnostic value of endoscopic ultrasonography in chronic pancreatitis

Author	No. of pts	No. of EUS criteria	Threshold for CP diagnosis	Comparison	Sn	Sp	PPV	NPV	Acc
Wiersema *et al*[4]	69	11	> 3 = dg	EUS *vs* ERCP	100	79			
				EUS *vs* ERCP + secretin test	70	33			
				EUS *vs* ERCP + history	90	66			
Catalano *et al*[2]	80	11	1-2 mild	EUS *vs* secretin test	84	78			
			3-5 moderate	EUS *vs* ERCP	86.1	95.4			
			> 5 severe	EUS *vs* ERCP + secretin test	84.2	97.6			
Sahai *et al*[8]	126	9	> 2 for any CP	EUS *vs* ERCP			> 85	< 85	
			< 3 = fibrosis						
			> 6 = severe				> 85		
Conwell *et al*[14]	56	9	4-5 = equivocal	EUS *vs* ePFT	36	94	93	41	
			> 6 = definite		26	100	100	39	
Stevens *et al*[13]	83	9	3-5 = dg	EUS *vs* ERCP	68	79	83	62	
			6-9 = severe						
Stevens *et al*[10]	100	9	> 4	Radial EUS *vs* ePFT	68	95			84
				Linear EUS *vs* ePFT	44	95			74
Stevens *et al*[12]	50	9	> 4	EUS *vs* secretin ePFT	71	92			
				EUS *vs* CCK ePFT	63	85			
Zimmermann *et al*[23]	21	9	> 4	EUS *vs* histology (surgery)	78	73			
Varadarajulu *et al*[24]	21	9	> 4	EUS *vs* histology[1] (surgery)	90	85.7			88.1
Chong *et al*[25]	71	9	> 3 = dg	EUS *vs* histology[1] (surgery)	83.3	80			
			> 4 = severe fibrosis						
Bhutani *et al*[22]	11	9	> 3	EUS *vs* histology (autopsy)					

[1]Non-calcific chronic pancreatitis. ePFT: Endoscopic pancreatic function test; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; Sn: Sensitivity; Sp: Specificity; Acc: Accuracy; CCK: Cholecystokinin; PPV: Positive predictive value; NPV: Negative predictive value.

the best negative predictive value for diagnosis, six criteria were needed, however, the sensitivity was only 26%[8,14]. Secretin-stimulated EUS detected the features of CP better than EUS without secretin (12/13 patients) and the sensitized EUS seemed to be able to predict a favorable outcome or success of endoscopic treatment[15] (Table 1).

Using ERCP as gold standard, more than two criteria or three criteria, respectively, were found to be optimal for diagnosis[4,8]. The EUS sensitivity for diagnosis varied between 68% and 100% and the specificity was 78%-97% when ERCP was considered the gold standard (Table 1). The overall agreement with ERCP was $k = 0.51$, but the concordance for mild forms on EUS was only 83%. The factors most predictive for abnormal ERCP were ductal stones and parenchymal calcifications[4]. Among patients with a normal pancreatogram, 84.2% were found to have parenchymal changes of CP (accentuation of lobular pattern, focal areas of reduced echogenicity, hyperechoic foci) or increased ductal wall echogenicity. During follow-up (median 18 mo), 68% of patients with initially normal findings on ERCP progressed to an abnormal pancreatogram, supporting the importance of EUS description for early CP. However, this evolution was not confirmed in a second study of alcoholic chronic cirrhosis and CP[16,17]. Evaluation of images can be improved by computer-assisted image analysis[18].

The patient's history may be suggestive of CP. More than five features of CP were seen in 49.9% of 156 patients with persistent or non-specific dyspepsia[19]. Another study showed that there were more criteria for CP in the group with pain and steatorrhea than in the group with pain but no steatorrhea, so they concluded that history can be helpful in diagnosing CP[20].

Table 2 Correspondence between standard endoscopic ultrasonography criteria and pathologic features in chronic pancreatitis (adapted from Sahai AV 2002[21])

Standard EUS criteria	Pathologic features
Parenchymal criteria	
Hyperechoic foci	Small calcifications
Hyperechoic strands	Fibrosis
Lobularity	Edema or fibrosis
Cysts	Pseudocysts
Calcifications	Calcifications
Ductal criteria	
MPD dilatation	MPD dilatation
MPD irregularity	MPD irregular
Hyperechoic MPD walls	Ductal fibrosis or edema
Visible side branches	Dilated secondary branches

EUS: Endoscopic ultrasonography; MPD: Main pancreatic duct.

Pathologic diagnosis, the ideal gold standard, is rarely obtained from surgical specimens, EUS fine needle aspiration (EUS-FNA) or Tru-Cut core biopsies. The correspondence of EUS criteria to pathologic changes is shown in Table 2[21,22]. One recent paper showed that in postmortem pancreatic specimens the presence of more than three EUS standard criteria of CP correlated with the histologic diagnosis, but these features were also present in elderly persons dying of diseases other than CP[22] and in 59% of asymptomatic alcohol abusers[5].

Comparing the EUS standard criteria with the histologic findings from specimens obtained during surgery, fulfillment of five or more criteria was associated with sensitivity of 60% and specificity of 83%, compared with 87% and 64% respectively when three criteria were

Table 3 Rosemont consensus definitions

Rank	Features		Definition	Location
	Parenchymal features			
1	Major A	Hyperechoic foci with shadowing	Echogenic structures ⩾ 2 mm in length and width that shadow	Body and tail only
2	Major B	Lobularity with honeycombing	Well-circumscribed, ⩾ 5 mm structures with enhancing rims and relatively echo-poor centers, with ⩾ 3 lobules	Body and tail only
	Minor	Lobularity with honeycombing	Well-circumscribed, ⩾ 5 mm structures with enhancing rims and relatively echo-poor centers, with non-contiguous lobules	Body and tail only
3	Minor	Hyperechoic foci without shadowing	Echogenic structures ⩾ 2 mm in length and width with no shadowing	Body and tail only
4	Minor	Cysts	Anechoic, rounded/elliptical structures with or without septations	Head, body and tail only
5	Minor	Stranding	Hyperechoic lines ⩾ 3 mm in length in at least two different directions with respect to the imaged plane	Body and tail only
	Ductal features			
1	Major A	MPD calculi	Echogenic structures within the MPD with acoustic shadowing	Head, body and tail only
2	Minor	Irregularity of MPD contour	Uneven or irregular outline and ectatic course	Body and tail only
3	Minor	Dilated side branches	3 or more tubular anechoic structures each measuring ⩾ 1 mm in width, budding from MPD	Body and tail only
4	Minor	MPD dilation	⩾ 3.5 mm in body or > 1.5 mm in tail	Body and tail only
5	Minor	Hyperechoic duct margin	Echogenic, distinct structure greater than 50%of the entire MPD	Body and tail only

MPD: Main pancreatic duct.

used[23]. Good correlation with histology obtained during surgery of non-calcific CP was also found for the presence of four pancreatic features and for EUS findings of foci, stranding, lobulation, or ductal modifications. A limitation of this study was its use of surgical specimens secondary to neoplastic pancreatic disease[24]. Using surgical specimens obtained after preoperative EUS, three criteria were shown to differentiate abnormal from normal pancreatic tissue, but four criteria represented the limit for identification of severe fibrosis[25]. Again, the use of four EUS criteria compared with the association of ERCP, surgical pathology, and/or long-term clinical follow-up showed that EUS was more sensitive than MRCP but equally specific, and when both tests were abnormal the specificity was 100%[26]. Therefore, three or four criteria seems to suffice to rule out CP, but to establish the diagnosis at least six criteria are necessary[27].

The diagnosis of autoimmune pancreatitis is based on the same criteria, but for early stages (corresponding to Cambridge grade 0 to 2) the characteristic criteria are lobularity and hyperechoic pancreatic duct walls[28]. One study found diffuse hypoechoic areas, diffuse enlargement of the parenchyma, focal hypoechoic areas, and bile duct wall thickening as supplementary features characterizing autoimmune pancreatitis; these manifestations resolved after steroid treatment and were helpful in differentiation from ductal adenocarcinomas[29]. EUS-FNA is able to show a stromal structure with high lymphoid cellularity[30]. Lymphoplasmocytic sclerosing pancreatitis can be more accurately detected in tissue samples obtained by Tru-Cut biopsy[31]. With regard to the assessment of severity, preliminary data have pointed to significant diagnostic EUS features: hyperechoic foci for mild CP; hyperechoic foci, visible side branches, and duct dilatation for moderate CP; and visible side branches, duct dilatation, duct irregularity, and calcifications for severe CP[32].

Table 4 Rosemont diagnostic stratification

Stratum	Criteria
Consistent with CP	1 major feature A + ⩾ 3 minor features 1 major feature A + major feature B 2 major feature
Suggestive of CP	1 major feature A + < 3 minor features 1 major feature B + ⩾ 3 minor features ⩾ 5 minor features (any)
Indeterminate for CP	3 or 4 minor features major feature B alone or with < 3 minor features
Normal	⩽ 2 minor features[1]

[1]Excludes cysts, dilated main pancreatic duct, hyperechoic non-shadowing foci, dilated side branch. CP: Chronic pancreatitis.

Because the different pathological characteristics of CP vary in importance, the nine-criteria scheme assigning each criterion the same importance is insufficiently reliable and its diagnostic accuracy doubtful. The Rosemont classification, elaborated by international consensus, uses parenchymal and ductal criteria divided into major and minor features (Table 3). On this basis the findings are classified as "consistent with CP", "suggestive of CP", "indeterminate for CP", or "normal" (Table 4)[33]. This system, quite complicated and more restrictive in diagnosing CP, proved to agree with the diagnostic classification of the nine-criteria scheme in 74% of cases, increasing to 84% when "suggestive of CP" was included[34,35]. Using this system, the findings were similar for radial and linear EUS, with good results for parenchymal criteria (cysts 100%, hyperechoic foci 98%, lobularity/dilated ducts 94%) and modest results for dilated side branch, irregular pancreatic duct and hyperechoic wall of MPD[36]. In a recent multicenter study, 14 experts evaluated 50 recorded videos using the standard nine EUS criteria (diagnostic: > 4 criteria) and the Rosemont criteria (diagnostic: suggestive of CP or consistent

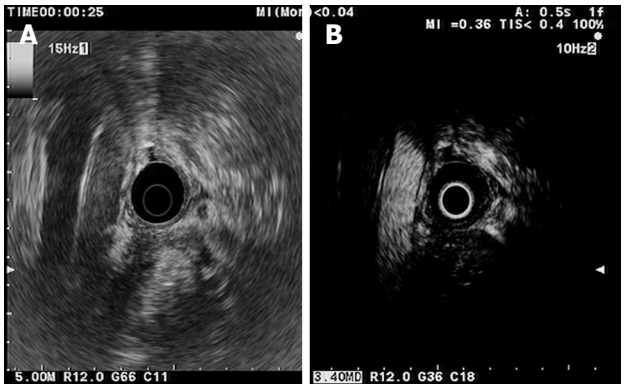

Figure 3 Mass resembling chronic pancreatitis. A: Conventional endoscopic ultrasonography (EUS). Hypoechoic inhomogeneous mass in the pancreatic head. Aorta and inferior caval vein are also seen; B: Contrast-enhanced harmonic-EUS. During the arterial phase (25 s after contrast injection) the abdominal aorta becomes hyperechoic and the mass is hypovascular compared with surrounding parenchyma.

with CP). They obtained substantial interobserver agreement for the Rosemont classification (k = 0.65) and moderate agreement for the standard classification (k = 0.54); the difference was not significant. The best agreement was noted for calcifications (standard scoring), pancreatic duct calcifications (Rosemont classification) and pancreatic duct dilation (both systems). The least agreement was seen for lobularity without honeycomb (Rosemont classification). This study used computed tomography (CT) and endoscopic pancreatic function test (ePFT) as gold standard, without histology. The patients were correctly classified as "definite CP" in 91.2% of cases (standard scoring) and 83.5% (Rosemont scoring); as "mild CP" in 50% (standard scoring) and 42.9% (Rosemont scoring); and "no CP" in 83.3% and 95.2% of cases respectively[37]. Further validation of the Rosemont classifications is needed.

Using EUS-FNA for diffuse CP, the negative predictive value increased to 100% against 75% for EUS, the specificity increased to 67% *vs* 60%, with higher concordance for severe disease than for mild CP[38]. Tru-Cut biopsy should not be recommended for non-focal CP because of complications[39], but its utility has been proved in autoimmune pancreatitis[31,40].

DIFFERENTIAL DIAGNOSIS

If focal hypoechoic lesion are found in the pancreatic parenchyma, the differential diagnosis includes primary or secondary pancreatic tumor, focal CP, and autoimmune pancreatitis. Several methods have been developed for this purpose.

Elastography

Elastography evaluates tissue strain resulting from compression and that strain is smaller in harder tissue than in softer tissue. Different tissue elasticity patterns are marked supplementary on the grey-color scale with different colors (blue for hard tissue and red for soft tissue). EUS elastography in CP shows a honeycomb aspect with predominantly hard strands, corresponding to fulfillment of four standard diagnostic criteria. The sensitivity, specificity, and accuracy were found to be 66%, 57% and 60%, respectively, and the method was considered useful in cases of equivocal EUS (three criteria or fewer)[41,42]. Further studies overcame the limitations of qualitative image analysis by means of digital image quantification, which helps to differentiate benign (normal pancreas and chronic pseudotumoral pancreatitis) from malignant lesions (pancreatic cancer and neuroendocrine tumors) with higher sensitivity, specificity, and accuracy (91.4%, 87.9% and 89.7%, respectively)[43]. Using a scoring system based on different color patterns in the images, the differentiation between benign and malignant pancreatic masses had sensitivity of 92.3% and specificity of 80%[44]. However, another study concluded that elastography did not allow complete delineation of the border of lesions greater than 35 mm in diameter or of lesions situated at some distance from the transducer, yielding poor sensitivity (41%), specificity (53%), and accuracy (45%) for predicting the nature of pancreatic focal lesions[45]. Because elastographic images are still difficult to obtain and interpret, although interobserver agreement is good (k = 0.725)[44], further improvement of the equipment with the possibility of quantification is expected. EUS elastography could have a special role in autoimmune pancreatitis, where the whole pancreas shows a typical, unique homogeneous stiffness, distinct from the circumscribed mass lesion in ductal adenocarcinoma[46].

Contrast-enhanced EUS

Ultrasound contrast agents increases the signal from the blood and improves the detectability of small vessels flow during ultrasound examinations. Before and after injection of Sonovue® (Bracco), the focal pancreatitis shows no detectable vascularization or the vessels appear regular over a distance of at least 20 mm, with detection of both arterial and venous vessels in the contrast-enhanced phase[47] (Figure 3). Based on the perfusion characteristics of microvessels, contrast-enhanced US facilitates differential diagnosis between inflammatory lesions and ductal adenocarcinoma. The specificity of the discrimination between benign and malignant focal pancreatic lesions was found to be 93.3% using power Doppler contrast-enhanced EUS (CE-EUS) compared with 83.3% for conventional EUS[47]. The hypovascular aspect of lesions under power Doppler CE-EUS seemed highly sensitive and specific (91.1% and 93.3%, respectively) for adenocarcinoma[48]. During power Doppler CE-EUS examinations the ultrasound frequency returned to the transducer is the same with that transmitted, but the method is associated with artifacts resulting from turbulent flow (blooming and overpainting). The use of contrast agents is preferred using harmonic frequencies which result from non-linear and non-symmetrical oscillation of the microbubbles. This yields an image with complete "subtraction" of the tissue-derived signal, optimized by using a low mechanical index, which allows continuous real-time assessment of the microvascularization during contrast medium uptake.

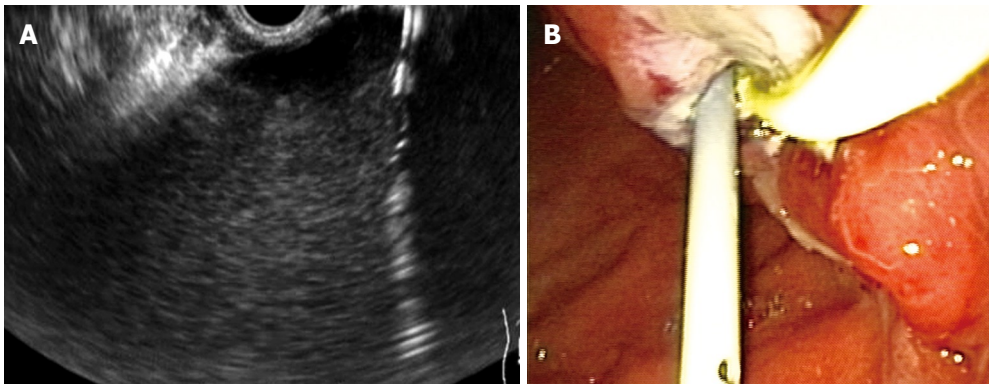

Figure 4 Endoscopic ultrasonography-guided pseudocyst drainage. A: The cystostomy is seen as a hyperechoic parallel structure inside the hypoechoic well-delineated pseudocyst; B: Endoscopic view of a stent and a nasocystic drainage placed transgastric into a pseudocyst.

Harmonic CE-EUS shows an iso vascular homogeneous pattern of CP[49] or, in severe forms, a hypovascular pattern, due to extensive fibrosis[50] (Figure 4A). Our results confirmed that severe CP may be hypovascular on harmonic CE-EUS, and quantitative assessment of images can improve differentiation between adenocarcinomas and chronic pancreatitis (accuracy of 86%) (unpublished data), but, similar to elastography, cannot replace the use of EUS-FNA.

EUS-FNA of focal lesions

The EUS sensitivity for detection of suspected pancreatic mass in a parenchyma with CP modifications was 100%, but the positive predictive value of pancreatic malignancy in these situations was only 60%, because some malignant masses present internal or peripheral calcifications, similar to focal CP[51]. The sensitivity of EUS-FNA for malignancy in parenchymal masses with features of CP is only 54%-74%, compared with 89% when the surrounding parenchyma is normal[51-55]. However, in the event of high suspicion of malignancy with negative EUS-FNA, repeated FNA yields a positive diagnosis in 84% of cases, whereas half of the failures of first biopsies are attributed to the presence of CP[56]. Kras mutation and allele deletion of the microsatellite or of the tumor suppressors can be reliably detected in EUS-FNA samples from pancreatic masses, improving the diagnostic accuracy[57,58]. The search for codon-12 Kras mutation revealed no cases in patients with pseudotumor CP, in contrast to the adenocarcinoma group, although 6%-12% of patients with diffuse CP and PanIN lesions had presented Kras mutations in a previous meta-analysis[59,60].

EUS THERAPY

EUS-guided celiac block

One of the therapeutic uses of EUS in CP is celiac plexus blockade, i.e. temporary inhibition of the celiac plexus using a combination of local anesthesia and steroids, with the aim of reducing pain and improving the quality of life[61]. This guidance is preferred to CT-guided blockade because the details of the region are better appreciated and the side effects are fewer and less severe[62]. Frequently the celiac ganglia can be seen as a unique or concatenate hypoechoic structure, less well delineated, with some whitish strands inside[63].

Some issues regarding EUS-guided celiac block remain to be resolved. The indication is pain in CP, but some studies included pain accompanying moderate pancreatitis[64] or patients with pain that had not responded to other forms of treatment[65]. Another unclarified issue is the technique of injection (central or bilateral) and the quantity of steroid needed. The majority of studies used the bilateral injection technique, considered equal in safety to central injection, but the results of the two techniques concerning the alleviation of pain were close and contradictory[64,66], showing the need for a placebo-controlled trial[67]. Direct injection of triamcinolone within the celiac ganglia (13 patients) compared with alcohol injection (5 patients) yielded disappointing results in respect of pain alleviation for steroid use (38% *vs* 80%)[68]. A comparative study of results between the celiac region injection and celiac ganglia injection for EUS-guided celiac block is still lacking.

The question of cost-effectiveness remains unresolved. Some studies followed up the patients for only 1-4 wk[66,68]. The only study with an extended follow-up period showed duration of pain relief of up to 673 d. This raises the question of whether the natural course of the disease may have been responsible, because there were no data indicative of the level of severity of CP: duration of disease from onset of pain, presence of diabetes, or calcifications[64].

In many studies, the alleviation of pain varied from 55% to 70% with a short duration of follow-up[64-66,69]. Persistence of pain alleviation for as long as 24 wk was seen in no patients[65] or in only 10% of patients[69]. Two meta-analyses showed efficacy in managing chronic abdominal pain in 51.46%[70] and 59.45%[71] of patients respectively. The rate of major complications seemed very low (0.6%), being represented by retroperitoneal abscess[72].

EUS-guided drainage of pseudocysts

Therapeutic intervention in patients with chronic pancreatic pseudocysts is indicated when at least one complica-

tion is present (compression of large vessels, obstruction of duodenum, stomach, or common bile duct, infection, hemorrhage into pancreatic pseudocyst, pancreatico-pleural fistula) or when symptoms occur (satiety, pain, nausea or vomiting, upper gastrointestinal bleeding)[73,74]. Since 1996, several series of EUS-guided drainage have been reported, especially for collections without bulging onto the gut wall or with parietal vessels due to portal hypertension[75-77]. The main limitation is location of the fluid collection further than 1 to 1.5 cm from the gut wall[78-80] (Figure 4).

This method is preferred to surgical drainage, which is associated with a high rate of mortality and morbidity[81]. However, a non-randomized case-control study showed the same rates of treatment success, complications, and reinterventions for surgical and EUS-guided drainage, but with lower costs and shorter hospital stay for the EUS-guided procedure[82].

Conventional endoscopic drainage and EUS-guided drainage were compared in four papers. In a prospective non-randomized study the two approaches seemed equally safe and effective[83], but this was not confirmed in a second non-randomized study, where EUS represented a salvage method in the case of failure of conventional endoscopic drainage owing to non-bulging pseudocysts or location in the tail of the organ, but was a more time-consuming procedure[84]. The conclusion of this second study was that EUS should be reserved for pseudocysts located in the tail of the pancreas, because these are unlikely to cause luminal compression or are technically difficult to access. Also, EUS assessment would identify a tumor in 5% of pseudocysts[84]. A third randomized clinical trial showed a significantly better success rate for EUS- than for conventional endoscopic-guided drainage (100% *vs* 33%), despite the small number of patients, even after statistical adjustment for luminal compression[85]. A fourth randomized study confirmed also a significant advantage for EUS over conventional endoscopic drainage (94% *vs* 72%); both were considered first-line methods for treatment of bulging pseudocysts, but the authors recommended that EUS-guided drainage should be preferred for non-bulging pseudocysts[86].

Several aspects of EUS-guided drainage remain to be elucidated. First among these is the issue of the means used to create the communication between gut and pseudocyst. There are two major techniques for obtaining this communication: (1) balloon dilatation of a previous puncture site, with a 93%-100% success rate[83,84,87-89]; and (2) coagulation of the communication site by means of a cystostomy (success rate of 95% when two procedures per patient were performed[90] and 71%-82% with one procedure per patient[91,92]), a Giovannini needle (success rate of 94%[93,94], but only 84% after the first attempt[86]), or a needle-knife, with the same success rate as balloon dilatation but a higher perforation rate[88,89,95,96]. Larger comparative studies will be necessary to assess the best device with the highest success rate and the lowest complication rate. The prototype "transluminal balloon accessotome", which combines a needle-knife and a dilating balloon, will probably allow easier drainage in one single step, reducing the exchange of accessories and simplifying the procedure[97]. Moreover, the use of the prototype three-layer puncture kit, which allows the simultaneous insertion of two guidewires at the initial puncture in one step, or the use of a larger working channel in the echo-endoscope, would allow safer and faster drainage[98]. Furthermore, the use of a forward-viewing echoendoscope seems promising for drainage of pseudocysts, even those inaccessible with a conventional therapeutic side-viewing EUS endoscope[99].

A further issue to be resolved is that of the morphological or biological factors that predict therapeutic success. Knowledge of such factors would facilitate selection of patients suitable for direct surgery. Moreover, to avoid pseudocyst relapse, described in 4%-17% of cases after 6-9 mo follow-up[94,96,100], communication with a secondary pancreatic duct, should be assessed very carefully.

EUS-guided drainage of main pancreatic duct

EUS-guided drainage of the MPD is a second-line procedure indicated when ERCP is unsuccessful owing to inability to cannulate the MPD (severe inflammation, previous surgery, postsurgical stricture) or difficult endotherapy (tight stenosis, large stone, MPD rupture, pancreas divisum). In practice, there are only few cases in which ERCP cannot be successfully performed by an experienced endoscopist, and recent studies suggests the superiority of surgery in managing pain. Thus, only a very small number of patients, namely those in whom ERCP fails and surgery cannot be performed safely, are good candidates for this procedure[101]. Using the transluminal approach or the transpapillary rendezvous approach, EUS-guided drainage of the MPD remains technically challenging because of difficulty in orienting the endoscope along the axis of the duct, difficult dilatation of the transmural tract due to pancreatic fibrosis, or the acute angle of the needle in relation to the MPD. Despite success rates of 68%-71%, the complication rates were important in all four series published (5%-43%); the complications included perforations, bleeding, pancreatitis, fever, and postprocedural pain[102-105]. EUS-guided drainage of the MPD should continue to be confined to tertiary care centers and very experienced endoscopists.

CONCLUSION

The diagnosis of CP is still accomplished using the standard scoring based on nine criteria each considered as having the same value. For diagnosis of any CP, at least three or four of these criteria must be present, but for diagnosis of severe CP more than six criteria must be fulfilled. The more restrictive Rosemont classification aims to standardize the criteria and assigns different values to different features, but requires further validation. EUS-FNA is less advisable for diagnosis of diffuse CP due to the possible side effects. Elastography and contrast-enhanced EUS are orientation in differentiating focal pancreatic mass, but they cannot replace EUS-FNA. The utility of EUS-guided celiac block for painful CP is still a matter of debate with

regard to best technique and the indications. EUS-guided drainage of pseudocysts is preferred especially in non-bulging pseudocysts or presence of portal hypertension. EUS-guided drainage of the MPD should be reserved for cases of unsuccessful ERCP caused by difficult cannulation of the papilla or difficult endotherapy. It should be performed only by highly skilled endoscopists, due to the high risk of complications.

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REVIEW

Intestinal epithelial cells in inflammatory bowel diseases

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Abstract

The pathogenesis of inflammatory bowel diseases (IBDs) seems to involve a primary defect in one or more of the elements responsible for the maintenance of intestinal homeostasis and oral tolerance. The most important element is represented by the intestinal barrier, a complex system formed mostly by intestinal epithelial cells (IECs). IECs have an active role in producing mucus and regulating its composition; they provide a physical barrier capable of controlling antigen traffic through the intestinal mucosa. At the same time, they are able to play the role of non-professional antigen presenting cells, by processing and presenting antigens directly to the cells of the intestinal immune system. On the other hand, immune cells regulate epithelial growth and differentiation, producing a continuous bi-directional cross-talk within the barrier. Several alterations of the barrier function have been identified in IBD, starting from mucus features up to its components, from epithelial junctions up to the Toll-like receptors, and altered immune responses. It remains to be understood whether these defects are primary causes of epithelial damage or secondary effects. We review the possible role of the epithelial barrier and particularly describe the role of IECs in the pathogenesis of IBD.

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Key words: Intestinal epithelial cells; Epithelial barrier; Tight junctions; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel diseases (IBDs) - mostly represented by Crohn's disease (CD) and ulcerative colitis (UC) - are a group of inflammatory disorders of the gastrointestinal tract characterized by an abnormal immune response to antigens of the intestinal content that leads to a persistent inflammatory state[1].

Intestinal homeostasis in healthy subjects is ensured by a complex system called "intestinal barrier", a dynamic structure that separates intestinal contents from the host tissues, regulates nutrient absorption and allows interactions between the resident bacterial flora and the mucosal immune system.

The intestinal barrier is composed of a thick mucus layer containing antimicrobial products, a monolayer of intestinal epithelial cells (IECs) and an underlying set of cells (mesenchymal cells, dendritic cells, lymphocytes and macrophages)[2].

IECs are exactly at the centre of this system because of their anatomical and functional position: on the lumi-

nal side they secrete and regulate the composition of the mucus layer, while on the basolateral side they interact and cross-talk with the underlying cells.

By putting the IECs at the centre of the barrier system we can divide it into an "upper barrier" and a "lower barrier". The former constitutes a physical barrier, which prevents bacterial adhesion and paracellular diffusion to the underlying host tissues, and a functional barrier, which is able to discriminate commensal bacteria from pathogens; the latter operates by regulating antigen traffic through an intensive cross-talking with immune cells of the lamina propria (Figure 1A).

Given the importance of the epithelium in intestinal immune regulation mechanisms, it is clear how defects at one of these levels could be the primary pathogenetic mechanism that causes the loss of oral tolerance and therefore the establishment of an inflammatory response against luminal antigens, as happens in IBDs (Figure 1B).

Our purpose is to review the state of the art in understanding the key role played by IECs within the epithelial barrier system, in both healthy subjects and IBD patients.

THE UPPER BARRIER

The "upper barrier" is the intestinal epithelial single layer of columnar cells consisting of four IEC types: the absorbent enterocytes, the goblet cells, the Paneth cells and the enteroendocrine cells[3]. Upper barrier features are similar in small and large bowel. The main difference is constituted by the presence of elevations and projections (circular folds, villi and microvilli) in duodenum, jejunum and ileum that allows the increase of the absorption surface. This is not observed in the colon, which instead shows a flat surface.

Amongst the mucous membrane protrusions termed villi, there are inflections called crypts of Lieberkühn, which are distinct glandular invaginations.

Enterocytes are the most representative type of cells, which present finger-like projections, known as microvilli. These arise on the luminal side of the cells, constituting the so-called buffy coat, and are completely coated by glycocalyx and joined by apical junctional complexes, which prevent the entry of pathogens by keeping the cells tight.

In addition, the enterocyte monolayer is interrupted by the presence of goblet cells, secreting mucus, and by the presence of enteroendocrine cells that produce peptide hormones. These hormones are involved in cellular trophism, tissue repair, angiogenesis, enterocyte differentiation and polarization along the crypt-villus axis.

The epithelial cells derive from multipotent stem cells located at the base of the crypts. When these cells reach their maturity, they migrate toward the top of the villus, where aged cells are expelled in the intestinal lumen.

In the depth of the crypts there are also the Paneth cells that have regulatory functions since they produce antimicrobial peptides - called defensins - which constitute an inducible system against pathogens.

Hence, it is clear that the epithelial layer represents an anatomical and functional barrier, an upper interface,

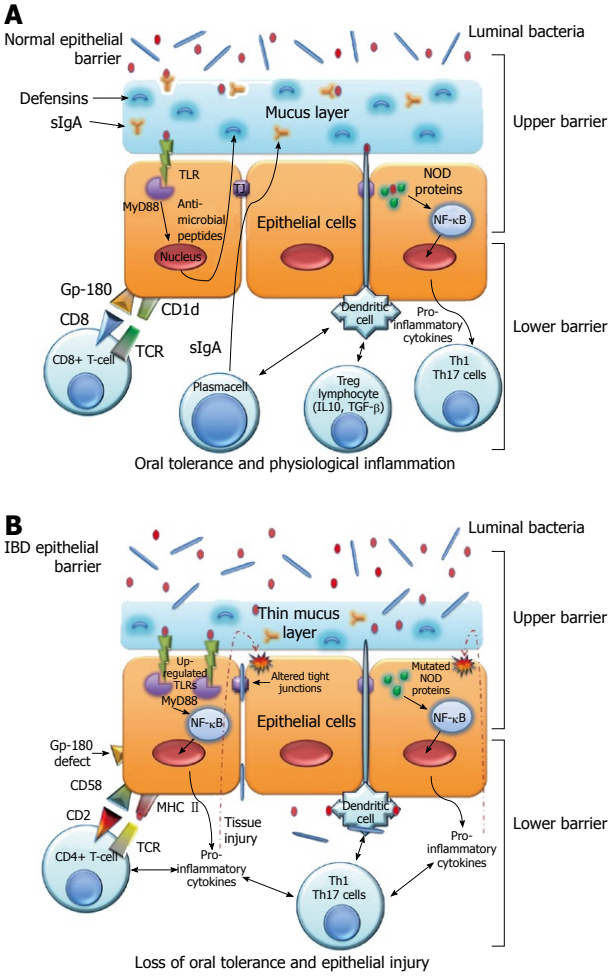

Figure 1 The epithelial barrier system. A: Normal epithelial barrier; B: Inflammatory bowel disease (IBD) epithelial barrier. TLR: Toll-like receptors; MyD88: myeloid differentiation factor 88; TJ: Tight junctions.

which is needed to maintain the whole intestinal homeostasis.

Evidence demonstrates that impairment of the upper barrier plays a key role in the pathogenesis of IBD. In fact, alterations of the mucus layer, disabled epithelial junctions, increased permeability and a defective production of antimicrobial peptides have been observed in CD as well in UC[2].

Mucus layer

The upper barrier presents a mechanical external protection, which consists of a thick mucus layer (about 150 μm) synthesized by goblet cells and full of several molecular peptides. The latter allow mucus to act as a chemical boundary, preventing pathogen invasion.

The most important role of mucus in protecting the mucosa is carried out by its viscosity, due to the concomitant presence of glycosylated mucins and trefoil factors (TFFs) which modulate the defensive properties of the mucus layer, most likely through the check of mucin polymerization and by trapping microbes, which would otherwise be conveyed by the peristaltic process[4]. TFFs are a family of small, yet abundant, secreted proteins

which maintain the epithelial continuity and restitution[5].

Furthermore, Mashimo *et al*[6] demonstrated that mice lacking intestinal trefoil factors (ITF) had impaired mucosal healing and died after oral administration of dextran sulfate sodium (DSS).

Taken together, these findings highlight the central role for TFFs in the maintenance and repair of the intestinal mucosa.

As mentioned previously, mucus is composed of a large and complex variety of molecules. The most representative element is mucin, a glycoprotein encoded by the *MUC2* gene and synthesized by goblet cells. Another important component is constituted by trefoil factors (mainly TFF3), which are small protease-resistant peptides, secreted together with the mucin by the same kind of cells[7,8]. A recent finding demonstrates that TLR2 activation induces synthesis of TFF3 which protects the inflamed mucosa during acute intestinal injury, such as IBD[9].

The other constitutive elements of the mucus are secretory immunoglobulins, especially sIgA, produced by B lymphocytes; antimicrobial peptides such as defensins and lectins, secreted by Paneth cells; antimicrobial protease inhibitors, synthesized by epithelial cells; and enterocyte hydrophobic phospholipids[2,10].

In IBD, a substantial reduction of trefoil factors, which leads to the production of less viscous mucus, has been found. This fact proves that mucus viscosity and not its thickness, as thought previously, is the most important factor in the protection of the epithelium[11]. For instance, in CD we observe goblet cell hypertrophy with an increase of mucus production and a weakness of antimicrobial activity of defensins and peptides[12].

UC is instead characterised by a reduction of MUC2 expression, by a thinning of the mucus layer and by a decreased goblet cell number. Despite this, UC is often clinically represented by a mucus diarrhea, because of a worsening of mucus quality, probably due to an accumulation of non-glycosylated mucin[13].

These alterations could lead to a lessened capability of the mucus layer to limit antigenic traffic and bacterial translocation in the lamina propria, but it is still to be clarified if they are primary defects or secondary effects of the inflammatory state[14].

Epithelial junctions

The absorbent IECs regulate intestinal permeability through the epithelial junctions, by limiting access of microbes to host tissues and mediating the antigenic traffic from the lumen to the lamina propria, where antigens are processed, presented and eliminated. The junctions consist of desmosomes, adherent junctions (AJs) and tight junctions (TJs), necessary for maintenance of intercellular adhesion and to regulate paracellular transport[3].

The adhesive junctional complexes are characterized by transmembrane proteins that interact with adjacent cells and with intracellular adaptor proteins, which are linked to the cytoskeleton. All together they form a connecting network[15].

The most representative structure of AJs is formed by cadherin-catenin interactions, which not only connect the junctional complex to the cellular cytoskeletal network but also help to maintain cell polarity by regulating epithelial migration and proliferation[16,17].

A dysfunction of AJ proteins has been described and consists of a down-regulation of E-cadherin, which weakens intercellular adhesion. This could be responsible for promoting intestinal inflammation, such as in IBD[18]. However, the most important impact on IBD pathogenesis seems to be the impairment of the tight junctions[19].

The tight junctions, located at the apical end of the intercellular space, consist of a complex structure composed of different proteins, such as hyperphosphorylated occludins, proteins of the zonula occludens and proteins of the claudin family[20]. Far from being static structures, tight junctions are highly regulated by cytokines, which play a central role in modulating intestinal barrier function.

Recent studies have showed that proinflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, induce a downregulation of the constituent proteins of the tight junctions, mainly zonula occludens-1. This phenomenon can produce an actin-cytoskeletal disarrangement. Probably these proinflammatory cytokines induce internalization of the apical-junctional complex due to an increase in macropinocytosis of the tight junction proteins[21-23].

Other recent studies have demonstrated that IFN-γ and TNF-α can cause a reorganization of numerous tight junction proteins such as zonulin-1, JAM-A, occludin, claudin-1 and claudin-4, increasing intestinal permeability[24].

A downregulation of occludin, claudin-5 and claudin-8 has been found in CD; an upregulation of claudin-2 has been observed in UC. The expression of this pore-forming protein is due to the stimulation of IECs by interleukin (IL)-13. This cytokine induces an increase of barrier permeability and promotes the epithelial apoptotic events in UC[25-27].

However, the extent of defects in the barrier is more significant in UC than in CD, because of an early presence of apoptotic foci that degenerate into erosion and ulcer-type lesions, which has already occurred in the mild stage of disease[28,29].

Overall, the increase of proinflammatory cytokines leads to an impairment of tight junctions and consequently to a loss of barrier function.

Transforming growth factor (TGF)-β, prototype of anti-inflammatory cytokines and produced by Th3 regulatory cells, can preserve the integrity of the tight junctions by acting directly on IECs, probably through a cAMP-dependent mechanism[30]. Hence, regulatory T cells are involved not only in suppressing inflammatory responses but also in preserving the integrity of the tight junctions.

Many studies have shown that TGF-β also plays a role in epithelial restitution, which occurs after an injury, and its secretion is promoted by the wounded epithelium itself[31].

Defensins

The intestinal mucosa produces antimicrobial peptides, called defensins, which contribute to maintaining host im-

munity and protect from pathological flora. The antimicrobial activity of defensins is expressed by the formation of micropores in the bacterial membranes that cause the lost of pathogen integrity.

In IBD there is a deficiency in defensin expression; however it is not clear whether this alteration contributes to the pathogenesis or is a secondary phenomenon[32].

The gastrointestinal mucosa produces ten types of defensins that help protect the epithelium from microbes. They play an important role, especially in protecting epithelial stem cells, thanks to their location in small bowel at the base of the crypts of Lieberkühn[33].

Defensins can be differentiated into two groups: α-defensins and β-defensins. Their type-expression is modulated along the different intestinal sections. For instance, α-defensins (HD) - especially HD5 and HD6 - are synthesized by Paneth cells positioned in the crypts, while β-defensins (HBD) are produced by colonic epithelial cells[34,35].

These peptides are produced as pro-peptides, a precursor form that needs an enzymatic digestion by trypsin to turn them into their active form. This process is necessary to allow a conformational folding of these proteins, helpful to accomplish their function.

Evidence demonstrates that in CD there is a defective expression of HD5 and HD6 with a release of non-functional peptide that forms an unfolded structure, probably due to a defective enzymatic digestion[36]. A decreased concentration of defensins is responsible for the presence of a less efficient mucus membrane as a biochemical barrier for pathogenic bacteria.

The cause of deficient defensin production has not been clearly determined but the NOD2 signalling pathway could be involved in this process. In fact, NOD2 receptors are highly expressed in Paneth cells and an association between *NOD2* gene mutation and a reduced expression of HD5 mRNA in Paneth cells of CD patients has been found by Wehkamp *et al*[37]. However, it should be pointed out that the primary cause of the α-defensin deficiency is due to an epithelial cell loss[38].

As stated above, HBD are produced by colonic epithelial cells. The type HBD1 is constitutively expressed in all subjects and its concentration does not change, in spite of the presence of inflammatory cytokines or bacteria. In contrast, HBD3 and HBD4 are minimally represented in normal intestinal mucosa and their expression is maintained in CD, and increased in UC. The most important difference in IBD patients with respect to controls is provided by HBD2 production, which is significantly increased in these patients, especially in active UC. This fact is probably due to an increase of pro-inflammatory cytokines[39].

Conversely, in CD, it seems that genetic factors induce a lower expression of inducible β-defensins by means of a suppression of nuclear factor (NF)-κB, which is caused by a direct mechanism or a *NOD2* mutation[40].

IECs as sentinels of innate immunity: Toll-like receptors and NOD signaling

The innate immune system is able to recognize a limited set of conserved bacteria and viral motifs known as pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs), including above all the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) families.

TLRs are a family of receptors which recognize specific PAMPs and activate signal transduction through the NF-κB pathway. As a consequence, a pro-inflammatory cascade initiates to induce cytokine and chemokine genes[41]. Activation of the TLR pathway occurs through an adapter molecule, myeloid differentiation factor 88 (MyD88)[42,43].

IECs express several members of the TLR family: TLR2, which recognizes peptidoglycan (PGN), a component of the bacterial cell walls of Gram+ bacteria; TLR3, a receptor for viral double-stranded RNA; TLR4, which recognizes lipopolysaccharide (LPS), the major component of the outer membrane of Gram- bacteria; and finally, TLR5, which binds bacterial flagellin. TLR1, TLR3 and TLR4 are all located on the apical surface of IECs; alternatively, TLR5 is restricted to the basolateral surface of IECs and it is only activated when bacteria invade the epithelium[42].

These findings suggest that PRRs are positioned in order to trigger a response in the event of bacterial penetration of the epithelium. Therefore, pathogens, which unlike commensals are able to penetrate the barrier, are recognized by basolateral TLRs[44]. Moreover, TLRs bind saturated fatty acids in acetylated form, which are essential for the agonistic activity.

Wolowczuk *et al*[45] report that saturated fatty acids are able to induce the activation of TLR2 and TLR4, whereas unsaturated fatty acids - such as PUFAs - inhibit the TLR-mediated signaling pathway and gene expression by suppressing NF-κB activation and inflammation. These data suggest the protective role of unsaturated fatty acids such as omega-3 and the regulation of immune responses by fatty acid types.

Recognition by TLRs protects against pathogens and is carefully regulated to shut down a proinflammatory response to commensal organisms[41].

An interesting observation is that various TLRs are also expressed in cells of the adaptive immune system, such as B and T cells and dendritic cells (DCs), inducing differentiation and cytokine production, connecting innate to adaptive immunity. We can consequently consider the TLRs as a link from upper to lower barrier function.

For all these reasons it is clear that TLRs may have a dual role. Under normal conditions they maintain oral tolerance and eliminate pathogens, while in IBD they can also amplify inappropriate immune responses.

Several polymorphisms of TLRs have been associated with IBD, such as those of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9. However, the functional effects of these variants are not well defined[43].

Szebeni *et al*[46] have demonstrated that in IECs of IBD patients, there is an abnormal production of certain TLR subtypes with a significant upregulation of TLR2 and TLR4 expression in the inflamed mucosa. These alterations could compromise the capability to distinguish

commensals from pathogens, or amplify inappropriate immune responses. It is still to be clarified whether this upregulation is one of the causes of IBD or just one of the consequences or even a concomitant factor.

NOD2 is a gene encoding for a cytoplasmatic protein (also known as CARD15), which recognizes bacterial muramyl dipeptide (MDP). This is the major component of peptidoglycan (PGN) and it is present in both Gram+ and Gram- bacteria. The binding of MDP to NOD2 results in the activation of the NF-κB pathway and IL-12 production[47].

In recent years researchers have identified a large number of *NOD2* polymorphisms (SNPs) and the most common are associated with susceptibility to CD[48].

It seems that NOD2 variations lead to an impaired intracellular microorganism recognition and a consequent perpetual nuclear translocation of NF-κB, which results in an inadequate phlogosis state.

Using transgenic mice, Watanabe *et al*[49] have demonstrated that mice overexpressing *NOD2* exhibit greatly decreased IL-12 responses to systemic administration of PGN but not to LPS, indicating that defects in NOD2 signaling lead to excessive TLR2-dependent inflammatory responses. Indeed, under normal conditions, PGN from commensal bacteria leads to innate immune responses, which are subsequently made weak by NOD2 modulation and other regulatory responses[49]. In the case of a NOD2 signaling defect, a TLR2-dependent inflammatory response cannot be controlled, leading to mucosal injuries[50].

Strober's group demonstrated that mice with cells with increased NOD2 function have decreased responses to TLR stimulation, resulting in protection against DSS-induced colitis. They showed that prestimulation of cells with NOD2 ligand renders them unresponsive to TLR stimulation, because of an inhibitor of TLR-induced inflammatory pathways (IRF4)[51,52].

Greater understanding of the relationship between NOD2 variations and the pathomechanisms of IBD is required, but recent studies indicate that these mutations could participate, together with other barrier dysfunctions described previously, in the progression to CD[48].

Bacterial-epithelial interactions

TLRs and NOD proteins are also key sensors of bacterial-epithelial interactions. The intestinal microbiota contribute to protecting the host against invasions by pathogenic bacteria, competing for nutrients and stimulating immune responses, and play a crucial role in correct epithelial cell development. In return, commensal bacteria derive benefits from this association with the host since they can inhabit a protected environment from which they receive nutrients[53].

Within the bacterial-epithelial interface there is a continuous cross-talk which is enabled by PRRs; components of the mammalian innate immune system continuously sample the composition of commensal communities. However, only pathogens can activate the innate immune system since they can survive within host tissues.

A recent study showed that the immune status of the host can influence the composition of the commensal community. For instance, in the intestinal epithelium of *Drosophila melanogaster*, the dysregulation of NF-κB-dependent expression of antimicrobial peptides results in the outgrowth of a pathogenic commensal community[54].

In turn, scientific evidence also shows that commensal bacteria modulate IEC function by inhibiting the NF-κB pathway, through blocking the ubiquitylation and degradation of IκB or by hijacking the peroxisome-proliferation-activated receptor-γ (PPARγ) pathway[44].

Several pathologic features of IBD suggest that they derive in part from dysregulated control of bacterial interactions with the mucosal surface. For example, as reported by Duerkop *et al*[55] demonstrated that IBD patients exhibit increased numbers of mucosal surface-associated bacteria. This evidence suggests a failure of the mechanisms to prevent the microbiota from direct contact with the surface epithelium.

In 2006, Mizoguchi found that chitinase 3-like1 (CHI3L1), a molecule characterized by a strong binding affinity to chitin (a polymer of N-acetylglucosamine richly found in microorganisms), is specifically up-regulated under intestinal inflammatory conditions; it plays a pathogenic role in acute colitis by enhancing bacterial adhesion and invasion into colonic epithelial cells[56].

Many studies have investigated the presence of specific potentially pathogenic microorganisms in the mucosa of IBD patients; Darfeuille-Michaud *et al*[57] have demonstrated the association between ileal CD and adherent-invasive *Escherichia coli* (AIEC), not only as secondary invaders but also as possibly responsible for the initiation of the inflammatory process.

Moreover, it was demonstrated that CD-associated AIEC strains adhere to the brush border of primary ileal enterocytes isolated from CD patients but not from controls; AIEC adhesion is dependent on type 1 pili expression on the bacterial surface and on carcinoembryonic antigen (CEA)-related cell adhesion molecule 6 (CEACAM6) expression on the apical surface of ileal epithelial cells in CD patients. CEACAM6 is up-regulated in intestinal epithelial cells of CD patients and acts as a receptor for AIEC adhesion. Finally, this study suggests that AIEC can promote its own colonization in CD patients, since it is able to increase CEACAM6 expression in infected epithelial cells[58].

THE LOWER BARRIER

A continuous epithelium: lymphocytes cross-talking

Recent advances regarding an active role of intestinal epithelial cells within the mucosal immune system have revealed that they act as non-professional antigen presenting cells (APCs), activating subsets of T-cells with regulatory function. On the other hand, lamina propria lymphocytes are able to influence epithelial cell growth and differentiation along the crypt/villus axis by mediating intercellular interactions and by secreting cytokines and other fac-

tors[41]. In IBD patients, the cross-talking between IECs and mucosal lymphocytes is changed by altered production of these factors. This complicated dialogue contributes to promoting mucosal inflammation.

IECs are able to directly process and present antigens to lymphocytes by a highly polarized system with apical antigen sorting, processing and exclusively basolateral presentation.

IECs do not express conventional costimulatory molecules such as CD80 and CD86, but express new B7 family members, such as B7h [inducible costimulatory ligand (ICOS-L)] and B7-H1 [programmed death ligand (PD-1 L)], as well as several non-classical MHC class Ib molecules such as MICA/B, HLA-E, and CD1d[42,54].

By using *in vitro* IEC: peripheral blood T-cells (PBT) co-cultures, Allez *et al*[59,60] have demonstrated that intestinal epithelial cells preferentially activate CD8+ regulatory T-cells, in particular a CD28- and CD101/CD103+ subset, characterized by the biased usage of the T cell receptor (TCR) Vβ5.1 chain. These data have been confirmed by the same group *in vivo* by using CD8+ T-cells isolated from the lamina propria (LP) of healthy subjects.

Hence, under normal conditions, luminal antigens presented by IECs cause a suppression rather than an increase of the immune response.

For the activation of the restricted regulatory T-cell subset, the role is crucial of a unique complex formed by gp-180 (a CEA family member glycoprotein) and by CD1d, which bind CD8 and the TCR on the T-cell surface, respectively. Indeed, blocking gp-180 with a monoclonal antibody (B9) suppresses the proliferation of regulatory T-cells[61-63]. This helps to explain the oral tolerance and controlled inflammation phenomena.

Results from both *in vitro* IEC:PBT co-cultures and from *in vivo* within the lamina propria of IBD patients demonstrated a reduced amount of CD8+ regulatory T cells, that might be linked to glycoprotein gp-180. Indeed, the frequency of Vβ5.1 cells among the LP CD8+ T cells is significantly decreased in IBD patients with respect to healthy subjects[64].

In IBD IECs, especially in CD, the same group observed a defective expression of gp-180, and moreover, IECs from IBD patients preferentially stimulate CD4+ T-cell proliferation and secretion of IFN-γ, through MHC class Ⅱ[65,66].

In a subsequent study, by using freshly isolated IECs and lamina propria lymphocytes (LPLs), as well as T84 cell lines, we have correlated SOX9 to *CEACAM5* gene expression: SOX9 is able to downregulate CEACAM5. The former is a transcription factor involved in the differentiation of several tissues such as chondrocytes, male gonads, neural crest and spinal cord glial cells, while the latter is a member of the CEA family. We speculate that LPLs, the main source of cytokines within the gastrointestinal mucosa-associated lymphoid tissue (GALT), influence the nuclear translocation of SOX9 in IECs and consequently the downregulation of the CEA family member gp-180, together with a lack of activation and/or expansion of regulatory cells[67,68].

As mentioned previously, the described cross-talk between epithelium and LPLs has also a role from the standpoint of IECs, in particular affecting their proliferation and differentiation along the crypt/villus axis in the colon. Indeed, starting from the concept that IECs can promote regulatory T-cell responses in the mucosa, Dahan *et al*[41] have demonstrated that lympho-epithelial interactions occur and play a role in IBD. By using freshly isolated LPLs derived from healthy subjects and CD patients, they suggest that cross-talk leads to an enhanced IEC differentiation, a pattern restricted to CD, which seems to involve the transcription factor CDX2 and PI3K/p38 MAPK pathways.

Moreover, T84 cells co-cultured with CD LPLs display a greater increase of differentiation and CDX2 mRNA levels with respect to normal LPLs.

These data were confirmed *in vivo*, through immunostaining both in human colonic mucosa and in RAG1-/- mice lacking lymphocytes; studies in which an altered IEC differentiation was observed[41,69,70].

CONCLUSION

Our purpose was to review the state of the art in understanding the key role of intestinal epithelial cells in maintaining gut homeostasis and the possible role in the pathogenesis of IBD.

Intestinal epithelial cells, because they act as a functional barrier and as non-professional antigen presenting cells, represent important elements in the development and maintenance of immune oral tolerance.

In IBD, we observe a global defect in the mucosal immune system: barrier function, innate and adaptive responses.

Two main strands of research on these defects exist: one is focused on the impairment of the epithelial barrier, the other on defects of epithelial-lymphocyte cross-talk. In both these lines of investigation, IECs occupy a prominent place within the complex and dynamic system of the intestinal barrier. Despite much progress in this area of research, it remains to be clarified whether defects involving IECs are fundamental or a consequence of abnormal signals coming from the lamina propria.

A better understanding of these regulatory mechanisms, which allow us to see intestinal epithelial cells at the interface between an "upper" and "lower" barrier, could help to identify new therapeutic targets.

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ORIGINAL ARTICLE

α,β-amyrin, a natural triterpenoid ameliorates L-arginine-induced acute pancreatitis in rats

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Abstract

AIM: To study the beneficial effects of triterpene α,β-amyrin and the underlying mechanisms in an experimental pancreatitis model.

METHODS: Acute pancreatitis was induced in five groups of rats (n = 8) by L-arginine (2 × 2.5 g/kg, intraperitoneal, 1 h apart) and 1 h later, they received a single oral dose of α,β-amyrin (10, 30 and 100 mg/kg), methylprednisolone (30 mg/kg) and vehicle (3% Tween 80). A saline (0.9% NaCl) treated group served as a normal control. Efficacy was assessed at 24 h by determination of serum levels of amylase, lipase and pro-inflammatory cytokines [tumor necrosis factor (TNF)-α and interleukin (IL)-6], pancreatic myeloperoxidase (MPO) activity, lipid peroxidation [thiobarbituric acid reactive substances (TBARS)], nitrate/nitrite levels, and the wet weight/body weight ratio. Tissue histology and the immunoreactivity for TNF-α and inducible nitric oxide synthetase (iNOS) were performed.

RESULTS: α,β-amyrin and methylprednisolone treatments significantly (P < 0.05) attenuated the L-arginine-induced increases in pancreatic wet weight/body weight ratio, and decreased the serum levels of amylase and lipase, and TNF-α and IL-6, as compared to the vehicle control. Also, pancreatic levels of MPO activity, TBARS, and nitrate/nitrite were significantly lower. Histological findings and TNF-α and iNOS immunostaining further confirmed the amelioration of pancreatic injury by α,β-amyrin.

CONCLUSION: α,β-amyrin has the potential to combat acute pancreatitis by acting as an anti-inflammatory and antioxidant agent.

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Key words: Acute pancreatitis; L-arginine; Cytokines; Lipid peroxidation; α,β-amyrin

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INTRODUCTION

Acute pancreatitis (AP) is a life-threatening inflammatory disorder with a significant impact on patient health. Although its pathogenesis is not fully understood, microcirculatory disturbances, leukocyte activation, and oxidative stress are the main events in AP that is characterized by activation of digestive proteases, a widespread inflammatory cell infiltration, leukocyte activation and the release of various kinds of inflammatory mediators and reactive oxygen and nitrogen species[1-4]. Repeated attacks of acute pancreatitis have the potential to evolve into chronic disease that is characterized by fibrosis and loss of pancreatic function[5]. There are no specific therapies for acute pancreatitis. Medical management is aimed at the control of symptoms with anti-inflammatory agents, steroids, and analgesics. As a result of the limitations of conventional therapy, many ethnobotanical agents are being pursued as alternative sources to develop novel and safe therapeutic agents to treat pancreatitis[6-11].

The resin (oily amorphous exudate) obtained from the trunk wood of *Protium heptaphyllum* (*P. heptaphyllum*) (Aubl.) March (Burseraceae) that grows abundantly in Brazil and South America is a reputed folk medicinal agent because of its analgesic and anti-inflammatory properties[12,13]. Chemical investigations have revealed the presence of α,β-amyrin, a pentacyclic triterpene as the major component of resin, and pharmacological studies have revealed its anti-inflammatory, antipruritic, gastroprotective and hepatoprotective effects[14-17]. Also, a few other studies have shown its efficacy in suppressing the acute visceral and orofacial nociception and bladder inflammation[17,18]. Recently, Vitor *et al*[19] have demonstrated that α,β-amyrin exerts a marked and rapid suppression of inflammatory cytokines and cyclooxygenase-2 levels in a murine model of trinitro-benzene-sulfonic acid (TNBS)-induced colitis. Since these studies have established the anti-inflammatory, antinociceptive, and antioxidant properties of α,β-amyrin at non-toxic doses, which range from 10 to 100 mg/kg, the present study evaluated its potential to ameliorate pancreatic injury in a rat model of acute pancreatitis induced by L-arginine, wherein inflammation and oxidative stress play a pathogenic role.

MATERIALS AND METHODS

Plant material and isolation of α,β-amyrin

The resinous exudate from the trunk wood of *P. heptaphyllum* (March.) was collected from the municipal areas of Timon, Maranhão state of Brazil, after its identification by botanist Professor Roseli Farias de Melo Barros. A voucher sample (#18247) has been deposited at the Herbarium Graziela Barroso of the Federal University of Piauí, Teresina, Brazil. The extraction and isolation of α,β-amyrin from the crude resin of *P. heptaphyllum* (March.) was carried out as described earlier[20] and its structural identity was confirmed by ^{1}H- and ^{13}C-NMR spectral analysis, based on the method developed by Gallegos *et al*[21] and in comparison to literature data[22]. The ratio of α- and β-amyrin in this mixture was 63:37.

Animals and animal procedures

Forty-eight male Wistar rats obtained from the Central Animal House of Federal University of Ceara, Fortaleza were maintained at a constant room temperature (23 ± 2℃) with light-dark cycles of 12/12 h and free access to water and standard laboratory chow. The rats were randomly divided into six groups of eight in each and experiments were performed after 12 h of fasting. Their body weights ranged between 180 and 200 g at the time of experimentation.

Experimental protocols were approved by the Institutional Committee on Care and Use of Animals for experimentation (No. 84/08) in accordance with the guidelines of the National Institutes of Health, Bethesda, MD, USA.

Chemicals

L-arginine, hexadecyltrimethylammonium bromide (HETAB), *o*-dianisidine dihydrochloride, thiobarbituric acid and methylprednisolone were from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and reagents were of the highest commercial grade available.

L-arginine-induced pancreatitis model

Acute pancreatitis was induced in five groups of rats (n = 8/group) by two intraperitoneal (ip) injections of L-arginine (2.5 g/kg, 1 h apart)[23]. One hour following the last injection of L-arginine, the rats were treated orally as follows: group 1 received the vehicle (3% Tween 80) of α,β-amyrin (vehicle control); groups 2, 3 and 4 were treated with α,β-amyrin (10, 30 and 100 mg/kg, respectively); and group 5 acted as positive control and received methylprednisolone (30 mg/kg), all in a volume of 10 mL/kg.

A sixth group (n = 8) of rats that received saline (0.9%, NaCl, ip) in place of L-arginine served as a normal control. Twenty-four hours after the last injection of L-arginine or saline, a midline laparotomy was performed in rats under ketamine anesthesia and blood samples were collected from the inferior vena cava, the rats were then exsanguinated, the whole pancreas was quickly removed and stored at -70℃ until use. The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema (mg/g)[24].

Serum analysis

For serum assays, blood samples were centrifuged at 3000 × g at 4℃ for 10 min. The serum amylase and lipase were determined by routine colorimetric methods using the commercial kits for amylase (Labtest Diagnostica SA, Lagoa Santa, Brazil) and lipase (Bioclin-Quibasa, Belo Hor-

izonte, Minas Gerais, Brazil) and expressed as U/dL and U/L, respectively. Serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured using an ELISA kit according to the manufacturer's instructions (Quantikine®; R&D Systems, Minneapolis, MN, USA). The cytokine levels were calculated from the standard curve and expressed as pg/mL.

Determination of myeloperoxidase activity and thiobarbituric acid-reactive substances

The degree of neutrophil infiltration was quantified by the measurement of pancreatic myeloperoxidase (MPO) activity[25]. Pancreatic tissue (50 mg) was minced and homogenized in 0.5 mL of 50 mmol/L phosphate buffer solution (PBS) (pH 6) that contained 0.5% HETAB. The homogenate was subjected to three cycles of freezing (-30℃) and thawing (37℃) and brief periods (15 s) of sonication, after which, they were centrifuged at 12000 × *g* for 15 min at 4℃. The supernatant (0.1 mL) was mixed with 2.9 mL of 50 mmol/L PBS, pH 6, which contained 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 470 nm was then measured for 5 min using a Beckman spectrophotometer (Beckman DU 640B; CA, USA).

Thiobarbituric acid-reactive substances (TBARS) level in the pancreatic tissue was determined as an indicator of lipid peroxidation according to a previously described method[26]. Briefly, 500 μL of 10% tissue homogenate in 0.15 mol/L KCl was mixed with 200 μL 8.1% SDS, and then incubated at room temperature for 5 min. The reaction mixture was heated at 95℃ for 1 h after the addition of 1.5 mL 20% acetic acid (pH 3.5) and 1.5 mL 0.8% thiobarbituric acid. After the mixture had cooled, 1.0 mL distilled water and 5.0 mL butanol/pyridine (15:1) solution were added under agitation using a vortex. This solution was centrifuged at 1000 × *g* for 15 min, and the resultant colored layer was measured at 532 nm using a Beckman DU 640B spectrophotometer.

Determination of nitrate/nitrite levels

Total nitrate/nitrite levels were determined as a measure of nitric oxide with the use of Griess reagent[27]. The pancreatic tissue was homogenized in 50 mmol/L potassium phosphate buffer (pH 7.8) and centrifuged at 11000 × *g* for 15 min at 4℃. One hundred microliters of the supernatant was mixed with 100 μL Griess reagent [0.1% N-(1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid], and after 10 min, the absorbance was measured at 540 nm using a Beckman DU 640B spectrophotometer. The standard curve was obtained by using sodium nitrite. The results were calculated from a standard curve by using sodium nitrite and expressed as micromoles of nitrate/nitrite.

Pancreatic histology and immunohistochemistry

Samples of pancreatic tissue were fixed in 10% buffered formalin solution, embedded in paraffin by standard methods, cut into 5-μm sections, stained with hematoxylin-eosin, and then assessed under light microscopy and examined blind by a morphologist for grading the histological alterations. Pancreatic edema, leukocyte infiltration, hemorrhage, acinar vacuolization and necrosis were described with scores ranging from 0 to 3 as described previously[28].

Immunohistochemical analysis of the expression of TNF-α and inducible nitric oxide synthase (iNOS) was performed. Sections of pancreas (4 μm) were transferred to a gelatin-coated slide. The tissue sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide (30 min). Non-specific protein binding was blocked by incubating the tissue sections with goat serum (0.5% in PBS for 45 min). The slides were then incubated overnight with primary rabbit anti-TNF-α or rabbit anti-iNOS (Sigma), diluted 1:400 in PBS plus bovine serum albumin. For TNF-α, the slides were incubated with avidin-biotin-horseradish peroxidase conjugate (Vectastain® ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 min, and TNF-α was visualized with the chromogen 3,3' diaminobenzidine and counterstained with Harry's hematoxylin. For iNOS, the slides were incubated with alkaline-phosphatase-conjugated secondary antibody (EnVisionTM/AP, K1396; Dako, Carpinteria, CA, USA). The reaction was developed by applying on the slides a solution containing levamisole and Fast Red Substrate (EnVisionTM/AP, K1396; Dako).

Statistical analysis

Statistical analysis was performed by analysis of variance followed by Kruskal-Wallis or Student Newman Keul's as post-hoc tests using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). The non-parametric data are expressed as median (with low and high ranges), and parametric data, expressed as mean ± SE. Differences were considered to be statistically significant when P was < 0.05.

RESULTS

Serum biochemical parameters and pancreatic edema

Figure 1 shows the levels of serum amylase and lipase activities and the pancreatic edema in rats under different treatments. When compared to the saline-treated group, the levels of serum amylase and lipase were significantly (P < 0.05) higher in the L-arginine-induced acute pancreatitis group. However, L-arginine caused a much higher increase in lipase as compared to amylase. Besides, L-arginine markedly increased the pancreatic wet weight/body weight ratio, an index of pancreatic edema. While treatment with α,β-amyrin (10, 30 and 100 mg/kg) significantly (P < 0.05) lowered the L-arginine-induced elevation of serum amylase and lipase (Figure 1A and B) at all doses, a significant decrease in pancreatic edema was observed only at 30 and 100 mg/kg (Figure 1C). Methylprednisolone (30 mg/kg), the reference anti-inflammatory drug included in the study, manifested similar reductions in serum amylase and lipase activities, as well as in pancreatic edema (Figure 1).

Figure 2 depicts the serum levels of pro-inflammatory cytokines TNF-α and IL-6 in rats under different treatments. Both TNF-α and IL-6 were significantly elevated

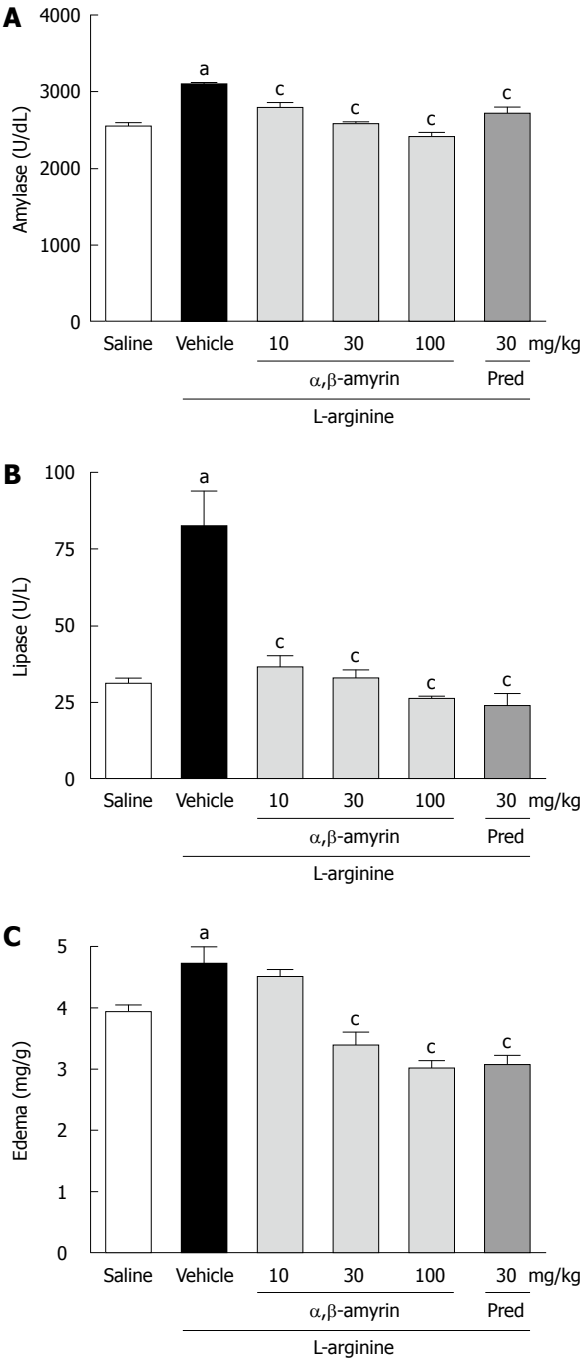

Figure 1 Effects of α,β-amyrin treatment on serum amylase (A), lipase (B) and on pancreatic edema (C) in rats on L-arginine-induced acute pancreatitis. Each column represents mean ± SE (n = 8). $^{a}P < 0.05$ *vs* saline control group; $^{c}P < 0.05$ *vs* vehicle control group. Pred: Methylprednisolone.

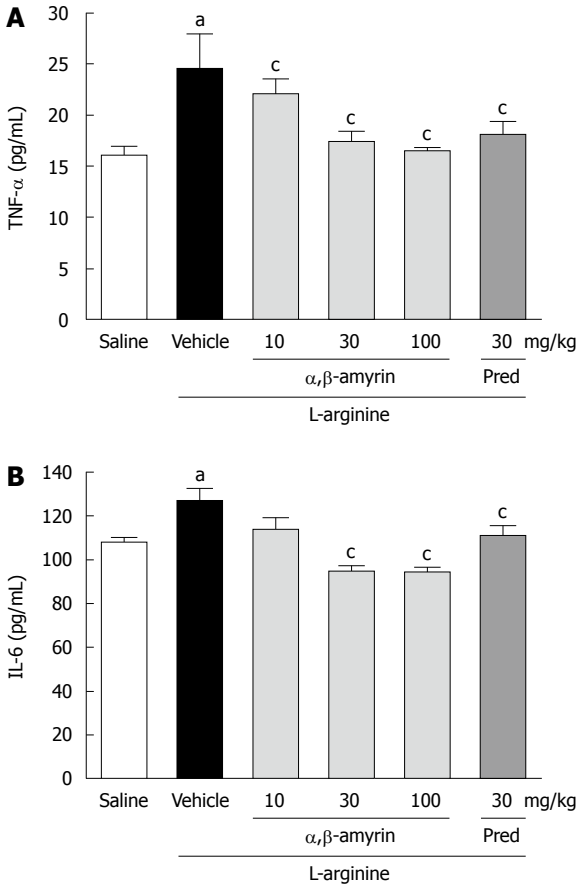

Figure 2 Effect of α,β-amyrin treatment on the serum tumor necrosis factor α (A) and interleukin-6 (B) in L-arginine induced acute pancreatitis. Each column represents mean ± SE (n = 8). $^{a}P < 0.05$ *vs* saline control group; $^{c}P < 0.05$ *vs* vehicle control group. Pred: Methylprednisolone; TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6.

in the L-arginine-induced pancreatitis control group when compared to the saline group. Treatment with α,β-amyrin (10, 30 and 100 mg/kg) effectively decreased the enhanced levels of TNF-α at all doses, however, IL-6 inhibition was statistically significant only at 30 and 100 mg/kg of α,β-amyrin.

Pancreatic MPO activity, and levels of TBARS and nitrate/nitrite

In the L-arginine-induced acute pancreatitis group, pancreatic MPO activity, TBARS, and nitrate/nitrite levels were significantly elevated when compared to the saline-treated group (Figure 3A-C). Treatment with α,β-amyrin (10, 30 and 100 mg/kg) and methylprednisolone significantly ($P <$ 0.05) reduced the L-arginine-evoked increase in pancreatic MPO activity, TBARS and nitrate/nitrite levels.

Pancreatic histology and immunostaining

Representative TNF-α and iNOS immunostaining of the pancreas for different treatments are shown in Figures 4 and 5. In saline-treated control rats, the pattern of TNF-α and iNOS staining was very mild (Figures 4A and 5A). On the other hand, there was a high intensity staining for TNF-α and iNOS in the acinar cells, inflammatory cells and blood vessels of the pancreas in the L-arginine-induced acute pancreatitis group that received only the vehicle (Figures 4B and 5B), however, in rats treated with α,β-amyrin (100 mg/kg) or methylprednisolone (30 mg/kg), immunostaining intensity for TNF-α and iNOS was much less (Figures 4C, D and 5C, D).

Histological examination of the saline-treated controls showed normal architecture and absence of edema, leukocyte infiltration, acinar vacuolization, hemorrhage and necrosis (Figure 6A and Table 1). In contrast, pancreatic sec-

Table 1 Effects of α,β-amyrin treatment on morphological signs of pancreatic damage

Group	Edema (0-3)	Inflammatory infiltration (0-3)	Acinar vacuolization (0-3)	Hemorrhage (0-3)	Necrosis (0-3)
Saline control	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Vehicle + L-arginine	3 (2-3)[a]	2 (2-3)[a]	3 (3-3)[a]	2 (2-3)[a]	3 (3-3)[a]
α,β-amyrin (100 mg/kg) + L-arginine	0 (0-0)[c]	0 (0-0)[c]	0 (0-1)[c]	0 (0-0)[c]	0 (0-0)[c]
Pred + L-arginine	0 (0-1)[c]	0 (0-0)[c]	1 (0-1)[c]	0 (0-1)[c]	0 (0-0)[c]

Median scores with ranges (min-max) of the results in six animals in each group are shown. [a]$P < 0.05$ *vs* saline control group; [c]$P < 0.05$ *vs* vehicle + L-arginine group. Pred: Methylprednisolone.

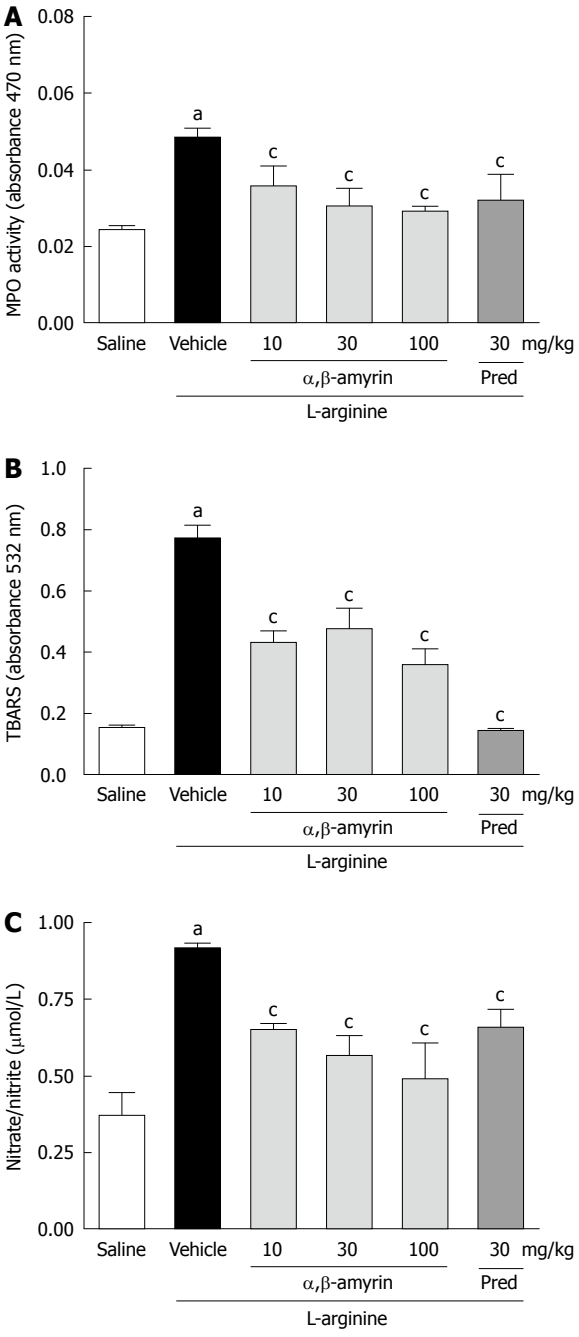

Figure 3 Effects of α,β-amyrin treatment on the pancreatic myeloperoxidase activity (A), thiobarbituric acid-reactant substances (B) and nitrate/nitrite levels (C) in L-arginine induced acute pancreatitis. Each column represents mean ± SE ($n = 8$). [a]$P < 0.05$ *vs* saline control group; [c]$P < 0.05$ *vs* vehicle control group. Pred: Methylprednisolone; MPO: Myeloperoxidase; TBARS: Thiobarbituric acid-reactant substances.

tions from the L-arginine-induced acute pancreatitis group of rats revealed extensive tissue damage that was characterized by significant disruption of normal architecture, with massive edema, acinar cell vacuolization, necrosis, hemorrhage and inflammatory cell infiltration, and thus received significantly higher scores (Figure 6B and Table 1). Treatment with α,β-amyrin (100 mg/kg) and methylprednisolone (30 mg/kg) resolved the inflammation, and most strikingly the edema, and protected the pancreas from histological damage induced by L-arginine. In addition, the total pathological scores were significantly decreased by α,β-amyrin treatment (Figure 6C and D, Table 1).

DISCUSSION

Our study demonstrated that the natural triterpene α,β-amyrin has the potential to attenuate the severity of L-arginine-induced pancreatitis in rats. In agreement with previous studies[29,30], we found significant increases in serum amylase and lipase levels, neutrophil infiltration, massive edema, necrosis and hemorrhage in this experimental model of pancreatitis. Besides, we noticed an increase in the serum levels of pro-inflammatory cytokines TNF-α and IL-6, and a higher expression of TNF-α and iNOS in pancreatic tissue, consistent with earlier reports that have described similar increases in experimental pancreatitis and in clinical patients as well[23,31,32]. TNF-α plays a pivotal role in severe acute pancreatitis, acting early in the disease course[33], and IL-6 constitutes the principal mediator in the synthesis of acute-phase proteins, in addition to transitioning the acute inflammatory response to a chronic response[34]. These findings suggest that inflammatory cytokines and neutrophil-mediated oxidative stress have a central role in the pathogenesis of acute pancreatitis induced by L-arginine, and it also implies that compounds that combat inflammation and oxidative stress ameliorate acute pancreatitis. Treatment with triterpene α,β-amyrin and glucocorticoid methylprednisolone resulted in a significant decrease of serum amylase and lipase, pancreatic edema, and serum TNF-α and IL-6 levels, as well as the TNF-α and iNOS expression induced by L-arginine. Both drugs effectively improved the pancreatic morphology, and these results clearly point out their anti-inflammatory potential in obliterating the pancreatic inflammation and the associated tissue injury.

The glucocorticoids are useful for the treatment of a wide range of inflammatory and autoimmune condi-

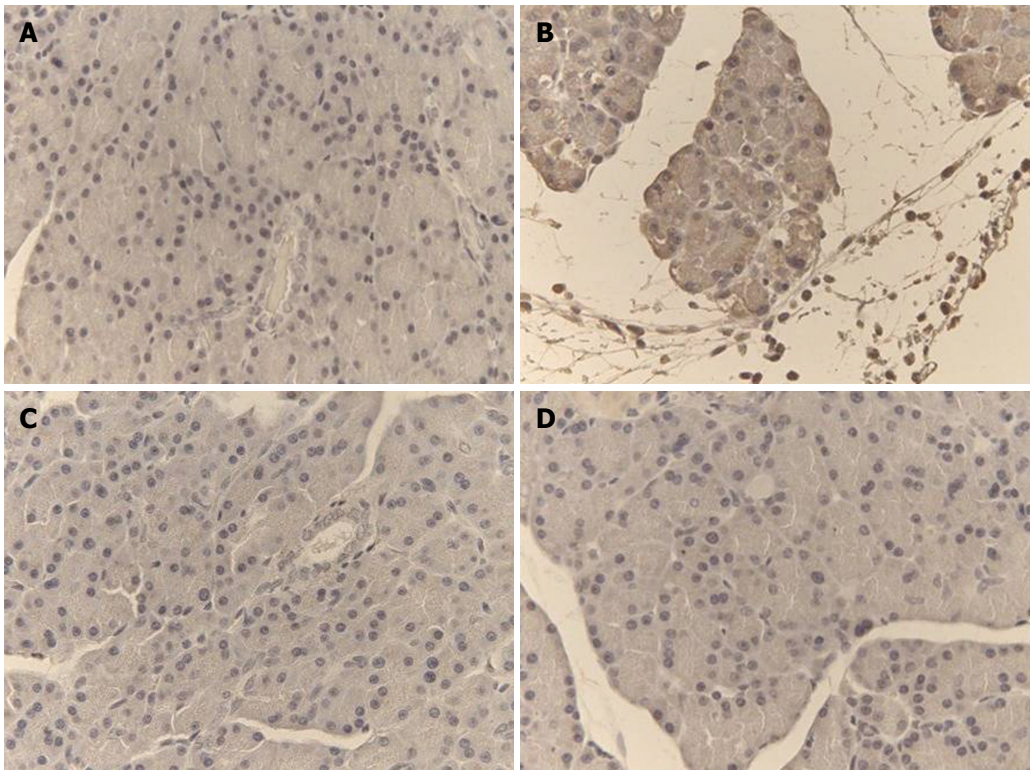

Figure 4 Effect of α,β-amyrin on tumor necrosis factor α immunoreactivity in L-arginine-induced acute pancreatitis (× 400). A: Normal control group; B: Vehicle + L-arginine; C: α,β-amyrin (100 mg/kg) + L-arginine; D: Methylprednisolone (30 mg/kg) + L-arginine.

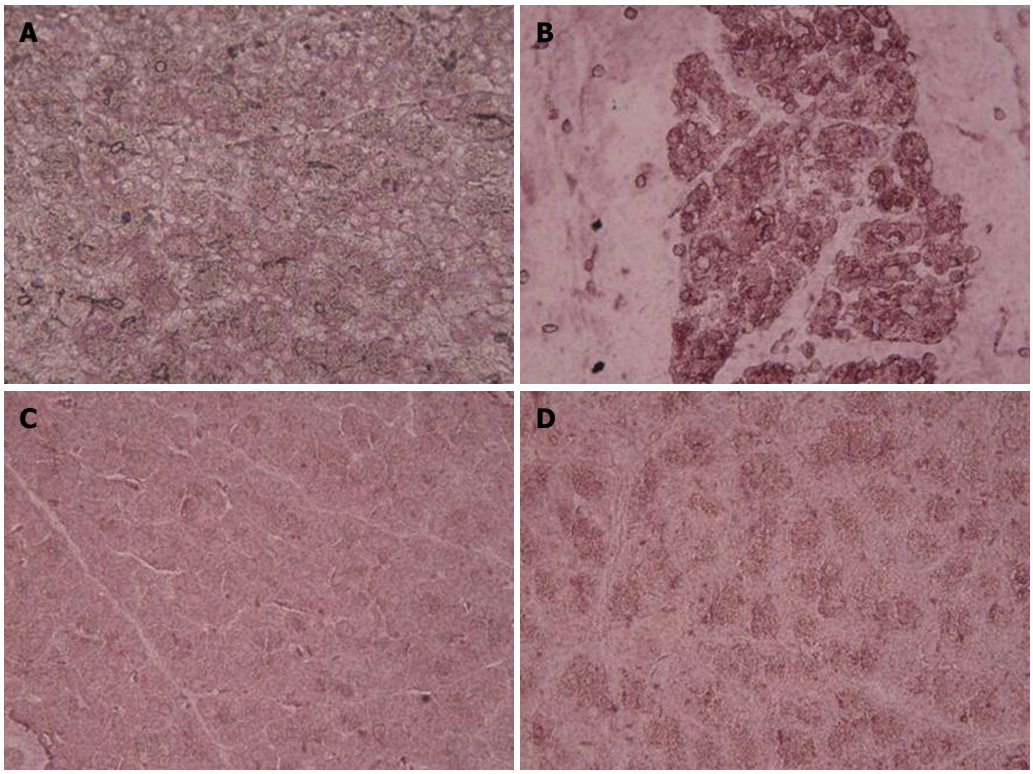

Figure 5 Effect of α,β-amirin on inducible nitric oxide synthetase immunoreactivity in L-arginine-induced acute pancreatitis (× 400). A: Normal control group; B: Vehicle + L-arginine; C: α,β-amyrin (100 mg/kg) + L-arginine; D: Methylprednisolone (30 mg/kg) + L-arginine.

tions[35]. The effect of α,β-amyrin treatment is the same as that of glucocorticoid methylprednisolone that has been previously shown to be effective in the L-arginine model of experimental pancreatitis[36]. Since α,β-amyrin

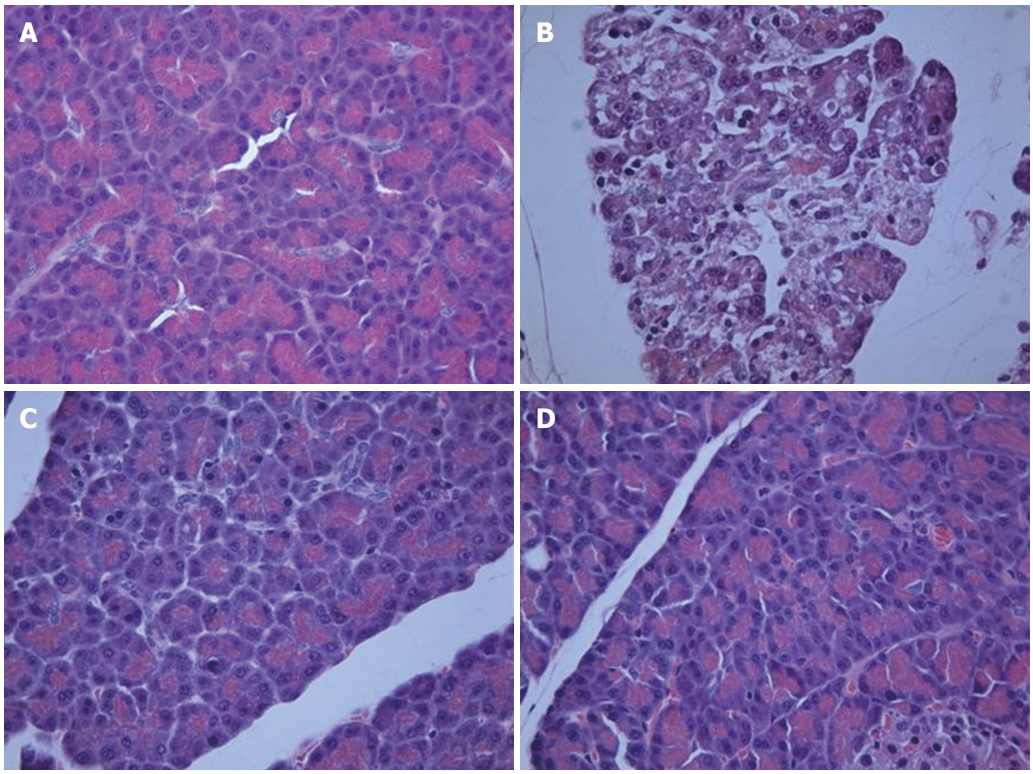

Figure 6 Representatives microphotographs of pancreatic sections (× 400). A: Normal control group; B: Vehicle + L-arginine; C: α,β-amyrin (100 mg/kg) + L-arginine; D: Methylprednisolone (30 mg/kg) + L-arginine.

belongs to the group of ursane and oleanane pentacyclic triterpenes that have a chemical structure that resembles glucocorticoids, it might exercise similar anti-inflammatory effects by affecting the transcription of inflammatory mediators[37].

α,β-amyrin therapy significantly reduces the extent of edema and the total pathological score, possibly due to its anti-inflammatory action[17-19]. NO produced *via* activity of NOS is one of the factors that is involved in the regulation of the rate of perfusion of the pancreatic microvessels[38]. These authors have suggested that excess arginine can induce iNOS activity, which results in high tissue levels of NO that might cause a direct toxic effect on pancreatic acinar cells. The ability of NO to increase the vascular/microcapillary permeability might contribute to the occurrence of pancreatic edema. Total nitrate/nitrite, a marker of endogenous NO, was markedly increased by L-arginine treatment, and was found to be significantly reduced in rats pretreated with α,β-amyrin (100 mg/kg). Furthermore, immunohistochemical staining for iNOS showed that α,β-amyrin could inhibit their expression. It implies that inhibition of NO also participates in the protective effect of triterpenoid.

In the present study, possibly, α,β-amyrin inhibited neutrophil infiltration, TNF-α and IL-6 production, and iNOS expression in pancreatic tissue, probably *via* inhibition of nuclear factor (NF)-κB activity. In this context, recent studies have shown that α,β-amyrin can inhibit NF-κB activation and thereby the production of inflammatory mediators[19]. Oxidative stress plays an important role in the pathophysiology of acute pancreatitis and the beneficial effects of α,β-amyrin might also be associated with the inhibition of NF-κB activity. Excessive reactive oxygen and nitrogen species produced by NOS and isoforms of NADPH oxidase, or as by-products of the mitochondrial electron-transport chain, have been implicated in the pathogenesis of acute pancreatitis[4]. α,β-amyrin potently suppressed neutrophil-mediated MPO and lipoperoxidation, as demonstrated by reduced TBARS formation; events that reflect its antioxidant action. Thus, the present study reveals that α,β-amyrin ameliorates acute pancreatitis by suppressing pro-inflammatory cytokines TNF-α and IL-6, and iNOS expression.

In conclusion, the study provides the first evidence to show that α,β-amyrin attenuates the development of L-arginine-induced acute pancreatitis by reducing the infiltration of neutrophils, generation of inflammatory cytokines and iNOS. This study might provide a basis for future investigations of the therapeutic role of α,β-amyrin in severe necrotizing pancreatitis.

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COMMENTS

Background

The growing incidence of acute pancreatitis in developing and developed countries

has a significant impact on the healthcare system. As a result of the limitations of conventional therapy, many ethnobotanical agents are being pursued as alternative sources to develop novel and safe therapeutic agents for acute pancreatitis. The present experimental study investigated α,β-amyrin, a natural triterpenoid from *Protium heptaphyllum* as a treatment option for acute pancreatitis.

Research frontiers

α,β-amyrin exhibits anti-inflammatory and antioxidant effects. Both inflammation and oxidant stress play a pathogenic role in a rat model of acute pancreatitis induced by L-arginine. α,β-amyrin (100 mg/kg) effectively ameliorates L-arginine-associated pancreatic injury through inhibition of neutrophil infiltration, tumor necrosis factor α and interleukin (IL)-6 production, and inducible nitric oxide synthase expression in pancreatic tissue, probably *via* inhibition of nuclear factor-κB activity.

Innovations and breakthroughs

This is believed to be the first study that has demonstrated the beneficial effect of α,β-amyrin treatment in a rat model of L-arginine-induced acute pancreatitis. Inhibition of pro-inflammatory cytokine IL-6 by α,β-amyrin might arrest the transition of acute pancreatitis to a chronic state, and thus, progression to the more severe form of pancreatitis that is characterized by fibrosis and loss of pancreatic function.

Applications

In this study, acute pancreatic injury caused by L-arginine was ameliorated by α,β-amyrin treatment. This could represent a basis for future investigations on the therapeutic role of α,β-amyrin in severe necrotizing pancreatitis. Clinical studies have suggested that prophylactic administration of anti-inflammatory drugs is useful in preventing pancreatitis in patients undergoing therapeutic endoscopic retrograde cholangiopancreatography (ERCP). α,β-amyrin is an anti-inflammatory and antioxidant agent, therefore, it could also serve as a prophylactic agent in the prevention of ERCP. However, more in-depth experimental studies are warranted to support our observations of its beneficial effects.

Terminology

α,β-amyrin, is a pentacyclic triterpene that is isolated from the resin of the traditional medicinal plant, *Protium heptaphyllum*. α,β-amyrin has anti-inflammatory, antinociceptive, gastroprotective and hepatoprotective properties and is non-toxic at the doses employed in this study.

Peer review

The article describes the beneficial effects of two natural triterpenoids, α and β-amyrin on experimental acute pancreatitis induced by L-arginine in rats. The results presented are very clear, and the authors have been able to demonstrate that these triterpenoids produce anti-inflammatory, antinociceptive, and antioxidant properties at doses that range from 10 to 100 mg/kg, which are non-toxic.

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ORIGINAL ARTICLE

Antitumor effect of matrine in human hepatoma G2 cells by inducing apoptosis and autophagy

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Abstract

AIM: To study the antitumor effect of matrine in human hepatoma G2 (HepG2) cells and its molecular mechanism involved in antineoplastic activities.

METHODS: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect viability of HepG2 cells. The effect of matrine on cell cycle was detected by flow cytometry. Annexin-V-FITC/PI double staining assay was used to detect cellular apoptosis. Cellular morphological changes were observed under an inverted phase contrast microscope. Transmission electron microscopy was performed to further examine ultrastructural structure of the cells treated with matrine. Monodansylcadaverine (MDC) staining was used to detect autophagy. Whether autophagy is blocked by 3-methyladenine (3-MA), an autophagy inhibitor, was evaluated. Expression levels of Bax and Beclin 1 in HepG2 cells were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Matrine significantly inhibited the proliferation of HepG2 cells in a dose- and time-dependent manner, and induced G1-phase cell cycle arrest and apoptosis of HepG2 cells in a dose-dependent manner. The total apoptosis rate was 0.14% for HepG2 cells not treated with matrine. In contrast, the apoptosis rate was 28.91%, 34.36% and 38.80%, respectively, for HepG2 cells treated with matrine at the concentration of 0.5, 1.0 and 2.0 mg/mL. The remarkable morphological changes were observed under an inverted phase contrast microscope. Abundant cytoplasmic vacuoles with varying sizes were observed in HepG2 cells treated with matrine. Furthermore, vacuolization in cytoplasm progressively became larger and denser when the concentration of matrine was increased. Electron microscopy demonstrated formation of abundant autophagic vacuoles in HepG2 cells after matrine treatment. When the specific autophagic inhibitor, 3-MA, was applied, the number of autophagic vacuoles greatly decreased. MDC staining showed that the fluorescent density was higher and the number of MDC-labeled particles in HepG2 cells was greater in matrine treatment group than in control group. Fewer autophagic vacuoles were observed in the combined 3-MA and matrine treatment group when 3-MA was added before matrine treatment, indicating that both autophagy and apoptosis are activated when matrine-induced death of hepatoma G2 cells occurs. Real-time quantitative RT-PCR revealed that the expression levels of Bax gene, an apoptosis-related molecule, and Beclin 1 gene which plays a key role in autophagy were higher in matrine treatment group than in control group, indicating that Beclin 1 is involved in matrine-induced autophagy and the pro-apoptotic mechanism

of matrine may be related to its upregulation of Bax expression.

CONCLUSION: Matrine has potent antitumor activities in HepG2 cells and may be used as a novel effective reagent in treatment of hepatocellular carcinoma.

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Key words: Matrine; Autophagy; Apoptosis; Bax; Beclin 1; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an important global health issue. Owing to the dissemination of hepatitis B and C virus infection, the overall incidence of HCC remains alarmingly high in developing countries and increases steadily in most developed countries[1,2]. HCC is the sixth most common cancer and the third leading cause of cancer-related death worldwide[3]. Its incidence rate is different in various countries, accounting for 55% of all cases (and deaths) in China[3]. Its age-adjusted annual incidence is 5.5-14.9 people per 100 000 population in the world[4,5]. The prognosis of HCC patients is generally very poor and their 5-year relative survival rate is 3%-5% in most countries[3]. Current major treatment modalities for HCC include surgical resection, liver transplantation and local ablation therapies[6]. Many patients who are diagnosed with HCC at its advanced stage, are only candidates for palliative care[2,7]. Furthermore, since no effective palliative chemotherapy is available, the prognosis of advanced HCC patients is dismal. Therefore, it is necessary to find new effective medications for HCC. Development of pharmacologically effective agents with little toxicity or few side effects from natural products has become a new trend.

Matrine is one of the main alkaloid components extracted from the Sophora root, with a molecular formula of $C_{15}H_{24}N_2O$ (Figure 1)[8], which was first isolated and identified in 1958 from Sophora flavescens Ait (also known as kushen), subprostrata (shandougen) and alopecuroides (kudouzi)[9-12]. Matrine has been widely used in treatment of viral hepatitis, hepatic fibrosis, cardiac arrhythmia and skin diseases, such as atopic dermatitis and eczema in China, because it has a wide range of pharmacological effects, such as anti-inflammatory[13,14], antiviral[15,16], immunoinhibitory[17], antifibrotic[18,19], analgesic[20], antiarrhythmic[21-23] and anti-diarrhea effects[24]. Recently, interest has been generated in its antitumor activity. It has been reported that matrine exerts its antitumor effects by inhibiting proliferation and inducing apoptosis of gastric and cervical cancer cells, leukemia and glioma cells[10,12,25-29]. Matrine can also inhibit invasiveness and metastasis of human malignant melanoma cell line A375 and cervical cancer HeLa cells, and induce differentiation of leukemia K-562 cells[12,30]. In addition, matrine-induced autophagy in rat C6 glioma cells has been observed by electronic microscopy[29]. However, the precise mechanism underlying the anticancer activity of matrine remains unclear. Therefore, we designed this study to investigate the antitumor effect of matrine in human hepatoma G2 cells, and further elucidate its molecular mechanism involved in antineoplastic activities.

MATERIALS AND METHODS

Reagents

Fetal bovine serum was purchased from Sijiqing Biological Engineering Company Limited (Hangzhou, China). RPMI medium 1640 was bought from Gibco (USA). Sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-glutamine, Annexin V-FITC/PI (propidium iodide) apoptosis detection kit, and monodansylcadaverine (MDC) were purchased from Sigma (USA). TRIzol reagent was bought from Invitrogen (USA). Primescript™ reverse transcription (RT) reagent kit and SYBR® Premix Ex Taq™ Ⅱ were purchased from TaKaRa (Dalian, China).

Matrine was purchased from Xi'an Tianyuan Biologics Plant (China), with a purity of over 99% as proved by high-performance liquid chromatography. Matrine was dissolved in sterile double distilled water at a stock concentration of 40 mg/mL, stored at -20℃ in the dark, and then diluted in RPMI-1640 medium to obtain the desired concentration. 3-methyladenine (3-MA) (Sigma, USA) was dissolved in heated sterile double distilled water to make a 100 mmol/L stock solution and then added to the medium after heated for a final concentration of 2 mmol/L. Three hours later, matrine was added for treatment.

Cell line and cell culture

Human hepatoma G2 (HepG2) cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37℃ in a 5% CO_2 incubator. The cells in mid-log phase were used in experiments.

Cell viability assay

Viability of HepG2 cells was assessed by MTT assay as previously described[31]. The cells were seeded in 96-well flat bottom microtiter plates (Costar 3599, Corning Inc., Corning, NY) at a density of 5×10^3 cells per well, allowed to adhere overnight, and then treated with matrine at the con-

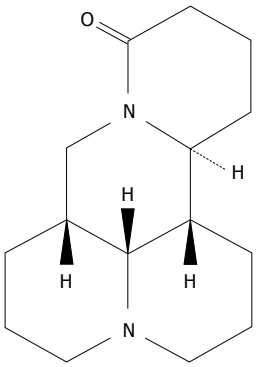

Figure 1 Chemical structure of matrine with a molecular formula of $C_{15}H_{24}N_2O$ and a molecular weight of 248.37.

centration of 0.25, 0.5, 1.0, 2.0 mg/mL for 24, 48 and 72 h, respectively. Control group and zero adjustment well were set as well. A MTT solution (5 mg/mL) was added 4 h before the end of incubation and the reaction was terminated by adding 10% acidified SDS. The absorbance value per well at 570 nm was read using an automatic multiwell spectrophotometer (PowerWave x, Bio-Tek Instruments Inc, USA). All MTT assays were performed in triplicate. The inhibitory rate for the proliferation of HepG2 cells was calculated according to the formula: (1-experimental absorbance value/control absorbance value) × 100%.

Cell cycle analysis

To determine cell cycle distribution, HepG2 cells were treated with matrine at the concentration of 0, 0.5 and 1.0 mg/mL, respectively. After 24 h of treatment, both floating and attached cells were collected and centrifuged before washed with cold phosphate-buffered saline (PBS), and then fixed in 70% cold ethanol overnight at 4℃. A fluorochrome solution containing 50 μg/mL PI, 3.4 mmol/L sodium citration, 20 μg/mL RNase A and 1% Triton X-100 was added and then incubated in the dark at room temperature for 30 min. Cell cycle analysis was performed using an EPICS XL flow cytometer (Beckman Coulter, California, USA). All experiments were performed in triplicate.

Detection of apoptosis

Annexin-V-FITC/PI double staining assay was performed to detect apoptosis of HepG2 cells. The cells were exposed to matrine at the concentration of 0, 0.5, 1.0 and 2.0 mg/mL, respectively, for 24 h, then harvested and resuspended in Annexin-V binding buffer. The suspension was incubated with 5 μL of Annexin V-FITC and 10 μL of PI for 10 min at room temperature in the dark, followed by cytometric analysis (EPICS XL, Beckman Coulter, USA) within 30 min of staining (as soon as possible). All experiments were performed in triplicate.

Morphologic observation under inverted phase contrast microscope

HepG2 cells were equally seeded in 24-well flat bottom microtiter plates (Costar 3524, Corning Inc., Corning, NY), and then treated with matrine at the concentration of 0, 0.25, 0.5, 1.0 and 2.0 mg/mL, respectively. After 24 h of treatment, the morphology of HepG2 cells was observed under an inverted phase contrast microscope (Olympus, Tokyo, Japan).

Observation of cell ultrastructure under transmission electron microscope

HepG2 cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L PBS (pH 7.4) for 90 min at room temperature, and post-fixed in 1% osmium tetraoxide for 30 min. After washed with PBS, the cells were progressively dehydrated in a 10% graded series of 50%-100% ethanol and propylene oxide, and embedded in Epon 812 resin. The blocks were cut into ultrathin sections with a microtome, which were then stained with saturated uranyl acetate and lead citrate. The ultrastructure of the cells was then observed under a transmission electron microscope (JEM-1230, JEOL, Japan).

MDC staining of autophagic vacuoles

MDC staining of autophagic vacuoles was performed for autophagy analysis as previously described[32]. HepG2 cells were divided into control group, 3-MA treatment group, matrine treatment group, and combined 3-MA and matrine treatment group. The cells were incubated for 48 h on coverslips. Autophagic vacuoles were labeled with 0.05 mmol/L MDC in PBS at 37℃ for 10 min. After incubation, the cells were washed three times with PBS and immediately analyzed under a fluorescence microscope (IX-81; Olympus, Japan). Fluorescence of MDC was measured at the excitation wavelength 380 nm with an emission filter at 530 nm.

Real-time quantitative RT-polymerase chain reaction

HepG2 cells were cultured in 35 mm dishes and then collected after treatment with matrine for 24 and 72 h, respectively. Total RNA was isolated from cells using Trizol reagent (Invitrogen, USA) according to its manufacturer's protocol. RNA concentration and purity were measured with a spectrophotometer at A260 and A260/280, respectively. RNA was reverse-transcribed into cDNA using a Primescript™ RT reagent kit (TaKaRa, Dalian, China) according to its manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) was carried out with the SYBR Green I fluorescent dye method (SYBR® Premix Ex Taq™ Ⅱ, TaKaRa, Dalian, China) and a Rotor Gene 3000 real-time PCR apparatus (Corbett Research Company, Australia). The sequences of primers used are as follows: forward: 5'-TGCTTCAGGGTTTCATCCAG-3' and reverse: 5'-GGCGGCAAT CATCCTCTG-3' for Bax; forward: 5'-GAGGGATGGAAGGGTCTAAG-3' and reverse: 5'-GCCTGGGCTGTGGTAAGT-3' for Beclin 1; forward: 5'-TGGCACCCAGCACAATGAA-3' and reverse: 5'-CTAAGTCATAGT CCGCCTAGAAGCA- 3' for β-actin. β-actin was used as an internal control to evaluate the relative expressions of Bax and Beclin 1. The PCR conditions were as follows:

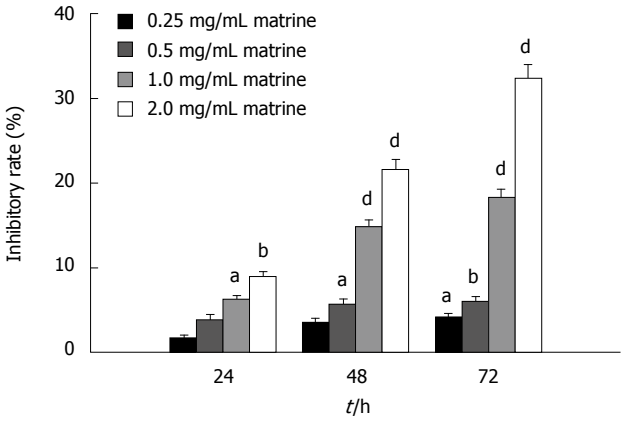

Figure 2 MTT assay showing the inhibitory effect of matrine on growth of HepG2 cells. HepG2 cells were treated with matrine at the concentration of 0.25, 0.5, 1.0 and 2.0 mg/mL, respectively for 24, 48 and 72 h. Matrine inhibited the growth of HepG2 cells in a dose- and time-dependent manner. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control group.

a pre-denaturing at 95℃ for 2 min, followed by 45 cycles of denaturation at 95℃ for 10 s, annealing/extension at 60℃ for 20 s. The amplification specificity was checked by melting curve analysis. The PCR products were visualized by gel electrophoresis to confirm the presence of a single product with a correct size. The $2^{-\Delta\Delta C_T}$ method was used to calculate the relative abundance of target gene expression generated by Rotor-Gene Real-Time Analysis Software 6.1.81. For each cDNA, the target gene mRNA level was normalized to β-actin mRNA level. Results were expressed as the ratio of normalized target gene mRNA level in cells treated with matrine to that in cells not treated with matrine. The experiments were performed in triplicate.

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using the SPSS 16.0 for Window. One-way analysis of variance (ANOVA) was used to analyze statistical differences between groups under different conditions. $P < 0.05$ was considered statistically significant.

RESULTS

Matrine inhibited proliferation of HepG2 cells in a dose- and time-dependent manner

The antiproliferative effect of matrine on HepG2 cells was detected by MTT assay. The results showed that matrine inhibited the proliferation of HepG2 cells in a dose-dependent and time-dependent manner. The inhibitory rate of matrine on growth of HepG2 cells was 6.28% ± 0.42%, 14.81% ± 0.81%, and 18.25% ± 0.99%, respectively, after the cells were treated with matrine at the concentration of 1.0 mg/mL for 24, 48 and 72 h (Figure 2).

Matrine induced G1-phase cell cycle arrest in HepG2 cells

To better understand the inhibitory effect of matrine on growth of HepG2 cells, cell cycle distribution was analyzed by flow cytometry. Matrine significantly increased the number of cells in G0/G1 phase and decreased the number of cells in the S phase in a dose-dependent manner (Figure 3), indicating that matrine can induce the G0/G1 phase cell cycle arrest in HepG2 cells.

Matrine induced apoptosis of HepG2 cells

Annexin-V-FITC/PI double staining assay showed that matrine induced apoptosis of HepG2 cells in a dose-dependent manner (Figure 4A). Flow cytometry showed that the total apoptosis rate was 0.14% in HepG2 cells not treated with matrine, and was 28.91%, 34.36%, and 38.80%, respectively, in HepG2 cells treated with matrine at the concentration of 0.5, 1.0 and 2.0 mg/mL (Figure 4B-E). Early apoptosis was observed in HepG2 cells treated with matrine at the concentration of 0.5 and 1.0 mg/mL, while late apoptosis was observed in HepG2 cells treated with matrine at the concentration of 2.0 mg/mL. The late apoptosis rate of HepG2 cells treated with matrine at the concentration of 2.0 mg/mL increased from 1.06% to 16%, which may be due to the direct cytotoxic effect of matrine on HepG2 cells.

Observation of vacuolization in cytoplasm by inverted phase contrast microscopy

Inverted phase contrast microscopy showed the morphological characteristics of HepG2 cells. Control cells not treated with matrine were well adhered, showing the normal morphology of HepG2 cells, while the tumor cells treated with matrine for 24 h demonstrated remarkable morphological changes (Figure 5). Abundant cytoplasmic vacuoles with varying sizes were observed. Vacuolization in cytoplasm progressively became larger and denser when the concentration of matrine was increased. Moreover, the majority of cells treated with matrine at the concentration of 2.0 mg/mL became round and shrunken, and could not be affixed to the wall and suspended in culture medium.

Transmission electron microscopy revealed formation of autophagosomes in matrine-treated HepG2 cells

To further clarify whether the cell vacuolization induced by matrine is involved in autophagy, transmission electron microscopy (TEM) was performed to detect the cells treated with matrine at the concentration of 1.0 mg/mL. The HepG2 cells not treated with matrine exhibited the normal ultrastructural morphology of cytoplasm, organelles and nuclei (Figure 6). The most prominent morphological change in matrine-treated cells was the formation of abundant autophagic vacuoles sequestrating cytoplasm and organelles, such as mitochondria and endoplasmic reticulum. Double-membranes, giant autophagosomes filled with degraded organelles and autolysosomes were frequently observed. TEM, the standard method to detect autophagy[33], was performed to detected the formation of autophagosomes, demonstrating that matrine could induce HepG2 cells to generate autophagy, which was consistent with the vacuolization obtained by inverted phase contrast microscopy. Moreover, 3-MA, a specific inhibitor

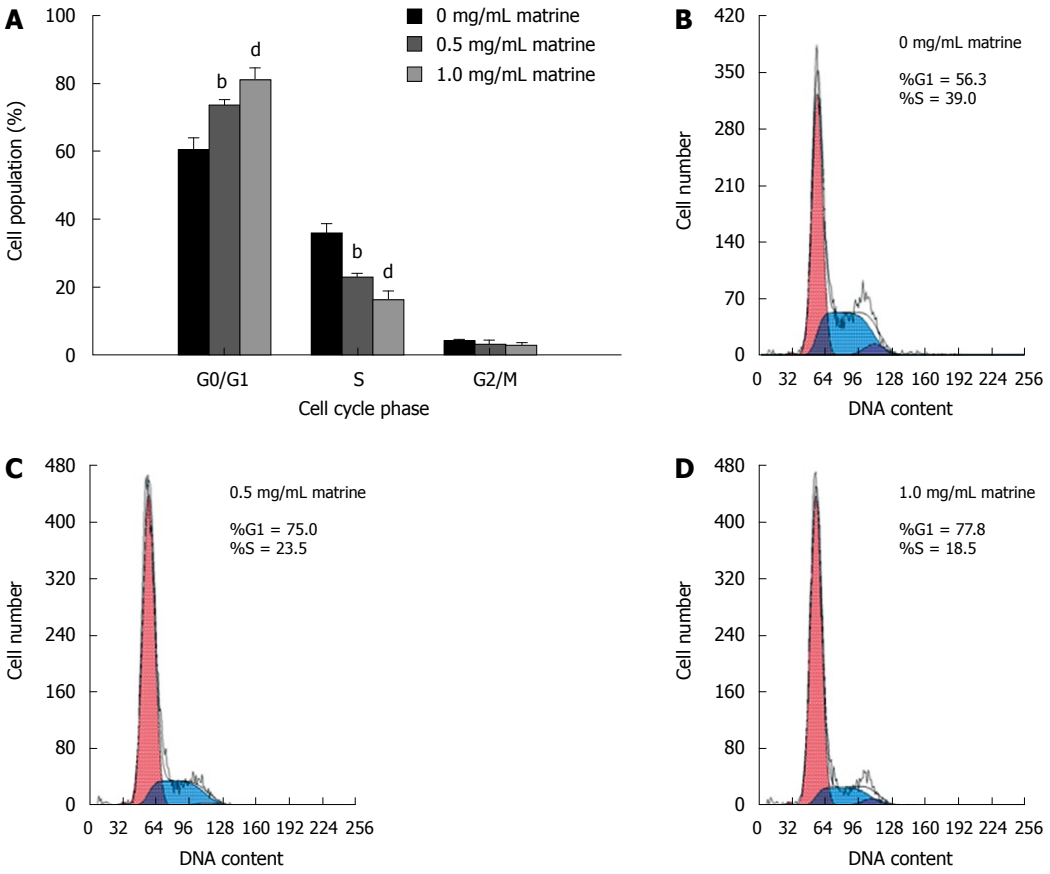

Figure 3 Effect of matrine on cell cycle distribution in HepG2 cells. A: Matrine treatment significantly increased the proportion of HepG2 cells in G0/G1 phase while decreased the number of HepG2 cells in the S phase. Results were expressed as mean ± SD (n = 3); B-D: Histograms of HepG2 cells incubated with matrine at the concentrations of 0, 0.5 and 1.0 mg/mL. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control group.

of autophagy, potently suppressed the matrine-induced autophagy (Figure 6E). The number of autophagic vacuoles was significantly lower while the formed vacuoles were smaller and appeared to be less developed in combined 3-MA and matrine treatment group than in single matrine treatment group.

MDC-labeled vacuoles in matrine-treated HepG2 cells

It has been reported that MDC is a specific marker for autophagic vacuoles[34]. When the cells were viewed under a fluorescence microscope, MDC-labeled autophagic vacuoles appeared as distinct dot like structures distributing in cytoplasm or in perinuclear. The fluorescent density and MDC-labeled particles of HepG2 cells were higher in matrine treatment group than in control group (Figure 7), indicating that matrine induces formation of MDC-labeled vacuoles. Fewer autophagic vacuoles were observed in combined 3-MA and matrine treatment group when 3-MA was added before matrine treatment, showing that 3-MA exerts its inhibitory effects on matrine-treated autophagy.

Matrine up-regulated mRNA expression of Bax and Beclin 1 in HepG2 cells

In order to understand the molecular mechanism underlying the apoptosis induced by matrine, the mRNA expression level of Bax gene, an apoptosis-related molecule, in HepG2 cells treated with matrine was measured by real-time quantitative RT-PCR, which showed that matrine up-regulated the Bax mRNA expression in HepG2 cells in a dose- and time-dependent manner (Figure 8A). Following 24 h of treatment, the Bax mRNA expression level increased nearly 3-fold and 30-fold, respectively, when the cells were treated with matrine at the concentration of 1.0 mg/mL for 24 and 72 h. At the same time, matrine gradually increased the Bax mRNA expression level in HepG2 cells when the concentration of matrine was increased.

To elucidate the mechanism underlying the autophagy induced by matrine, real-time quantitative RT-PCR was performed to evaluate the effect of matrine on mRNA expression of Beclin 1, which plays a key role in autophagy[35]. Real-time quantitative RT-PCR showed that matrine activated the Beclin 1 gene expression in a dose- and time-dependent manner (Figure 8B). In other words, the Beclin 1 mRNA expression level steadily increased with the increasing drug concentration and action time. It was interesting that the mRNA expression level of Bax and Beclin 1 was lower in HepG2 cells treated with matrine at the concentration of 2.0 mg/mL than in those treated with matrine at the concentration of 1.0 mg/mL, indicating that matrine at the concentration of 2.0 mg/mL exerts its direct cytotoxic effect on necrosis of HepG2 cells.

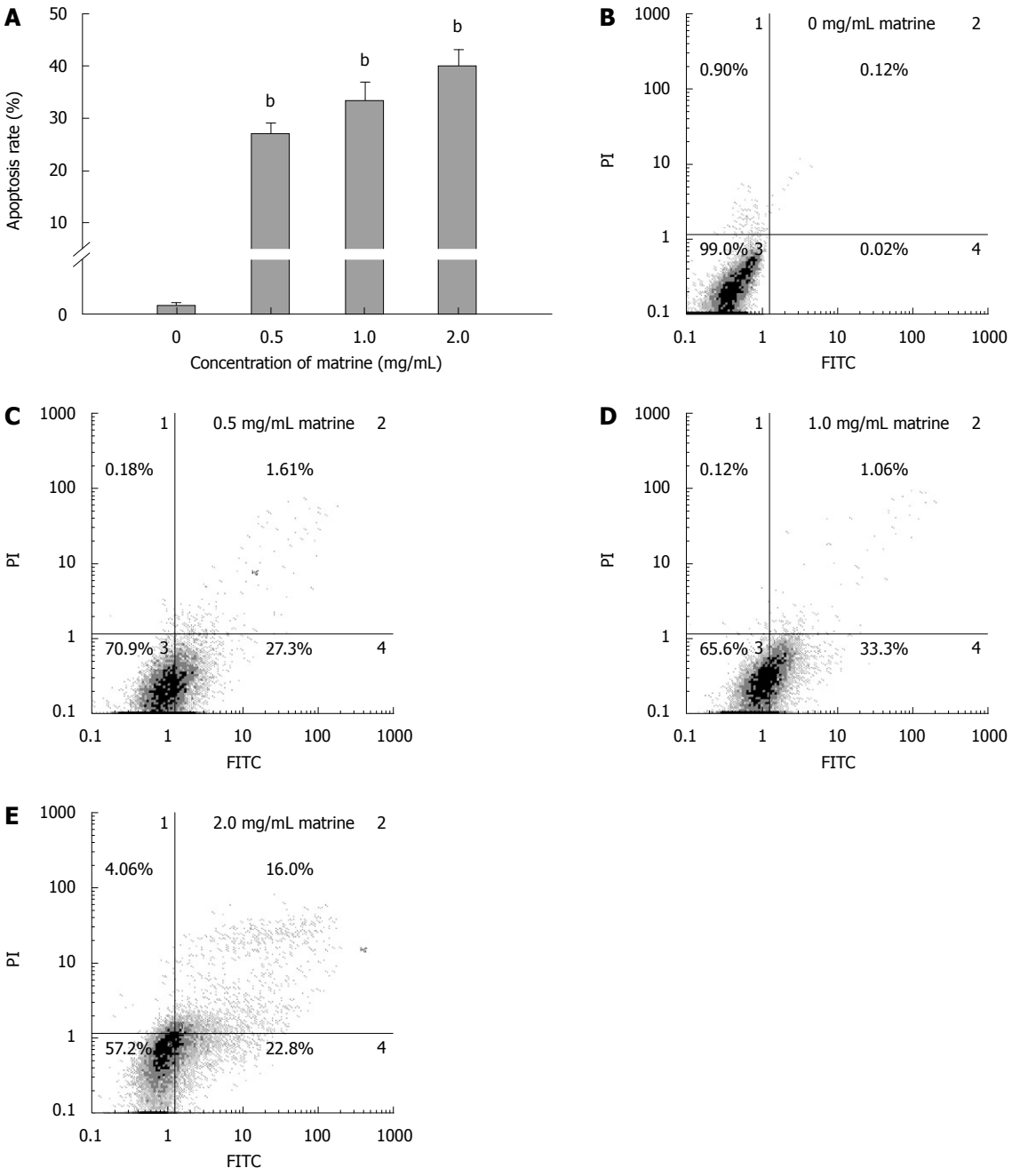

Figure 4 Matrine induces apoptosis of HepG2 cells. A: Apoptosis rate of HepG2 cells treated with matrine at the concentration of 0.5, 1.0 and 2.0 mg/mL, respectively, were significantly different from that in control group. Furthermore, the percentage of apoptotic cells increased when the concentration of matrine was increased. The data are expressed as mean ± SD (n = 3); B-E: Histograms of HepG2 cells incubated with matrine at the concentration of 0, 0.5, 1.0 and 2.0 mg/mL, respectively. Early apoptotic HepG2 cells were observed after incubated with matrine at the concentration of 0.5 and 1.0 mg/mL, the late apoptotic HepG2 cells were observed after incubated with matrine at the concentration of 2.0 mg/mL. $^{b}P < 0.001$ *vs* control group.

DISCUSSION

Both *in vivo* and *in vitro* studies showed that matrine inhibits the proliferation of tumor cells[10,27-29]. In this study, matrine obviously inhibited the growth of HepG2 cells in a dose- and time-dependent manner (Figure 2). Flow cytometry showed that matrine markedly arrested HepG2 cells in G0/G1 phase of cell cycle (Figure 3), which is consistent with the reported findings[8], suggesting that retardation of cell cycle progression may be one of the mechanisms underlying the antiproliferative effect of matrine.

It has been reported that matrine induces apoptosis in gastric cancer MKN45 and SGC-7901 cells, leukemia U937 and K562 cells, and C6 glioma cells[25,26,28,29,36]. In this study, matrine induced apoptosis of HepG2 cells in a dose-dependent manner (Figure 4). Consistent with the ability of matrine to kill cells *via* apoptotic processes, matrine up-regulated the expression of proapoptosis gene, Bax, in a dose- and time-dependent manner (Figure 8A), indicating that the increased Bax expression may trigger matrine-induced apoptosis of HepG2 cells, which is in agreement with the reported findings[28].

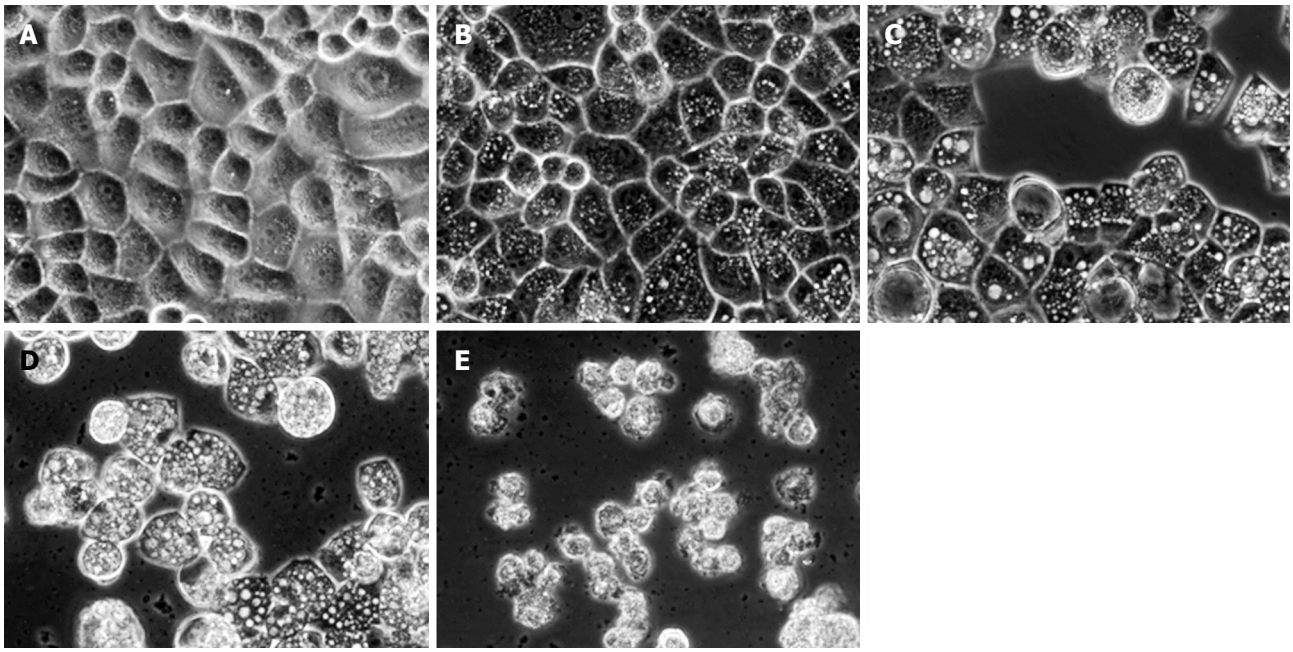

Figure 5 Inverted phase contrast microscopy showing matrine-induced morphologic changes of HepG2 cells. The control cells were well adhered, displaying the normal morphology of HepG2 cells. In contrast, abundant cytoplasmic vacuoles were observed in cells treated with matrine. Moreover, vacuolization in cytoplasm progressively became larger and denser when the concentration of matrine was increased. The majority of HepG2 cells treated with matrine at the concentration of 2.0 mg/mL became round and shrunken and could not be affixed to the wall and suspended in culture medium (× 400 magnification). A: 0 mg/mL matrine; B: 0.25 mg/mL matrine; C: 0.5 mg/mL matrine; D: 1.0 mg/mL matrine; E: 2.0 mg/mL matrine.

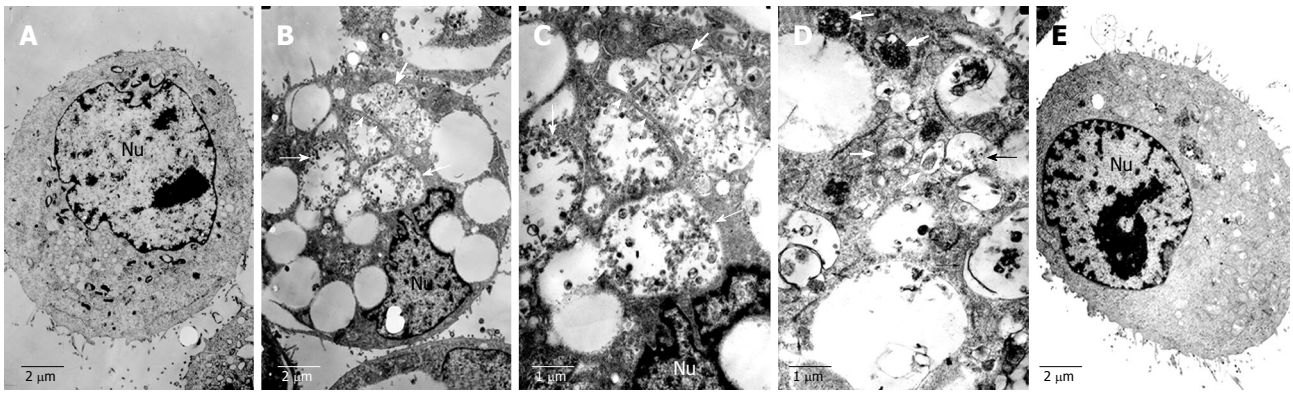

Figure 6 Transmission electron microscopy showing normal morphology of cytoplasm, cell organelles, and nuclei of HepG2 cells not treated with matrine (A), characteristic ultrastructural morphology of autophagy (B), double-membrane (C) and a large number of autophagic vacuoles (D) of HepG2 cells treated with matrine, and sharply decreased autophagic vacuoles (E) of HepG2 cells treated with combination of 3-MA and matrine (A, B, E, × 4000; C, D, × 8000). Arrowheads represent double-membrane, thick arrows represent autophagosomes, and thin arrows represent autolysosomes. Nu: Nucleus.

In this study, abundant cytoplasmic vacuoles were observed in matrine-treated HepG2 cells under an inverted phase contrast microscope (Figure 5). Electron microscopy showed autophagosomes in HepG2 cells treated with matrine at the concentration of 1.0 mg/mL (Figure 6). When the specific autophagic inhibitor, 3-MA, was applied, the number of autophagic vacuoles greatly decreased. MDC staining demonstrated independent evidence supporting the conclusion that matrine triggers autophagocytosis (Figure 7). The results of our study demonstrated that both autophagy and apoptosis were activated when death of HepG2 cells occurred after matrine treatment, revealing that the mechanism underlying the cytocidal action of matrine may be more complex than it has been reported. Autophagy, an evolutionarily conserved process, regulates cell death in both physiological and pathophysiological conditions[37-39]. In normal cells, autophagy contributes to the turnover of long-lived proteins and elimination of damaged or aged organelles to maintain cell homeostasis[40,41]. While under pathological conditions, autophagy is generally considered to play a prosurvival role. However, extensive autophagy or inappropriate activation of autophagy results in autophagic cell death (type II programmed cell death), which is an important cell death process besides apoptosis. Recently, increasing evidence indicates that autophagy is closely associated with tumors and plays an important role in human tumor suppression[35,41,42]. Autophagy has been

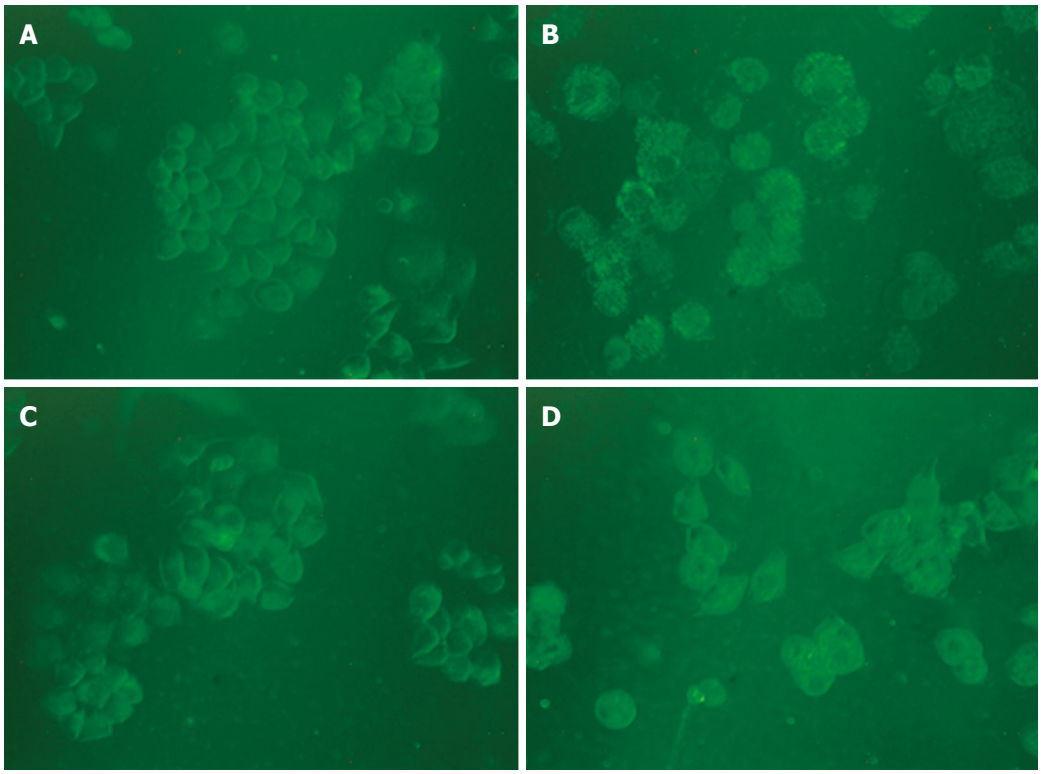

Figure 7 Monodansylcadaverine-labeled vacuoles in HepG2 cells. Autophagic vacuoles were labeled with 0.05 mmol/L monodansylcadaverine (MDC) in phosphate-buffered saline (PBS) at 37℃ for 10 min. The fluorescent density and the MDC-labeled particles in HepG2 cells were higher in matrine treatment group than in control group. The number of MDC-labeled particles in HepG2 cells was significantly lower in combined 3-MA and matrine treatment group than in single matrine treatment group (× 400 magnifications). A: Control; B: Matrine; C: 3-MA; D: 3-MA + matrine.

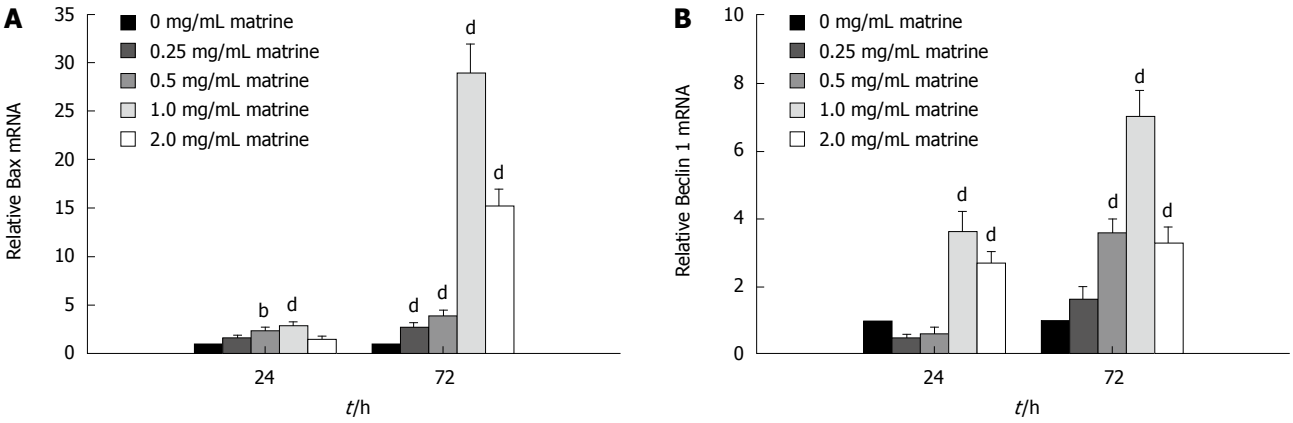

Figure 8 Matrine up-regulates the mRNA expression level of Bax (A) and Beclin 1 (B) in HepG2 cells. Data are shown as mean ± SD of three independent experiments. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control group.

observed in response to anticancer agents, such as vitamin D analogues[43], resveratrol[44], arsenic trioxide[45], tamoxifen[46], temozolomide[47] and rapamycin[48], indicating that autophagy can be potentially used in treatment of cancer. Furthermore, it has been reported that agents can directly lead to autophagic cell death. For example, caspase inhibitor induces autophagic cell death of L929 and U937 cells [49], which is consistent with that of human leukemia HL60 cells treated with Eupalitin A[50]. In addition, 5-fluorouracil induces autophagic cell death of Bax or PUMA deficient human colon cancer cells[51], indicating that induction of autophagy may be a promising new approach to treatment of tumor cells. In this study, a novel activity of matrine was identified in HepG2 cells, namely the ability of matrine to induce autophagy. In line with our data, matrine-induced autophagy in rat C6 glioma cells has been reported[29], suggesting that autophagic cell death induced by matrine underlines its potential utility as a new cancer treatment modality.

In this study, the mRNA expression level of Beclin 1 in HepG2 cells was measured. Beclin 1, a mammalian orthologue of the yeast Apg6/Vps30 gene, is the first identified mammalian gene to induce autophagy[35]. The Beclin 1 gene is mapped to the human chromosome 17q21[52],

which is monoallelically deleted in 40%-75% of human prostate, breast, and ovarian cancers[53]. Ectopic expression of Beclin 1 restores full autophagy potential in Beclin 1 deficient MCF-7 cells[35]. Moreover, the incidence of spontaneous tumors is high in Beclin $1^{+/-}$ heterozygous mice and Beclin $1^{-/-}$ homozygous embryonic stem cells exhibit a decreased number of autophagic vesicles, establishing that Beclin 1 is a critical component of mammalian autophagy and a haploinsufficient tumor suppressor gene[42,54]. Beclin 1 functions in autophagy as part of class Ⅲ phosphatidylinositol 3-kinase (PI3k) complex, which is necessary for the formation of autophagosome during the autophagic sequestration process[55,56]. In our study, matrine treatment increased the expression of Beclin 1 in HepG2 cells in a dose- and time-dependent manner (Figure 8B), indicating that Beclin 1 is involved in matrine-induced autophagy.

Additionally, although matrine simultaneously induced both apoptosis and autophagy in HepG2 cells in our study, the relation between apoptosis and autophagy remains unknown. Further study is needed to analyze the relation between related molecules at protein level.

In conclusion, matrine is a potent antitumor agent and exerts its antineoplastic action by inhibiting cell proliferation and inducing cell apoptosis and autophagy. Autophagic cell death induced by matrine underlines its potential utility as a new cancer treatment modality. In particular, autophagy may provide leverage to treat HCC that is chemoresistant on the basis of ineffective apoptosis. Beclin 1 is involved in matrine-induced autophagy and the pro-apoptotic mechanism of matrine may be related to its upregulation of Bax expression.

COMMENTS

Background

The incidence of hepatocellular carcinoma (HCC) increases in the world while many patients are diagnosed with HCC at its advanced stage. Since no effective palliative chemotherapy is available for HCC, the prognosis of patients with advanced HCC is dismal. Recently, interest has been generated in the antitumor activity of matrine, which has been widely used in treatment of various diseases.

Research frontiers

Autophagy is closely associated with tumors and plays an important role in human tumor suppression. Furthermore, it has been reported that agents directly cause autophagic cell death. Matrine induces autophagy in human hepatoma G2 (HepG2) cells. This study investigated the effect of matrine on the proliferation, cell cycle, apoptosis and autophagy of HepG2 cells and its molecular mechanism involved in antineoplastic activities.

Innovations and breakthroughs

In this paper, the authors identified a novel activity of matrine in HepG2 cells, namely the ability of matrine to induce autophagy, revealing that the mechanism underlying the cytocidal action of matrine may be more complex than it has been previously reported. The authors further demonstrated that Beclin 1 was involved in matrine-induced autophagy and the pro-apoptotic mechanisms of matrine might be related to its upregulation of Bax expression. Autophagic cell death induced by matrine underlines its potential utility as a new cancer treatment modality.

Applications

Matrine may be used as a potentially promising reagent in treatment of hepatocellular carcinoma. In particular, autophagy may provide leverage to treat HCC that is chemoresistant on the basis of ineffective apoptosis.

Terminology

Autophagy, an evolutionarily conserved process, regulates cell death in both physiological and pathophysiological conditions. Autophagic cell death (type Ⅱ programmed cell death) is an important cell death process besides apoptosis. Beclin 1, a mammalian orthologue of the yeast Apg6/Vps30 gene, is the first identified mammalian gene to induce autophagy and a haploinsufficient tumor suppressor gene. Bax, a pro-apoptotic molecule, is closely related to apoptosis.

Peer review

This is an interesting study. The study investigated the effect of matrine, one of the main alkaloid components extracted from the Sophora root, on the proliferation, apoptosis and autophagy of HepG2 cells. Matrine has been widely used in treatment of various diseases. The authors showed that matrine inhibited the proliferation and stimulated the apoptosis and autophagy of HepG2 cells, indicating that matrine can be used as a potentially promising agent in treatment of cancer.

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BRIEF ARTICLE

Peripancreatic collections in acute pancreatitis: Correlation between computerized tomography and operative findings

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Abstract

AIM: To evaluate the ability of contrast-enhanced computerized tomography (CECT) to characterize the nature of peripancreatic collections.

METHODS: Twenty five patients with peripancreatic collections on CECT and who underwent operative intervention for severe acute pancreatitis were retrospectively studied. The collections were classified into (1) necrosis without frank pus; (2) necrosis with pus; and (3) fluid without necrosis. A blinded radiologist assessed the preoperative CTs of each patient for necrosis and peripancreatic fluid collections. Peripancreatic collections were described in terms of volume, location, number, heterogeneity, fluid attenuation, wall perceptibility, wall enhancement, presence of extraluminal gas, and vascular compromise.

RESULTS: Fifty-four collections were identified at operation, of which 45 (83%) were identified on CECT. Of these, 25/26 (96%) had necrosis without pus, 16/19 (84%) had necrosis with pus, and 4/9 (44%) had fluid without necrosis. Among the study characteristics, fluid heterogeneity was seen in a greater proportion of collections in the group with necrosis and pus, compared to the other two groups (94% *vs* 48% and 25%, P = 0.002 and 0.003, respectively). Among the wall characteristics, irregularity was seen in a greater proportion of collections in the groups with necrosis with and without pus, when compared to the group with fluid without necrosis (88% and 71% *vs* 25%, P = 0.06 and P < 0.01, respectively). The combination of heterogeneity and presence of extraluminal gas had a specificity and positive likelihood ratio of 92% and 5.9, respectively, in detecting pus.

CONCLUSION: Most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue. CECT has a somewhat limited role in differentiating the different types of collections.

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Key words: Contrast-enhanced computerized tomography; Correlation; Pancreatic necrosis; Pancreatitis; Peripancreatic fluid collection; Surgery

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INTRODUCTION

Pancreatic necrosis and so called peripancreatic "fluid" collections are local complications of acute pancreatitis (AP) defined according to the Atlanta Criteria of AP, which categorizes AP as severe if these complications are present. It has been estimated that about 15% (4%-47%) of patients with AP will have necrotizing pancreatitis[1]; and 21%-46%[2-4] of patients will develop a peripancreatic "fluid" collection, including acute collections (during the first 4-6 wk), pseudocysts (after 4-6 wk of an acute attack with a defined wall with no or little necrotic tissue), necrotic collections and abscesses (collection after 4-6 wk with pus and a defined wall), according to the definitions of the Atlanta Classification. Earlier studies have demonstrated good correlation between contrast-enhanced computerized tomography (CECT) and operative findings with regard to pancreatic parenchymal necrosis in patients with AP[5-8]. Lack of vascular-based enhancement of the pancreatic parenchyma is the most characteristic finding on CECT that would suggest the presence of pancreatic parenchymal necrosis; and it has been suggested that the degree of pancreatic necrosis has important prognostic implications[9]. Problems, however, have been evident with much of the nomenclature of the Atlanta Classification with our more current understanding of the spectrum of necrotizing pancreatitis. The nomenclature of peripancreatic fluid collection is confusing due to various terms (as described above) and their clinical significance. It is not always possible in the first 4-6 wk to distinguish on CECT simple fluid collections (associated with acute edematous pancreatitis and better outcomes) from necrotic collections (associated with necrotizing pancreatitis and worse outcomes). No specific CT features that could detect necrosis of peripancreatic tissues (so called peripancreatic necrosis) have been reported so far. Moreover, studies evaluating CECT and operative correlation in the detection of peripancreatic necrosis and peripancreatic fluid collections are scant. Sarr *et al*[7] suggested that peripancreatic necrosis may not be seen on CECT and that a normal enhancement of the pancreas does not necessarily rule out the presence of peripancreatic necrosis. Therefore, assessment of the nature of these peripancreatic collections can be difficult even though these collections are fairly well imaged by CECT.

In this study, we evaluate the correlation between CT findings of peripancreatic collections and findings at operation and thereby assess the ability of CECT to characterize the nature of these peripancreatic collections. We have consciously and purposely elected to call the peripancreatic collections, not peripancreatic "fluid" collections, but peripancreatic "collections" because of the apparent difficulty of differentiating true fluid collections from necrotic areas and necrotic areas with some liquefaction necrosis. Thus, we will not refer to these collections as peripancreatic fluid collections as was done in the Atlanta Classification.

MATERIALS AND METHODS

In this retrospective study, we evaluated 25 patients who had one or more peripancreatic collections on CECT and who underwent operative intervention for severe acute pancreatitis (SAP) between 1995 and 2001 at the Mayo Clinic, Rochester. Severity of acute pancreatitis was determined according to the Atlanta Criteria. The CT slice thickness was 7 mm. Operative findings were used as an objective, gold standard for defining the nature of the peripancreatic collections. A single surgeon (MGS), blinded to the CT findings, interpreted the surgical notes and classified the peripancreatic collections into the following three groups: (1) necrosis without frank pus; (2) necrosis with pus; and (3) fluid without necrosis. The nomenclature of pancreatic collections is currently undergoing a revision (International Working Group); indeed the above three terms could mean the previous entities, peripancreatic necrosis, peripancreatic necrosis with pus, and pseudocysts. The presence of necrosis was confirmed from the operative findings and histopathologic review of the surgical specimens. Following this, a single radiologist (JGF), blinded to the operative findings, assessed the preoperative CTs of each patient for necrosis and peripancreatic collections. Pancreatic parenchymal necrosis was assessed in terms of extent (no necrosis, 30% necrosis, 30%-50% necrosis and > 50% necrosis), and location. Peripancreatic collections were described in terms of volume, location, number, heterogeneity, fluid attenuation, wall perceptibility, wall enhancement, presence of extraluminal gas, and vascular compromise. The volumes of the collections on CECT were measured using the formula $\pi/6 \times (d1 \times d2 \times d3)$, with d representing the maximum diameters in three different planes. Thereafter, the locations of the collections seen on CECT were matched to those identified at operation as per the operative notes. When two collections on CECT matched with only one collection seen at operation, then one of the collections on CT was randomly selected for further evaluation. If collections seen on CT did not match with collections seen at operation, they were excluded from further consideration.

Other parameters that were retrieved from the charts included demographic variables, presence of organ failure, presence of infection in the different groups of peripancreatic collections, and mortality. Approval by the Mayo Institutional Review Board approval was obtained prior to the study.

Statistical analysis

A database was generated in Microsoft Excel, and subsequently all statistical analyses were performed using the JMP software (version 7) from the SAS Institute, NC, USA. Continuous variables were expressed as median (IQR) and categorical variables as percentage. The different CT characteristics between the three groups of peripancreatic collections were compared by the chi square test (with Yates correction wherever applicable) and ordinal variables were analyzed with the Wilcoxon rank test. The 't' test was applied to compare continuous variables. A 'P' value of less than 0.05 was considered statistically significant.

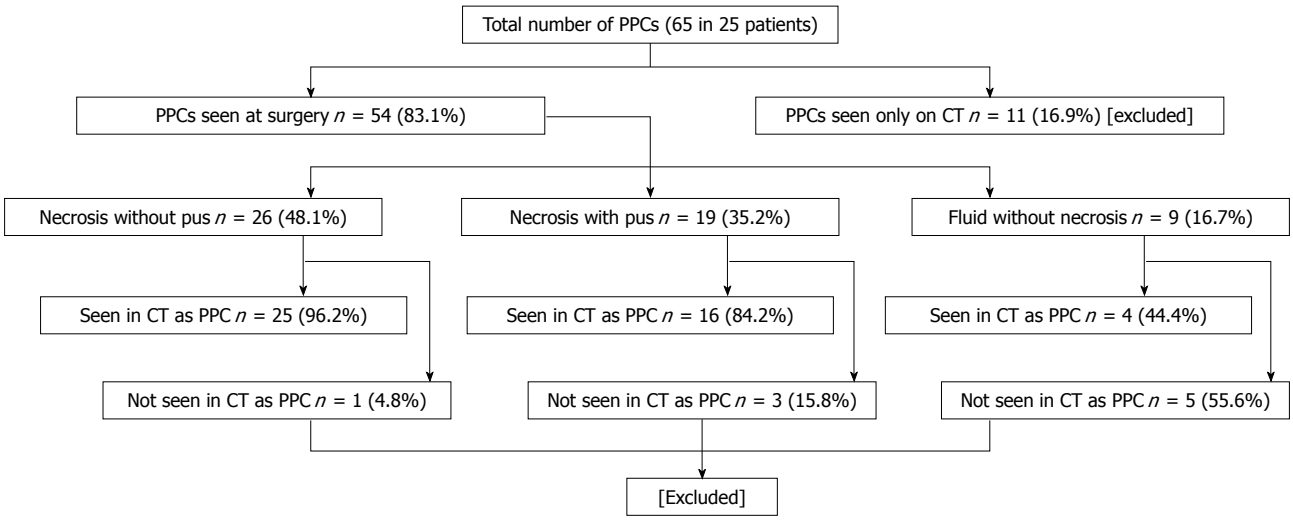

Figure 1 Distribution of different types of peripancreatic collections. PPCs: Peripancreatic collections; CT: Computerized tomography.

Table 1 Characteristics of the study population

Parameters	
Median (IQR) age (yr)	54 (50-67)
Male patients, *n* (%)	20 (80)
Contrast-enhanced CT, *n* (%)	23 (92)
Median (IQR) No. of PPCs	
Necrosis without pus	2 (1-2)
Necrosis with pus	1 (1-2)
Fluid without necrosis	1 (1-3)
Infected PPC, *n* (%)	12 (48)
OF in patients with PPC	
Total, *n* (%)	19 (76)
Persistent OF (> 48 h), *n* (%)	15 (60)
Multiple OF, *n* (%)	2 (8)
Mortality, *n* (%)	3 (12)

NB: Two patients in the group with necrosis and pus did not have a contrast injection prior to computed tomography scan due to renal failure. PPC: Peripancreatic collections; OF: Organ failure.

RESULTS

We evaluated 25 individuals who had a total of 65 collections, of which 54 were identified specifically at operation. These patients underwent surgery because they had either deterioration in clinical and laboratory parameters or they had evidence of sepsis. Table 1 shows the patient characteristics and Figure 1 shows the distribution of the different types of peripancreatic collections.

Peripancreatic collections

We analyzed only the peripancreatic collections that were evaluated at operative exploration. Of the 54 seen at operation, 26 had necrosis without pus, 19 had necrosis with pus, and 9 had fluid without necrosis. Forty five (83%) of 54 collections were identified on CECT, among which 25/26 (96%) had necrosis without pus, 16/19 (84%) had necrosis with pus, and 4/9 (44%) had fluid without necrosis. Therefore, the majority (*n* = 41, 91%) of the collections seen on CT were actually peripancreatic necrosis

Figure 2 Representative computed tomography picture of a patient with peripancreatic necrotic collection observed at operation but which could not be identified as a discrete collection on preoperative computed tomography (i.e. false negative computed tomography).

when correlated with operative findings. Among the 54 collections seen at operative exploration, 9 (17%) could not be identified as discrete collections on CT (Figure 2). Five (55%) of these 9 collections had associated necrosis, and 4 (44%) had only fluid without necrosis and were read as ascites on CT.

CT characteristics of pancreatic necrosis

Eighteen (72%) out of 25 patients had pancreatic parenchymal necrosis. Among these patients, 9 (50%) had < 30% necrosis, 4 (22%) had 30%-50% necrosis, and 5 (28%) had > 50% necrosis. We did not observe a statistically significant relationship between the percentage and location of pancreatic necrosis and the number and size of peripancreatic collections.

CT characteristics of peripancreatic collections

All peripancreatic collections were seen around the pancreas, except for one collection in the group with necrosis without pus, which was present in the lower abdomen in the retroperitoneum. Among the characteristics studied,

Table 2 Computed tomography characteristics of peripancreatic collections

		Necrosis without pus (n = 25)	Necrosis with pus (n = 16)	Fluid without necrosis (n = 4)	'P' value
	Volume in cm^3, median (IQR)	193.4 (50-1129)	116.3 (88-389)	551.7 (324-937)	
Fluid characteristics	Heterogeneity, *n* (%)	12 (48)	15 (93.8)[c]	1 (25)[a]	0.004[1]
	Fluid attenuation, *n* (%)	21 (84)	13 (81.3)	4 (100)	0.69
Wall characteristics	Perceptible, *n* (%)	23 (92)	14 (88)	4 (100)	0.76
	Enhancing, *n* (%)	21 (84)	14 (88)	4 (100)	0.68
	Irregular, *n* (%)	18 (71)	14 (88)	1 (25)[a]	0.05
	Internal air, *n* (%)	4 (16)	9 (56)[c]	0 (0)[a]	0.009[1]
	Vessel involvement, *n* (%)	5 (20)	3 (19)	0 (0)	0.62

NB: [1]Indicates statistically significant value when all groups were compared. [a]$P < 0.05$ when compared individually with the group with necrosis and pus; [c]$P < 0.05$ when compared individually with the group with necrosis without pus. Difference between parameters in the group with necrosis without pus and the group with fluid without necrosis were not statistically significant ($P > 0.05$). Difference in volumes of peripancreatic collections in the three groups were not statistically significant ($P > 0.05$).

fluid heterogeneity was seen in a greater proportion of collections in the group with necrosis and pus, compared to the other two groups (94% *vs* 48% and 25%, $P = 0.002$ and 0.003, respectively); however, there was no difference in heterogeneity between the groups with necrosis without pus and fluid without necrosis (48% *vs* 25%, $P = 0.39$). Attenuation units were similar in all three groups (Table 2).

Among the characteristics of the "wall" of the collections studied, irregularity of the wall was seen in a greater proportion of collections in the groups with necrosis with and without pus, when compared to the group with fluid without necrosis (88% and 71% *vs* 25%, $P = 0.06$ and $P < 0.01$, respectively). This finding was not different between the groups with necrosis with and without pus (88% *vs* 71%, P = ns). The other wall characteristics, i.e. perceptibility and enhancement, were similar in all three groups. Two patients with necrosis and pus could not have intravenous contrast administered during CT due to renal failure; when these two patients were excluded from the group while comparing the wall characteristics, the results were unchanged.

The presence of internal, extraluminal gas was seen only in collections with necrosis and in a greater proportion of collections in the group with necrosis and pus when compared to the other two groups (56% *vs* 16% and 0%, $P < 0.01$ in each). In the group with necrosis and pus, the proportion of collections containing extraluminal gas was less than the proportion containing heterogeneity (56% *vs* 94%, $P = 0.04$). In the group with necrosis without pus, bacterial growth was present in cultures of the surgical specimens from 12/14 (86%) patients; while in the group with necrosis and pus, bacterial growth occurred in 10/12 (83%) patients. All these patients were on high-dose broad-spectrum antibiotics at the time of operation. Among the necrotic collections without pus, there were gram positive and gram negative infections in 50% and 14%, respectively, and mixed infections in 21%. On the other hand, among the necrotic collections with pus, there were gram positive and negative infections in 25% and 33%, respectively, and mixed infections in 25%. Table 3 shows the sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios of fluid heterogeneity and the presence of extraluminal gas on CT in predicting the presence of pus in peripancreatic collections.

Table 3 Predictive value of heterogeneity and presence of air within necrotic collection on computed tomography scan in diagnosing the presence of pus

	Heterogeneity	Presence of air	Both
Sensitivity (%)	93.8	47.3	47.4
Specificity (%)	52.0	84.0	92.0
Positive predictive value (%)	55.5	69.2	81.8
Negative predictive value (%)	92.9	67.7	23.3
Positive likelihood ratio	1.9	2.9	5.9
Negative likelihood ratio	0.1	0.6	0.6

DISCUSSION

Earlier studies have demonstrated good correlation between CECT and operative exploration in diagnosing pancreatic necrosis in patients with AP[5-8]. The CECT feature that characterizes pancreatic necrosis most reliably is lack of enhancement of pancreatic parenchymal tissue[9]. In contrast, no specific CECT features that can differentiate peripancreatic necrosis from peripancreatic fluid without necrosis, especially early after the onset of pancreatitis, have been reported so far. MRI has been reported to be a more powerful tool for detecting necrotic debris within peripancreatic collections after acute pancreatitis[10], however, MRI is substantially more expensive than CECT and can be difficult to perform in the very ill patient, and requires radiologic experience and expertise in its interpretation. These considerations regarding MRI *vs* CECT increase the likelihood of the utility of CECT in the assessment of patients worldwide with severe acute pancreatitis for years to come. In this study, we evaluated the correlation between CECT findings of peripancreatic collections with objective findings at operative exploration in order to assess the ability of CECT to predict the nature of these collections. Such differentiation would be important from the standpoint

of better clinical decision-making, and thereby, possibly altering the treatment of these collections.

Importantly in this study, we specifically avoided the term "peripancreatic fluid collection" as was adapted by the Atlanta Classification. This term, in our opinion, has stifled progress in this field, led to controversy in the discussion of acute fluid collections *vs* peripancreatic necrosis, and has led to unnecessary controversy as well as confusion as to the appropriate use and misuse of purely drainage procedures (radiologic, endoscopic, or operative) *vs* procedures allowing a true necrosectomy. Therefore, we use the term peripancreatic "collection" rather than peripancreatic "fluid collection", because early in the course of necrotizing pancreatitis, some (perhaps most) pancreatic collections are composed primarily of necrosis without substantial fluid components, thereby differentiating them from acute fluid collections that lack necrosis. This study was designed, in part, to see if CT would be able to differentiate areas of necrosis (with or without a fluid component) from collections of fluid without necrosis.

We found that CT had a false positive rate (peripancreatic collections seen on CT but not found at operation) of 17% (11/65) and a false negative rate (collections found at operation but not seen on CT) of 17% (9/54). The false positive and false negative collections on CT were excluded from the comparative analysis. Operative findings and histologic evidence confirmed that over 90% of the peripancreatic collections detected on CECT in patients with necrotizing pancreatitis undergoing operative exploration/necrosectomy contained necrosis. In the current study, we found all the CT parameters (heterogeneity, attenuation, wall perceptibility, wall enhancement, wall irregularity, and presence of extraluminal gas) to be similar in the groups with necrosis without pus and fluid without necrosis. In contrast, heterogeneity and presence of extraluminal gas were significantly more common in the group with necrosis and pus, compared to the collections without necrosis. All other parameters were similar. Therefore, CT did not have any specific feature that would reliably detect the presence of necrosis within the peripancreatic collections. The presence of irregularity of the wall of the collection was more common in those with necrosis (88% and 71% with and without pus, respectively) but not enough to reliably differentiate necrotic collections from fluid collections, 25% of which had an irregular wall.

If we consider the groups with necrosis with and without pus, the presence of heterogeneity on CT was seen in a significantly greater proportion in the former group with a sensitivity and positive likelihood ratio of detecting pus in the collection of 94% and 1.9%, respectively. The presence of extraluminal gas within fluid collections is well-recognized as a marker of infection in the absence of a history of prior endoscopic, radiologic, or operative intubation. In the current study, as expected, internal air was seen in a significantly greater proportion of collections with pus (indeed no fluid collection without necrosis had gas within it), although significantly less than those with heterogeneity ($P = 0.04$). When both heterogeneity and the presence of extraluminal gas were combined, then the positive likelihood ratio increased substantially to 5.9, and the absence of both reliably excluded the presence of pus (specificity 92%). From these observations, it is clear that heterogeneity and presence of extraluminal gas in the peripancreatic collections in a patient with severe acute pancreatitis can suggest the presence of pus, i.e. purulent infection. The characteristics of the progression of infection in necrotic tissue is a dynamic process, and pus formation tends to occur in the later stages of the disease. In this study, bacterial growth occurred in 86% patients with a peripancreatic collection containing necrosis but no obvious pus. None of the CT features could, therefore, detect the presence of infection in this group of collections, i.e. infected necrosis without pus.

To our knowledge, this analysis is among the very few studies in the literature that have assessed the direct correlation between CECT and operative findings in the diagnosis and characterization of peripancreatic collections in the presence of severe acute pancreatitis. We used operative exploration as the gold standard to detect the presence of necrosis within these collections which was confirmed histologically. The operative data and the CECT findings were interpreted and analyzed by a single, blinded surgeon and radiologist, respectively. This approach helped to eliminate the chance of bias in the interpretations. This study, however, has a few limitations. First, this is a retrospective study, and the number of collections in the group with fluid without necrosis was only 4. This small number of fluid collections could have potentially skewed the CECT findings towards an inability to detect necrosis within the collections. Moreover, the CECT scans were performed between 1995 and 2001, when older generation machines with thick sections were used. The use of modern, multidetector CECTs with thinner slice sections might increase the ability of CECT to detect the presence of infection in these collections without pus. Second, the gold standard for the presence of a collection was operative exploration. We believe that this method was most appropriate, because at operative exploration, the operative approach at our institution is to expose the entire pancreas and open all areas of the peripancreatic retroperitoneum to ensure complete necrosectomy. In addition, CECT serves as a roadmap for surgery, and all collections noted on CECT were specifically sought in the operating room. Finally, the patients we studied were a skewed population with necrotizing pancreatitis, all of whom required operative intervention and thus represent only patients with necrotizing pancreatitis.

In conclusion, this study shows that most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue. It appears that CECT has a somewhat limited role in differentiating the different types of collections into necrosis with pus, necrosis without pus, and fluid collections without necrosis. While hetero-

geneity of the collection can reliably suggest the presence of necrosis, and extraluminal gas defines the presence of infection in a collection with necrosis, no CECT feature could suggest the presence of infection in a necrotic collection without pus.

COMMENTS

Background

Earlier studies have demonstrated good correlation between contrast-enhanced computerized tomography (CECT) and operative exploration in diagnosing pancreatic necrosis in patients with acute pancreatitis. Studies evaluating CECT and operative correlation in the detection of peripancreatic necrosis and peripancreatic fluid collections are scarce. Vege *et al* suggested that peripancreatic necrosis may not be seen on CECT and that a normal enhancement of the pancreas does not necessarily rule out the presence of peripancreatic necrosis.

Innovations and breakthroughs

There are very few similar studies in the literature so far. In this study, the authors objectively correlated peripancreatic collections seen on CT with those actually seen at surgery. The use of surgery as a gold standard adds maximum value to this study.

Applications

This study shows that while heterogeneity of the collection can reliably suggest the presence of necrosis, and extraluminal gas can define the presence of infection in a collection with necrosis, no CECT feature can suggest the presence of infection in a necrotic collection without pus. However, since the authors' sample size was not very large, the data could be validated in centers where a large volume of surgery is still performed for peripancreatic collections in patients with acute necrotizing pancreatitis.

Peer review

The Authors reported an interesting study which correlates CECT and intraoperative findings in patients who required surgical drainage for peripancreatic collections following severe acute pancreatitis. The paper is clear, well written, the aim of the study is covered, the methodology of the study despite being retrospective is correct, the statistical analysis is well done and explores the most important topics and the discussion is complete.

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BRIEF ARTICLE

Pre-illness changes in dietary habits and diet as a risk factor for inflammatory bowel disease: A case-control study

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Abstract

AIM: To evaluate whether symptoms of inflammatory bowel disease (IBD), before diagnosis modify dietary habits, and to investigate the pre-illness diet in patients with recent IBD in comparison with an age-matched healthy control group.

METHODS: Overall, 83 new cases of IBD (41 ulcerative colitis, 42 Crohn's disease) and 160 healthy controls were studied. Portions per week of 34 foods and beverages before onset of symptoms were recorded using a validated questionnaire. Duration of symptoms before IBD diagnosis, presence of specific symptoms and their impact on subjective changes in usual dietary habits were also recorded. The association between diet and IBD was investigated by multiple logistic regression and dietary patterns were assessed by factor analysis.

RESULTS: Changes in dietary habits, due to the presence of symptoms, were reported by 38.6% of patients and were not significantly related to specific symptoms, rather to long duration of symptoms, only in Crohn's disease patients. In IBD patients who did not change dietary habits, moderate and high consumption of margarine (OR = 11.8 and OR = 21.37) was associated with ulcerative colitis, whilst high consumption of red meat (OR = 7.8) and high intake of cheese were associated with Crohn's disease.

CONCLUSION: More than one third of IBD patients change dietary habits before diagnosis. Margarine, red meat and cheese increase the risk of ulcerative colitis and Crohn's disease.

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Key words: Inflammatory bowel diseases; Diet; Symptoms; Factor analysis

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INTRODUCTION

The aetiology of inflammatory bowel disease (IBD) is

still unknown. Since genetically determined mechanisms have remained unchanged over historical time periods, the increase in incidence of ulcerative colitis (UC) and Crohn's disease (CD) during the 20th century suggests environmental influences. Epidemiological and clinical evidence support an association between IBD and many apparently unrelated environmental factors, including diet, smoking, geographical and social status, occupation, and microbial factors[1-4]. In particular, the role of dietary factors or components of diet in the pathophysiology have long since been taken into consideration, and immunological mechanisms linking food antigens to development of inflammation have also been postulated. However, this attractive explanation is far from proven, and studies investigating this potential link are few and unconvincing. In some reports, it has been suggested that increased consumption of sugar and refined carbohydrates might be a risk factor for CD[5-10], and has also been demonstrated in some UC patients[7,9,11-13], whereas protein and fat intake, as well as decreased consumption of fruit, vegetables and fibre, appear to increase the risk of IBD, but data reported have been more controversial[5,14-16]. Indeed, a causal relationship between diet and IBD is difficult to define, due to the possibility that early symptoms of the disease may lead to a modification in dietary habits and the inability of the patients to accurately remember their diet before the onset of symptoms. To date, only a few studies have examined the pre-illness diet, in incident cases, and have shown conflicting results[5,15-18].

The present study aimed to evaluate whether the signs and symptoms of IBD in patients before diagnosis led to a modification in their diet, and to investigate pre-illness dietary habits in patients with recent IBD in comparison to those in an age-matched control group.

MATERIALS AND METHODS

Between June and September 2003, and between September 2007 and June 2008, incident cases of UC and CD (diagnosis < 12 mo, median duration of symptoms: 5 mo, range: 0-84 mo) consecutively observed at the IBD Unit of the Gastroenterology Department of L. Sacco University Hospital were interviewed. Controls were recruited among healthy blood donors of the same Hospital and were frequency-matched (by quinquennial of age and sex) with the cases. None of the controls reported serious abdominal diseases that could have influenced their dietary habits. All patients were contacted by two L. Sacco University gastroenterologists (Cucino C and Bezzio C), who interviewed patients and controls, asking questions in an identical manner, with interviews lasting approximately 30 min each during which a validated questionnaire was employed which had been previously used for epidemiological studies concerning the relationship between cancer and diet[19].

This questionnaire included information on sociodemographic characteristics, anthropometric measures, lifestyle, including both tobacco smoking and alcohol habits, and personal and family medical history. Attention was focused, in particular, on long-term drinking, smoking, and dietary habits before diagnosis and onset of symptoms in IBD patients. Patients and healthy controls were asked about the number and size of portions of different food items consumed per week and whether 3 alcoholic and 4 non-alcoholic drinking items were consumed per day. IBD patients were also investigated to determine the duration of symptoms before diagnosis and the presence of specific symptoms such as diarrhoea, abdominal pain, rectal bleeding and weight loss > 3 kg. In IBD patients, an attempt was made to establish whether they had changed their usual dietary habits due to symptoms. The categorical answers to these questions were "yes", "no" or "don't know". The validated food frequency questionnaire investigated the usual dietary habits during the 5 years prior to diagnosis and onset of symptoms for IBD patients or during the last 5 years in healthy controls. The questionnaire included 34 food items and beverages commonly used in the Italian diet, grouped as follows: (1) bread and cereal dishes (first courses); (2) red meat and other main dishes such as fish, pork, poultry or rabbit (second courses); (3) vegetables (side dishes); (4) fruit; (5) sweets (including refined carbohydrates and sugar), desserts and soft drinks; (6) milk and hot beverages; and (7) alcoholic beverages. Furthermore, the consumption of coffee (non decaffeinated and decaffeinated), tea, olives and seed oils, margarine and butter were also investigated. Questions were also asked concerning the average weekly frequency of consumption of each dietary item; occasional intakes (< once a week but at least once a month) were coded as 0.5/wk.

Data analysis

Data were reported as mean and SD or as median and range, where appropriate. The association between symptoms and changes in dietary habit was evaluated by the Fisher exact test.

Consumption of foods and beverages, as well as other continuous variables, were subdivided into tertiles (low, moderate and high consumptions).

Odds ratios (OR) and corresponding 95% CI were defined using multiple logistic regression models. All the regression equations included terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ⩾ 25 cigarettes/d) and body mass index (BMI) (quintiles). Tests for trends were based on the likelihood ratio test between models with and without a linear term for the diet score.

Dietary patterns, based on nutrient intake, associated with IBD, were defined using factor analysis, a multivariate technique which analyses the underlying structure of a set of data in order to explain observed relationships between a large number of variables in terms of simpler relations.

Within a factor, negative loading indicates that foods

Table 1 Demographic and clinical features of inflammatory bowel disease cases and healthy controls

	Cases (n = 83)	Controls (n = 160)
Sex (M/F)	49/34	97/63
Age (mean ± SD)	37.5 ± 15.2	40.4 ± 14.6
Social status, n (%)		
Unmarried	50 (60.3)	83 (51.9)
Married	26 (31.3)	69 (43.1)
Divorced/widowed	7 (8.4)	8 (5.0)
Years of education (mean-SD)	12.4-3.5	11.5-4.2
Body mass index, kg^2/cm (mean-SD)	26.1-9.3	29.1-10.8
Smoking habit, n (%)		
Never	32 (38.6)	80 (50.0)
Smokers	29 (34.9)	41 (35.6)
Ex-smokers	22 (26.5)	39 (24.4)
No. cigarettes/d (smokers) mean (range)	13.4 (3-50)	14.7 (5-30)
Disease location (Crohn's disease) n = 42		
Ileum, n (%)	18 (42.9)	
Ileum and colon	13 (31.0)	/
Colon	11 (26.2)	
Disease location (ulcerative colitis) n = 41		
Proctitis	5 (12.2)	
Left sided colitis	22 (53.7)	/
Pancolitis	14 (34.1)	
Disease behaviour (Crohn's disease)		
Inflammatory (B1)	27 (64.3)	
Stricturing (B2)	8 (19.0)	/
Penetrating (B3)	7 (16.7)	

are inversely associated with the factor, while positive loading indicates a direct association with the factor. After varimax rotation, factor scores were saved from the principal components analysis for each individual. Factor scores were categorized into tertiles based on the distribution of the control.

RESULTS

The study population comprised 83 IBD patients (41 UC, 42 CD) and 160 sex- and age-matched healthy controls, comparable for social status, years of education, BMI and smoking habits. CD patients showed more frequent ileal localisation of disease and, as expected, an inflammatory behaviour, whilst UC patients showed more frequent left sided colitis (Table 1).

Symptoms and change of diet in IBD patients

The duration of symptoms before diagnosis was comparable in UC and CD patients [median (range): 5 (1-84) *vs* 6 (0-48) mo]. Weight loss, diarrhoea and abdominal pain at diagnosis were comparable, judging from the complaints referred to by the UC and CD patients. As expected, UC patients presented rectal bleeding more frequently than CD patients (Figure 1).

A conscious change of dietary habits due, before diagnosis, to the presence of symptoms, was reported by 32 patients (38.6%), 15 with UC and 17 with CD. The main dietary changes were reduction of fat and calorie intake (12 patients), while other patients reduced or stopped the consumption of fibre (18 patients) and milk or cheese (9 patients).

Change in dietary habits was not due to specific symptoms. However, in CD patients the long duration of symptoms was significantly correlated with changes in dietary habits (Table 2).

Table 2 Association between changes in pre-illness dietary habits and symptoms in inflammatory bowel disease patients

	Changes in diet			
	Ulcerative colitis		Crohn's disease	
	No (26)	Yes (15)	No (25)	Yes (17)
Duration of symptoms				
Short (≤ 3 mo)	14 (53.85)	4 (26.67)	8 (32.00)	4 (23.53)
Intermediate (4-10 mo)	7 (26.92)	4 (26.67)	13 (52.00)	4 (23.53)
Long (> 10 mo)	5 (19.23)	7 (46.66)	4 (16.00)	9 (52.94)[a]
Median, range (mo)	3 (1-15)	7 (1-84)	5 (1-30)	12 (1-48)[a]
Weight loss				
Absent/low (< 2 kg)	12 (46.15)	4 (26.67)	8 (32.00)	3 (16.65)
Moderate (2-6 kg)	8 (30.77)	3 (20.00)	11 (44.00)	8 (47.06)
Severe (> 6 kg)	6 (23.08)	8 (53.33)	6 (24.00)	6 (35.29)
Abdominal pain				
No	9 (34.61)	3 (20.00)	4 (16.00)	1 (5.88)
Yes	17 (65.39)	12 (80.00)	21 (84.00)	16 (94.12)
Diarrhoea				
No	5 (19.23)	1 (6.67)	4 (16.00)	1 (5.88)
Yes	21 (80.77)	14 (93.33)	21 (84.00)	16 (94.12)
Rectal bleeding				
No	9 (34.62)	5 (33.33)	13 (52.00)	12 (70.59)
Yes	17 (65.38)	10 (66.67)	12 (48.00)	5 (29.41)

[a]$P < 0.05$.

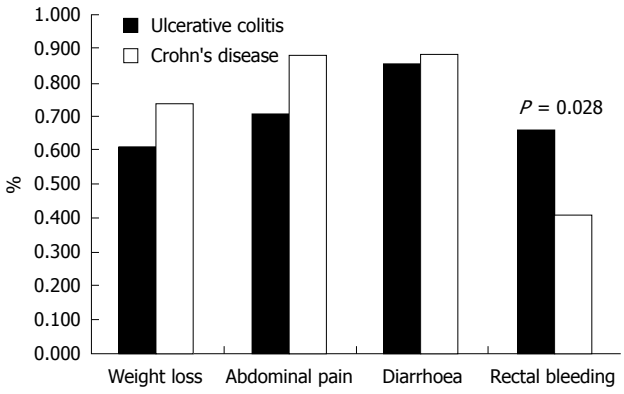

Figure 1 Prevalence of specific symptoms and changes in dietary habits in ulcerative colitis and Crohn's disease patients.

Food consumption and risk of IBD

To exclude any potential confounding influence of symptom-induced changes in diet (i.e. intake of milk in UC and fibre in CD) only patients who did not change dietary habits (26 UC and 25 CD patients) were taken into consideration (Tables 3 and 4). In these patients, an increased risk of UC (although not significant) was found for those reporting a high consumption of pasta and rice (OR = 3.38, 95% CI: 0.99-11.47) and with moderate or high consumption of margarine (OR = 11.8 and OR = 21.37, respectively). CD was significantly associated with moder-

Table 3 Association between pre-illness intake of foods and beverages and risk of ulcerative colitis in patients who did not modify the diet before diagnosis

	Controls	Ulcerative colitis	
	n (%)	*n* (%)	OR[1] (95% CI)
Pasta and/or rice			
Low	67 (41.88)	7 (26.92)	[2]
Moderate	68 (42.50)	12 (46.15)	1.89 (0.66-5.41)
High	25 (15.63)	7 (26.92)	3.38 (0.99-11.47)
Bread			
Low	60 (37.50)	6 (23.08)	[2]
Moderate	55 (34.38)	9 (34.62)	1.31 (0.42-4.13)
High	45 (28.13)	11 (42.31)	2.40 (0.80-7.26)
Sweets and cakes			
Low	52 (32.50)	10 (38.46)	[2]
Moderate	49 (30.63)	4 (15.38)	0.37 (0.10-1.33)
High	59 (36.88)	12 (46.15)	0.96 (0.36-2.51)
Red meat			
Low	73 (45.63)	12 (46.15)	[2]
Moderate	38 (23.75)	8 (30.77)	1.22 (0.45-3.32)
High	49 (30.63)	6 (23.08)	0.63 (0.20-1.94)
White meat			
Low	45 (28.13)	5 (19.23)	[2]
Moderate	70 (43.75)	17 (65.38)	2.04 (0.69-6.05)
High	45 (28.13)	4 (15.38)	0.75 (0.19-3.04)
Tuna fish			
Low	37 (23.13)	10 (38.46)	[2]
Moderate	48 (30.00)	6 (23.08)	0.43 (0.13-1.37)
High	75 (46.88)	10 (38.46)	0.49 (0.18-1.36)
Fish			
Low	61 (38.13)	17 (65.38)	[2]
Moderate	39 (24.38)	3 (11.54)	0.33 (0.09-1.26)
High	60 (37.50)	6 (23.08)	0.33 (0.11-0.92)
Processed meat			
Low	50 (31.25)	9 (34.62)	[2]
Moderate	57 (35.63)	10 (38.46)	0.82 (0.28-2.36)
High	53 (33.13)	7 (26.92)	0.63 (0.21-1.91)
Milk			
Low	45 (28.13)	13 (50.00)	[2]
Moderate	36 (22.50)	3 (11.54)	0.30 (0.08-1.19)
High	79 (49.38)	10 (38.46)	0.55 (0.21-1.40)
Cheese			
Low	43 (26.88)	8 (30.77)	[2]
Moderate	70 (43.75)	11 (42.31)	0.93 (0.33-2.63)
High	47 (29.38)	7 (26.92)	0.98 (0.31-3.12)
Eggs			
Low	44 (27.50)	11 (42.31)	[2]
Moderate	51 (31.88)	11 (42.31)	0.78 (0.30-2.06)
High	65 (40.63)	4 (15.38)	0.21 (0.06-0.73)
Potatoes			
Low	25 (15.63)	10 (38.46)	[2]
Moderate	78 (48.75)	11 (42.31)	0.39 (0.14-1.09)
High	57 (35.63)	5 (19.23)	0.24 (0.09-0.83)
Vegetables			
Low	44 (27.67)	11 (42.31)	[2]
Moderate	67 (42.14)	10 (38.46)	0.49 (0.18-1.35)
High	48 (30.19)	5 (19.23)	0.37 (0.11-1.21)
Fruits			
Low	48 (30.00)	9 (34.62)	[2]
Moderate	61 (38.13)	11 (42.31)	1.12 (0.42-3.01)
High	51 (31.88)	6 (23.08)	0.41 (0.13-1.35)
Refined sugar			
Low	57 (35.63)	9 (34.62)	[2]
Moderate	45 (28.13)	12 (46.15)	1.52 (0.57-4.09)
High	58 (36.25)	5 (19.23)	0.57 (0.17-1.87)
Butter			
Low	28 (17.50)	1 (3.85)	[2]
Moderate	88 (55.00)	18 (69.23)	6.02 (0.75-47.97)
High	44 (27.50)	7 (26.92)	5.18 (0.58-46.20)
Margarine			
Low	57 (35.63)	1 (3.85)	[2]
Moderate	84 (52.50)	19 (73.08)	11.80 (1.51-91.99)
High	19 (11.88)	6 (23.08)	21.37 (2.32-196.6)
Olive oil			
Low	47 (29.38)	6 (23.08)	[2]
Moderate	88 (55.00)	16 (61.54)	1.40 (0.50-3.93)
High	25 (15.63)	4 (15.38)	1.16 (0.29-4.63)
Seed oil			
Low	33 (20.63)	1 (3.85)	[2]
Moderate	97 (60.63)	22 (84.62)	7.26 (0.93-56.53)
High	30 (18.75)	3 (11.54)	3.82 (0.36-40.14)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ⩾ 25 cigarettes/d) and body mass index (quintiles); [2]Reference category.

ate consumption of meat (OR = 7.8, 95% CI: 1.61-37.89) and high consumption of cheese (OR = 3.7, 95% CI: 1.14-12.01). High intake of fish and potatoes reduced the risk of IBD while the consumption of vegetables and tuna fish was negatively correlated with the risk of CD and high intake of eggs was negatively correlated with risk of UC.

In particular, as far as any possible correlation of diet with disease location in CD is concerned, tuna fish consumption was negatively correlated with both pure colonic location (OR = 0.33, 95% CI: 0.11-0.98) and ileal or ileocolonic CD (OR = 0.48, 95% CI: 0.26-0.91), while vegetable consumption showed protective effects in patients with ileal or ileocolonic location of CD (OR = 0.43, 95% CI: 0.21-0.88). Interestingly, we also found that an increased risk of ileal or ileocolonic (but not pure colonic) CD associated with high bread or cheese consumption (OR = 2.61, 95% CI: 1.26-5.41 and OR = 2.61, 95% CI: 1.26-5.41, respectively) (Table 5).

No correlation was found between beverages and risk of IBD.

Factor analysis identified three dietary-intake factors that, overall, explained 94% of total variability, accounting for 59%, 20% and 14%, respectively. A first dietary pattern, called "refined", was mainly correlated to pasta, sweets, red and processed meat, butter and margarine. The second pattern (prudent) loaded heavily on white meat, tuna fish, fish, eggs and potatoes. The last pattern, designated "healthy", loaded heavily bread, cheese and, in particular, fruit and vegetables, as well as olive oil (which was found to be negatively correlated with the two previously described patterns). Evaluation of the association between these specific dietary intake patterns and IBD showed that a "refined" diet was associated with an increased risk of UC and CD. In contrast, the "prudent" pattern was significantly associated with a decreased risk (Table 6). The "healthy" pattern was not consistently associated with UC, moreover, a non-significant increase in risk was present for CD.

DISCUSSION

In this case-control study analysing the relationship be-

Table 4 Association between pre-illness intake of foods and beverages and risk of Crohn's disease in patients who did not modify the diet before diagnosis

	Controls	Crohn's disease	
	n (%)	*n* (%)	OR[1] (95% CI)
Pasta and/or rice			
Low	67 (41.88)	8 (32.00)	[2]
Moderate	68 (42.50)	14 (56.00)	1.59 (0.61-4.17)
High	25 (15.63)	3 (12.00)	1.01 (0.24-4.34)
Bread			
Low	60 (37.50)	6 (24.00)	[2]
Moderate	55 (34.38)	8 (32.00)	1.25 (0.38-4.04)
High	45 (28.13)	11 (44.00)	2.47 (0.81-7.56)
Sweets and cakes			
Low	52 (32.50)	8 (32.00)	[2]
Moderate	49 (30.63)	4 (16.00)	0.43 (0.11-1.63)
High	59 (36.88)	13 (52.00)	1.21 (0.44-3.29)
Red meat			
Low	73 (45.63)	8 (32.00)	[2]
Moderate	38 (23.75)	6 (24.00)	1.25 (0.38-4.07)
High	49 (30.63)	11 (44.00)	2.42 (0.85-6.85)
White meat			
Low	45 (28.13)	8 (32.00)	[2]
Moderate	70 (43.75)	15 (60.00)	1.33 (0.50-3.52)
High	45 (28.13)	2 (8.00)	0.25 (0.05-1.27)
Tuna fish			
Low	37 (23.13)	13 (52.00)	[2]
Moderate	48 (30.00)	6 (24.00)	1.31 (0.35-4.87)
High	75 (46.88)	6 (24.00)	0.25 (0.08-0.77)
Fish			
Low	61 (38.13)	16 (64.00)	[2]
Moderate	39 (24.38)	6 (24.00)	0.57 (0.19-1.72)
High	60 (37.50)	3 (12.00)	0.18 (0.05-0.67)
Processed meat			
Low	50 (31.25)	2 (8.00)	[2]
Moderate	57 (35.63)	18 (72.00)	7.80 (1.61-37.89)
High	53 (33.13)	5 (20.00)	1.97 (0.35-11.03)
Milk			
Low	45 (28.13)	7 (28.00)	[2]
Moderate	36 (22.50)	2 (8.00)	0.41 (0.07-2.18)
High	79 (49.38)	16 (64.00)	1.55 (0.57-4.19)
Cheese			
Low	43 (26.88)	5 (20.00)	[2]
Moderate	70 (43.75)	5 (20.00)	0.54 (0.14-2.06)
High	47 (29.38)	15 (60.00)	3.70 (1.14-12.01)
Eggs			
Low	44 (27.50)	10 (40.00)	[2]
Moderate	51 (31.88)	9 (36.00)	0.85 (0.30-2.40)
High	65 (40.63)	6 (24.00)	0.42 (0.14-1.29)
Potatoes			
Low	25 (15.63)	7 (28.00)	[2]
Moderate	78 (48.75)	13 (52.00)	0.60 (0.19-1.83)
High	57 (35.63)	5 (20.00)	0.24 (0.06-0.91)
Vegetables			
Low	44 (27.67)	14 (56.00)	[2]
Moderate	67 (42.14)	7 (28.00)	0.26 (0.09-0.78)
High	48 (30.19)	4 (16.00)	0.21 (0.05-0.78)
Fruits			
Low	48 (30.00)	9 (36.00)	[2]
Moderate	61 (38.13)	8 (32.00)	0.78 (0.26-2.30)
High	51 (31.88)	8 (32.00)	0.76 (0.26-2.21)
Refined sugar			
Low	57 (35.63)	8 (32.00)	[2]
Moderate	45 (28.13)	12 (48.00)	1.61 (0.58-4.42)
High	58 (36.25)	5 (20.00)	0.58 (0.17-1.97)
Butter			
Low	28 (17.50)	1 (4.00)	[2]
Moderate	88 (55.00)	20 (80.00)	6.07 (0.75-49.20)
High	44 (27.50)	4 (16.00)	1.84 (0.18-18.19)
Margarine			
Low	57 (35.63)	8 (32.00)	[2]
Moderate	84 (52.50)	13 (52.00)	1.07 (0.40-2.84)
High	19 (11.88)	4 (16.00)	1.17 (0.29-4.74)
Olive oil			
Low	47 (29.38)	10 (40.00)	[2]
Moderate	88 (55.00)	10 (40.00)	0.60 (0.22-1.61)
High	25 (15.63)	5 (20.00)	0.98 (0.29-3.35)
Seed oil			
Low	33 (20.63)	6 (24.00)	[2]
Moderate	97 (60.63)	15 (60.00)	0.84 (0.29-2.43)
High	30 (18.75)	4 (16.00)	0.66 (0.16-2.76)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ⩾ 25 cigarettes/d) and body mass index (quintiles). [2]Reference category.

Table 5 Odds ratios[1] and corresponding 95% CI for pre-illness intake of foods according to the disease location for Crohn's disease patients

	Small bowel	Colon
Pasta and/or rice	1.44 (0.71-2.92)	0.65 (0.20-2.13)
Bread	2.61 (1.26-5.41)[2]	0.27 (0.06-1.10)
Sweets and cakes	1.17 (0.64-2.14)	1.39 (0.53-3.63)
Red meat	1.58 (0.88-2.82)	1.23 (0.49-3.12)
White meat	0.74 (0.38-1.46)	0.40 (0.12-1.29)
Tuna fish	0.48 (0.26-0.91)[2]	0.33 (0.11-0.98)[2]
Fish	0.48 (0.24-0.93)[2]	0.41 (0.13-1.29)
Processed meat	1.31 (0.67-2.58)	0.74 (0.27-2.01)
Milk	1.30 (0.71-2.36)	1.13 (0.46-2.78)
Cheese	2.61 (1.26-5.41)[2]	1.94 (0.62-6.09)
Eggs	0.67 (0.36-1.23)	0.69 (0.27-1.80)
Potatoes	0.50 (0.23-1.07)	0.46 (0.15-1.41)
Vegetables	0.43 (0.21-0.88)[2]	0.60 (0.20-1.83)
Fruits	0.82 (0.42-1.58)	0.83 (0.29-2.36)
Refined sugar	0.80 (0.44-1.46)	0.77 (0.31-1.95)
Butter	0.83 (0.39-1.79)	1.44 (0.41-5.12)
Margarine	1.10 (0.51-2.36)	1.83 (0.58-5.76)
Olive oil	1.20 (0.58-2.51)	0.24 (0.06-1.00)[2]
Seed oil	0.81 (0.37-1.79)	1.05 (0.32-3.42)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ⩾ 25 cigarettes/d) and body mass index (quintiles) and according to an increase of 1 tertile level; [2]Significant associations.

tween pre-illness diet and the risk of IBD, in incident cases of UC and CD, results showed a positive relationship between the intake of margarine and a non-significant trend for pasta and rice in UC, and meat and cheese in CD. IBD patients reported a significant reduction in intake of potatoes and fish, and CD patients also have reduced consumption of vegetables and tuna fish compared with healthy controls. An association between pasta, sweets, red and processed meat, butter and margarine and IBD was also observed by factor analysis.

Several studies have focused on the role of dietary factors in IBD, some of which were already implicated as influencing the development of IBD, starting from the study by Martini & Brandes which demonstrated a

Table 6 Food group loadings for 3 dietary patterns extracted by factor analyses

	Factor 1	Factor 2	Factor 3
Pasta and/or rice	0.30	-0.25	0.32
Bread	-0.01	-0.40	0.51
Soft, sweets and cakes	0.45	-0.28	0.09
Red meat	0.57	0.30	0.23
White meat	0.45	0.49	-0.02
Tuna fish	0.43	0.60	-0.11
Fish	0.33	0.58	-0.12
Processed meat	0.46	0.04	-0.02
Milk	0.03	-0.33	0.04
Cheese	0.02	0.19	0.56
Eggs	-0.05	0.69	0.08
Potatoes	0.09	0.58	0.01
Vegetables	-0.52	0.22	0.32
Fruit	-0.53	-0.01	0.25
Refined sugar	-0.01	-0.03	0.60
Butter	0.47	0.20	0.27
Margarine	0.46	0.29	-0.02
Olive oil	-0.17	-0.32	0.44
Seed oil	0.39	0.13	0.07
	Refined OR (95% CI)	Prudent OR[1] (95% CI)	Healthy OR (95% CI)
Ulcerative colitis			
Low[2]	1[3]	1[3]	1[3]
Moderate	2.29 (0.70-7.32)	0.32 (0.11-0.90)	0.80 (0.28-2.28)
High	2.46 (0.77-7.83)	0.15 (0.04-0.51)	0.95 (0.33-2.76)
Crohn's disease			
Low	1[3]	1[3]	1[3]
Moderate	1.51 (0.45-4.99)	0.47 (0.17-1.27)	0.57 (0.18-1.78)
High	2.15 (0.67-6.87)	0.13 (0.03-0.51)	1.39 (0.49-3.95)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ≥ 25 cigarettes/d) and body mass index (quintiles); [2]Tertiles of factor scores; [3]Reference categories.

higher intake of refined carbohydrates in CD patients compared to controls[20]. This report was then followed by several studies in which other dietary factors, such as fibre, proteins, and total calorie intake, were evaluated in patients with UC and CD[5,6,8,11-17]. Despite the large number of papers on this issue, it has been shown that studying the association between diet and chronic disease presents methodological problems, since dietary habits could have already been influenced by the pathological condition itself. This has been demonstrated also by the present data, which revealed that UC and CD patients had already modified their dietary habits by themselves before diagnosis. We found that most IBD patients remembered having avoided vegetables, milk and/or cheese, or having reduced calorie intake before diagnosis and the onset of symptoms. The influence of symptoms on dietary habits and the difficulties encountered in recalling foods eaten 5 years before diagnosis are the main problems emerging in studies investigating diet and IBD.

In previous studies IBD patients, when asked about their dietary habits, described an increased consumption of refined sugar and a decreased intake of fruit and vegetables. These findings have also been interpreted as a consequence of the disease, rather than a factor which could be implicated in its aetiology. Indeed, refined sugar is rapidly absorbed in the small intestine, avoiding bulking effects and giving the possibility to compensate loss of energy and weight, which are typical characteristics of UC and CD. On the other hand, a decreased consumption of fibres could easily be the effect of trying to avoid symptoms caused by the bulk-rich food. Due to this methodological bias, in some case-control studies efforts have been made to retrieve data on the patients' dietary habits many years before onset of the illness, unfortunately obtaining less reliable answers, due to the weakness of the memory of those replying as well as the influence that the symptoms themselves could have had on the pre-illness dietary habits, the so called "recall bias". One condition which should be met is, therefore, to interview the patients about pre-illness dietary habits as soon as the diagnosis of IBD is made, in order to consider those subjects as incident cases. Even new patients might have had symptoms for several months or years prior to the diagnosis. To date, only a few studies by-passed the recall bias interviewing incident cases of IBD, as soon as possible after diagnosis, collecting information on pre-illness dietary habits[5,15-18].

The consumption of refined sugar was found to be positively associated with IBD in two of these studies[5,17], while another reported a positive association with CD but not with UC[20]. The total intake of proteins and a high consumption of eggs were shown to be positively related with the risk of IBD in one study[17]. This study also showed that a high consumption of fruit, vegetables and fibres, in general, helps to protect against the risk of IBD, while two other studies did not show any difference between patients and controls[5,20]. The study by Thornton *et al*[16] did not find any striking relationship in the intake of sugar, fibre, carbohydrates and proteins between the new cases of UC and controls.

In our study, only patients in whom the diagnosis had been made < 1 year prior to the interview and who had not modified dietary habits were considered. We found a significant intake of margarine and a high, although not significant, intake of rice and pasta in UC and a significant consumption of red meat and cheese in CD patients. We also identified a dietary pattern at higher risk of IBD correlated to pasta, sweets, red and processed meat, butter and margarine intake.

The differences in collecting and grouping foods and nutrients make it difficult to compare our results with those of other studies. On the other hand, while some authors reported an increased intake of protein in CD patients[21,22] others did not[17], furthermore, the pre-illness consumption of red meat, as well as cheese, has not been previously considered in detail. It is worthwhile pointing out that we found a relationship between both these nutrients and CD and that these foods are also related to *Mycobacterium avium* subspecies paratuberculosis, a candidate as an infectious aetiological agent in CD[23,24]. We also found a correlation between UC and margarine, pasta and rice intake, but not with other carbohydrates such as bread and sugar. These findings confirm the results of another Italian study showing that UC patients had a high intake

of total carbohydrates but not with those of a Japanese study in which the intake of bread for breakfast tended to be positively associated with the risk of UC[25,26]. However, the latter study, together with a recent multicenter study, showed that margarine and polyunsatured fatty acid intake was positively and significantly associated with UC, an association also found in our study[26,27].

In conclusion, although based on only a small number of cases, our study is one of the few in the literature providing information on the changes in dietary habits before diagnosis in IBD patients, thus explaining the difficulties and uncertainties encountered in epidemiological studies on diet and IBD. Furthermore, the study also revealed the role played by some environmental dietary factors and identified, by factor analysis, a specific dietary pattern at risk of IBD.

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COMMENTS

Background

The aetiology of inflammatory bowel disease (IBD) is still unknown, but the role of dietary factors in the pathophysiology has long since been taken into consideration. However, studies investigating this potential link are few and unconvincing.

Research frontiers

To date, only a few studies have examined the pre-illness diet, in incident IBD cases, showing conflicting results. In the present study the authors showed that signs and symptoms of IBD in patients before diagnosis led to a modification in their diet, and confirmed that pre-illness dietary habits, namely margarine, red meat, and cheese significantly increased the risk of ulcerative colitis and Crohn's disease.

Innovations and breakthroughs

A causal relationship between diet and IBD is difficult to define, due to the possibility that early symptoms of the disease may lead to a modification in dietary habits and the inability of the patients to accurately remember their diet before the onset of symptoms. The present case-control study evaluated whether the signs and symptoms of IBD patients before diagnosis led to a modification in their diet and assessed the association between diet and IBD onset in patients who did not change dietary habits by using multiple logistic regression and factor analysis.

Applications

The results of this study could contribute to a better understanding of the impact and significance of diet in the pathogenesis of inflammatory bowel disease.

Terminology

In the present study, the association between diet and dietary pattern and IBD has been assessed using multiple logistic regression and factor analysis. Factor analysis is a multivariate technique which analyses the underlying structure of a set of data in order to explain observed relationships between a large number of variables in terms of simpler relations.

Peer review

Although the number of patients is rather small, it is a well written and interesting study with some weaknesses which need to be addressed in order to strengthen the manuscript.

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BRIEF ARTICLE

Exertional esophageal pH-metry and manometry in recurrent chest pain

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Abstract

AIM: To investigate the diagnostic efficacy of 24-h and exertional esophageal pH-metry and manometry in patients with recurrent chest pain.

METHODS: The study included 111 patients (54% male) with recurrent angina-like chest pain, non-responsive to therapy with proton pump inhibitors. Sixty-five (59%) had non-obstructive lesions in coronary artery angiography, and in 46 (41%) significant coronary artery narrowing was found. In all patients, 24-h esophageal pH-metry and manometry, and treadmill stress tests with simultaneous esophageal pH-metry and manometry monitoring were performed. During a 24-h examination the percentage of spontaneous chest pain (sCP) episodes associated with acid reflux or dysmotility (symptom index, SI) was calculated. Patients with SI > 50% for acid gastroesophageal reflux (GER) were classified as having GER-related sCP. The remaining symptomatic individuals were determined as having non-GER-related sCP. During the stress test, the occurrence of chest pain, episodes of esophageal acidification (pH < 4 for 10 s) and esophageal spasm with more than 55% of simultaneous contractions (exercise-provoked esophageal spasm or EPES) were noted.

RESULTS: Sixty-eight (61%) individuals reported sCP during 24-h esophageal function monitoring. Eleven of these (16%) were classified as having GER-related sCP and 53/68 (84%) as having non-GER-related sCP. The exercise-provoked chest pain during a stress test occurred in 13/111 (12%) subjects. In order to compare the clinical usefulness of 24-h esophageal function monitoring and its examination limited only to the treadmill stress test, the standard parameters of diagnostic test evaluation were determined. The occurrence of GER-related or non-GER-related sCP was assumed as a "gold standard". Afterwards, accuracy, sensitivity and specificity were calculated. These parameters expressed a prediction of GER-related or non-GER-related sCP occurrence by the presence of chest pain, esophageal acidification and EPES. Accuracy, sensitivity and specificity of chest pain during the stress test predicting any sCP occurrence were 28%, 35% and 80%, respectively, predicting GER-related sCP were 42%, 0% and 83%, respectively, and predicting non-GER-related sCP were 57%, 36% and 83%, respectively. Similar values were obtained for exercise-related acidification with pH < 4 longer than 10 s in the prediction of GER-related sCP (44%, 36% and 92%, respectively) and EPES in relation to non-GER-related sCP (48%, 23% and 84%, respectively).

CONCLUSION: The presence of chest pain, esophageal acidification and EPES had greater than 80% specificity to exclude the GER-related and non-GER-related causes of recurrent chest pain.

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Key words: Chest pain; Diagnosis; Esophageal manom-

etry; Esophageal pH-metry; Treadmill test

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INTRODUCTION

Recurrent, effort-provoked chest pain is the most common among cardiac and esophageal symptoms. It is also one of the greatest problems in contemporary health care because of its prevalence, adverse effects on quality of life, morbidity, and the utilization of health care resources[1,2]. The other problem is the frequent overlapping of causes of chest pain[3]. The presence of gastroesophageal reflux (GER)-related chest pain was confirmed in about 60% of patients with normal coronary angiography[4] and in 35% of patients with coronary artery disease (CAD)[5]. On the other hand, patients with GER presented with many co-morbidities, originating both from cardiac and noncardiac sources, which may cause chest pain[6]. For this reason, in the diagnostic procedures of chest pain, both in patients with and without significant coronary artery narrowing, it is very important to evaluate the temporal relationship between symptoms and electrocardiographic signs of myocardial ischemia and/or the occurrence of esophageal abnormalities. It has been proven that analysis of the symptom association probability (SAP), symptom index (SI) or symptom sensitivity index reproducibly increases the yield of 24-h esophageal pH-metry, manometry and impedance examination[7,8]. As a result, some provocative tests inducing symptoms, could probably make these diagnostic procedures more efficient. It was reported that esophageal testing during exercise[9-16], dynamic position changing[17] and bending[18] made the 24-h esophageal pH-metry more informative and more efficient in the detection of significant GER. Exercise can provoke symptoms and abnormalities originating both from the heart and the esophagus[11,16]. Therefore, it was made a hypothesis that the simultaneous monitoring of clinical, electrocardiographic and hemodynamic parameters, as well as esophageal pH and pressure during the treadmill stress test, might also provide a more accurate means to evaluate the temporal interrelation between chest pain occurrence and myocardial and esophageal disturbances than separate tests[19]. Such a procedure might be useful, especially in patients non-responsive to empirical therapy with proton pump inhibitors (PPI)[7], both with and without significant coronary artery narrowing in coronary artery angiography, because of the above mentioned overlap in the causes of chest pain[5,20]. In addition it may allow the possibility of diagnosing myocardial ischemia in patients with non-obstructive coronary artery lesions due to microvascular angina[2] or the ischemic effect of the cardio-esophageal reflex[21]. The cardio-esophageal reflex is a vagal, visceral neural reflex, which may be activated by changes in intra-esophageal pH, pressure or temperature. Its stimulation may lead to a decrease in myocardial perfusion, proven in invasive[21,22] and non-invasive examinations[23], as well as to the occurrence of electrocardiographic signs of myocardial ischemia[5,24] or arrhythmia[19,25-29]. The mentioned effects were confirmed in about 56% of subjects with a normal coronarography[21] and in some subjects with significant coronary artery narrowing[24]. On the other hand, products of anaerobic myocardial metabolism, especially bradykinin[30], or invasive procedures on coronary arteries[22] *via* neural pathways may lead to esophageal dysmotility and reflux. These relationships connect ischemic heart disease and esophageal disorders in a vicious circle.

It is known that the activation of vagal reflexes may change the autonomic nervous system balance. In this way, abnormalities in intraesophageal pH[31,32] and pressure may also lead to a decrease in pain threshold and hypersensitivity[33]. This may explain why, in many studies, time-dependence between GER, esophageal dysmotility and chest pain episodes was relatively small and amounted to 22%-65%, and why many of the patients with noncardiac chest pain remained symptomatic in spite of detailed diagnosis and appropriate treatment[4]. These complicated interrelations assumed the planning of further studies to evaluate the new diagnostic tools in patients with recurrent chest pain of suspected noncardiac origin, as well as to determine more easily, and in a shorter time, the causal associations between esophageal disorders and patients' symptoms.

The aim of this study was to estimate the diagnostic efficacy of esophageal pH-metry and manometry monitoring during a treadmill stress test in comparison to 24-h esophageal pH-metry and manometry in patients with recurrent angina-like chest pain. In other words, this study addresses whether it is possible to replace 24-h esophageal function monitoring by an examination limited only to a treadmill stress test.

MATERIALS AND METHODS

One hundred and twenty-nine consecutive patients diagnosed with recurrent angina-like chest pain of suspected noncardiac origin were investigated. The symptoms were suspected of being of noncardiac origin by the leading doctor, independently of the researcher, who referred his patients for gastroenterological diagnosis after a cardiac work-up because of recurrent symptoms resistant to standard treatment oriented to coronary reserve improvement and empirical therapy with PPI. The pre-referral cardiac diagnostics procedures covered history, physical examination, electrocardiogram (ECG), treadmill stress test, and coronary artery angiography (Table 1). An extracardiac source of chest pain was suspected because none of the referred patients presented with an association between chest pain and ischemic changes during a treadmill stress

Table 1 Demographic and clinical data of investigated patients with a comparison of subjects without and with significant (> 50%) coronary artery narrowing *n* (%)

Parameter	Significant coronary artery narrowing	
	No (*n* = 65)	Yes (*n* = 46)
Males/females	29/36 (45/55)	31/15 (67/33)
Age (yr)	55.0 ± 8.8	55.0 ± 8.8
BMI (kg/m^2)	28.7 ± 4.2	27.8 ± 3.9
WHR	0.94 ± 0.9	0.93 ± 0.07
Smoking	11 (17)	10 (21)
History of PCI	0	21 (46)[a]
History of CABG	0	9 (19)[a]
History of myocardial infarction	0	18 (40)
Hypertension	24 (39)	24 (52)
Systolic blood pressure (mmHg)	122.8 ± 17.7	123.4 ± 17.4
Diastolic blood pressure (mmHg)	82.0 ± 11.2	81.1 ± 9.3
Diabetes mellitus	4 (5)	7 (13)
Total cholesterol (mg/dL)	195.7 ± 28.1	218.9 ± 48.3[a]
LDL cholesterol (mg/dL)	122.7 ± 25.9	136.1 ± 39.6
HDL cholesterol (mg/dL)	52.1 ± 11.9	48.5 ± 12.9
Triglycerides (mg/dL)	100.1 ± 33.0	166.1 ± 84.5[a]
Blood glucose (mg/dL)	100.2 ± 15.8	95.0 ± 17.7
Stress test duration (s)	455 ± 172	549 ± 191[a]
ST interval depression > 1 mm without chest pain during treadmill (silent ischemia)	28 (43)	17 (37)
Chest pain without ST interval depression during stress test	9 (14)	4 (9)
GER-related chest pain	7 (11)	4 (9)
Non-GER-related chest pain	35 (54)	22 (48)
Erosive esophagitis in endoscopy	16 (25)	17 (37)
Pathological GER	19 (29)	16 (35)
DES	13 (20)	10 (22)
epGER	12 (18)	4 (9)
epDES	14 (22)	9 (20)

Data presented as *n* (%) or mean ± SD. [a]$P < 0.05$ in an unpaired Student *t*-test or Fisher exact test. BMI: Body mass index; WHR: Waist/hip ratio; LDL: Low density lipoprotein; HDL: High density lipoprotein; PCI: Percutaneous coronary intervention; CABG: Coronary artery bypass graft; GER: Gastroesophageal reflux (> 4.5% time of esophageal monitoring with pH < 4); DES: Diffuse esophageal spasm, defined as esophageal motility abnormality with more than 30% of simultaneous contractions; epGER; Exercise-provoked GER, defined as a decrease in esophageal pH during a stress test for more than 10 s.

test. However, in spite of the results of the pre-referral cardiological diagnostic procedures, angina-like chest pain connected with electrocardiographic signs of myocardial ischemia was observed during the treadmill stress test conducted in the clinic in 18 subjects with significant coronary artery narrowing in angiography. These patients were excluded from the analysis because it would be impossible to distinguish between cardiac and extracardiac sources of chest pain, especially in patients with significant coronary artery disease. Finally, 111 consecutive subjects were included in the analysis, and fulfilled the following inclusion criteria: (1) age between 40 and 70 years; (2) prior coronary angiography performance not earlier than 3 mo before gastroenterological work-up; (3) angina-like chest pain to a degree of class Ⅱ in accordance with the Canadian Cardiovascular Society; such a pattern of chest pain was defined as precordial symptoms induced by exercise of less than, for example, marching for a distance under 200 m, and receding after rest or taking nitroglycerine; the occurrence of such defined chest pain during a treadmill stress test cannot be accompanied by signs of myocardial ischemia in the ECG; and (4) persistent symptoms despite adequate anti-anginal treatment (in patients with significant coronary artery lesions) and at least 1 mo-long therapy with a double dose of omeprazole, both in patients with and without significant coronary artery narrowing. Such a course of symptoms justified a suspicion of an extracardiac cause of chest pain, resistant to empirical therapy with PPI, and provided reasons for gastroenterological diagnostics to be undertaken. The exclusion criteria were: the presence of changes in the resting ECG, which made it impossible to estimate signs of myocardial ischemia (e.g. left bundle branch block or pre-excitation syndrome). All patients were asked not to take histamine receptor type 2 antagonists (e.g. ranitidine and famotidine), PPI or prokinetics (metoclopramide, cisapride, trimebutine and mebeverine).

Finally, the study group consisted of 46 (40%) patients with significant coronary artery changes, more than 50% of them with narrowing of the arteries, although not suitable for revascularization, and 65 (60%) subjects showing a normal coronary arteriography or no obstructive coronary lesions. Clinical and demographic data of the studied patients were divided according to the presence of significant narrowing of the coronary vessels (Table 1). Neither group differed in relation to the majority of these (Table 1). During the investigation, patients continued taking the stable doses of previously prescribed drugs (i.e. for CAD, hypertension and diabetes).

In all subjects, the medical history, physical examination, panendoscopy with gastric and esophageal biopsy, 24-h esophageal pH-metry and manometry were performed "off-therapy". An investigation of ambulatory esophageal function was carried out using a multi-use antimony probe (Synetics Medical AB, Sweden), a manometry catheter (Synectics, Medtronic) with 3 pressure sensors separated by 5 cm, and a Synectics micro-Digitrapper. An esophageal pH-metric sensor, after calibration to pH 7 and 1, using nasal and esophageal intubation, was positioned 5 cm above a monometrically-determined lower esophageal sphincter (LES). Pressure transducers were located through the other nostril at 3, 8 and 13 cm above the LES. During esophageal pH and pressure monitoring, all patients recorded occurring symptoms. None of the patients reported disturbances in nasal breathing. Every chest pain appearing during 24-h esophageal function monitoring was recorded by the micro-Digitrapper and labelled spontaneous chest pain (sCP).

The following day, when patients had become accustomed to the presence of the pH-metric and manometric probes in their nostrils, a treadmill stress test on a running track was carried out at approximately 7 am during continuous esophageal pH-metry and manometry

monitoring. The exercise test was performed using a device manufactured by Schiller, Switzerland, according to the Bruce protocol (the speed and gradient of the running track were increased every 3 min to 2.7, 4, 5.5 and 6.8 km/h, and by 10°, 12°, 14° and 16°). The start and finish of the exercise during the treadmill stress test as well as exercise-provoked angina-like chest pain (epCP) episodes were marked on the micro-Digitrapper.

The obtained data were downloaded to a personal computer and analyzed using GASTROSOFT software. Standard pH-metric and manometric parameters were calculated[34]. The GASTROSOFT software also analyzed the relationships between chest pain and the type of esophageal abnormality (a decrease in esophageal pH, changes in esophageal pressure or peristaltic wave coordination). This analysis concerned a period of 2 min prior to and during chest pain episodes. Patients were classified as having "GER-related" chest pain when the SI, defined as a percentage of sCP episodes associated with acid reflux during 24-h esophageal pH-metry, was $\geqslant$ 50%. Patients were classified as having "non-GER-related" chest pain if the percentage of sCP episodes during 24-h esophageal pH-metry and manometry associated with esophageal dysmotility was $\geqslant$ 50% and the individual did not fulfill GER-related chest pain criteria. Esophageal dysmotility was classified following esophageal manometry parameters presented during chest pain or in periods of 2 min prior to its appearance, non-peristaltic contractions, or contractions with amplitude or duration exceeding 95% of their daily average value.

Apart from types of sCP and esophageal abnormalities appearing within 24-h esophageal examination, additional symptoms and esophageal pH-metric and manometric abnormalities occurring during the treadmill stress test were determined. Angina-like chest pain (retrosternal pressing) appearing during the treadmill stress test was termed exercise-provoked chest pain (epCP). Gastroesophageal acid reflux provoked by exercise (epGER) was defined as a decrease in esophageal pH to below 4 for more than 10 s during the exercise stress test. Exercise-provoked esophageal spasm (EPES) was diagnosed when the percentage of simultaneous contractions during the treadmill stress test exceeded 55%. Simultaneous contractions, according to gastrosoft software settings, were defined as a sequence of contractions with less than 0.25 s delay between adjacent transducers separated by 5 cm (a propagation speed higher than 20 cm/s). The value of the cut-off at the level of 55% originated from the work by Stein *et al*[35], who proposed such diagnostic criteria for diffuse esophageal spasm in 24-h manometry.

Ethics

The study protocol was approved by the local Bioethics Committee of Nicolaus Copernicus University in Toruń and the Collegium Medicum in Bydgoszcz, Poland. All subjects gave their informed consent prior to the start of enrolment procedures. All procedures have been conducted in compliance with the Declaration of Helsinki.

Table 2 Parameters for the clinical usefulness of exercise-provoked chest pain, exercise-provoked gastroesophageal reflux, and exercise-provoked esophageal spasm in the diagnosis of gastroesophageal reflux-related and non-gastroesophageal reflux-related spontaneous chest pain based on 24-h esophageal function examination (n = 111) (%)

Parameter	epCP for both sCP	epCP for GER-related sCP	epCP for non-GER-related sCP	epGER for GER-related sCP	EPES for non-GER-related sCP
Accuracy	28	42	57	44	48
Sensitivity	35	0	36	36	23
Specificity	80	83	83	92	84
PPV	64	0	64	44	59
NPV	55	89	61	89	53
LR+	1.75	0	2.1	4.5	1.4
LR-	0.81	1.2	0.77	0.7	0.92

epCP: Exercise-provoked chest pain; sCP: Spontaneous chest pain; EPES: Exercise-provoked esophageal spasm; GER: Gastroesophageal reflux; PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio.

Statistical analysis

Statistical analysis was conducted using a licensed version of statistical software STATISTICA PL 8.0 for Windows. The results were mainly presented as the mean ± SD or n (%). The normal distribution of variables was estimated using the Kolmogorov-Smirnov test. The comparison of demographic and clinical data between patients with and without significant coronary artery narrowing (Table 1) was made using an unpaired Student t-test (for quantitative variables) and the Fisher exact test for qualitative variables. In addition, the standard parameters of diagnostic test usefulness according to evidence-based medicine (EBM), e.g. accuracy, sensitivity, specificity, positive and negative predictive values, as well as the positive and negative likelihood ratios, were calculated. The diagnosis of GER-related and non-GER-related chest pain acted as a "gold standard" (reference point) for this analysis. According to such assumptions, the parameters of diagnostic test usefulness expressed the relationships between the occurrence of exercise-related disturbances (epCP, epGER, EPES) and diagnoses of GER-related and non-GER-related sCP in 24-h esophageal pH-metry and manometry. This means that they expressed the ability of exercise-related esophageal abnormalities to predict the presence of GER-related or non-GER-related sCP. Accuracy was defined as the proportion of subjects with and without spontaneous chest pain and the presence or lack of evaluated esophageal function disorder (e.g. EPES). This represented the ratio of patients with true positive and true negative results to the total number of subjects. Sensitivity, i.e. the percentage of true positive results, was defined as the proportion of subjects with a respective kind of sCP in 24-h esophageal examination (GER-related or non-GER-related) and the simultaneous presence of epCP, epGER or EPES during the treadmill stress test (Table 2). Specificity, i.e. the percentage of true negative

results, was defined as the proportion of asymptomatic subjects during 24-h esophageal pH-metry and manometry in whom evaluated exercise-related disorders (e.g. epCP, epGER and EPES) did not appear during the treadmill stress test (e.g. epGER and EPES) (Table 2). The positive predictive value (PPV) was defined as the percentage of subjects with the presence of an evaluated parameter (e.g. EPES) having chest pain during 24-h esophageal pH-metry and manometry (true positive/true + false positives). The negative predictive value (NPV) was defined as the percentage of patients without an evaluated parameter (e.g. epCP, epGER or EPES) in whom chest pain during 24-h esophageal pH-metry and manometry did not appear (true negative/true + false negatives). The positive diagnostic likelihood ratio (LR+) was defined as an odds ratio of likelihood that a patient with chest pain during 24-h esophageal pH-metry and manometry would have an evaluated disorder to the probability that an individual without chest pain would have this esophageal disturbance [LR+ = sensitivity/(1-specificity)]. However, a negative likelihood ratio (LR-) represented the odds ratio that the lack of an evaluated esophageal disorder (e.g. EPES) would be observed in subjects with chest pain during 24-h esophageal function monitoring compared with whether the same results would be observed in individuals with spontaneous chest pain; LR- = (1-sensitivity)/specificity.

RESULTS

Patients with and without significant coronary artery disease had a similar prevalence of estimated esophageal abnormalities (Table 1). During 24-h esophageal pH and pressure monitoring, 68/111 (61%) individuals were symptomatic and presented with sCP. Among them, 11/68 (16%) experienced GER-related sCP and in 57/68 (84%) non-GER-related sCP was diagnosed. These frequencies in patients both with and without significant coronary artery narrowing were similar.

In only 13/111 (12%), epCP not connected with signs of myocardial ischemia was observed and appeared significantly less frequently than sCP during 24-h esophageal pH-metry and manometry ($P = 0.0001$). The prevalence of epCP was not significantly greater in patients without CAD (Table 1). Chest pain during the stress test occurred in 6 subjects who did not show symptoms during 24-h pH-metry and manometry. This corresponded with 5% of all subjects and 14% (6/43) of individuals who did not report sCP during daily monitoring.

The monitoring of intraesophageal pH and pressure during the treadmill stress test revealed some exercise-provoked esophageal abnormalities, i.e. intraesophageal acidification, labeled epGER, in 16/111 (14%) of all subjects and EPES in 23/111 (21%) (Table 1). Of these patients, epGER was diagnosed in 4 (4%) and EPES in 13 (12%), who had no esophageal abnormalities (i.e. erosive esophagitis, pathological gastroesophageal acid reflux or diffuse esophageal spasm) in panendoscopy and in 24-h esophageal pH-metry and manometry. However, these esophageal disorders were not significantly related to chest pain presence during the treadmill stress test. Symptomatic epCP was noted in only 14% of epGER episodes ($P > 0.05$) and in 30% of EPES ($P > 0.05$).

In the next part of the analysis, the clinical usefulness of a short protocol of esophageal examination, limited only to treadmill stress test duration, was estimated and compared to the diagnostic efficacy of 24-h pH-metry and manometry, expressed by the diagnosis of GER-related or non-GER-related (dysmotility-related) sCP. This acted as the "gold standard". The occurrence of epCP, epGER and EPES during the stress test had only acceptable specificity as did the NPV value in the diagnosis of GER-related or non-GER-related sCP (Table 2). A separate analysis performed in patients both with and without significant coronary artery narrowing was conducted with similar results.

DISCUSSION

This study has addressed the question of whether it is possible to replace 24-h esophageal pH-metry and manometry with a short protocol of these examinations limited only to stress test duration in the diagnosis of noncardiac chest pain originating from the esophagus. In other words, this investigation estimated exercise as a provocative test offering a greater possibility of correlating symptoms with esophageal abnormalities and excluding the potential life-threatening state connected with myocardial ischemia. The obtained results met the assumed requirements only in part.

The main finding of this study was that diagnoses of exercise-related esophageal disorders, such as epCP, epGER and EPES, had high values of specificity and NPV (Table 2). This makes them useful in excluding rather than confirming an esophageal source of recurrent angina-like chest pain, non-responsive to PPI, in patients both with and without significant coronary artery narrowing. This means in practice that 24-h pH-metry and manometry would not offer any important information concerning the cause of chest pain, if a patient, non-responsive to empirical therapy with PPI, did not present retrosternal symptoms during a treadmill stress test (e.g. conducted during a cardiologic work-up). Similar conclusions prompted the diagnosis of epGER and EPES during exertional esophageal pH and pressure monitoring during a treadmill stress test. A recognition of epGER or epCP in patients non-responsive to PPI was weak in this study (LR+ > 2 or LR- < 0.5) or uncertain (LR+ < 2 or LR- > 0.5), regarding parameters in the prediction of GER-related and non-GER-related spontaneous chest pain appearance during 24-h esophageal function monitoring.

The next observation of this study, as well as the next argument against recommending 24-h esophageal pH and pressure monitoring substitution by their examination only during a treadmill stress test, was that chest pain appeared during the stress test significantly and several times less frequently than during the 24-h investi-

gation. This did not correlate with esophageal pH-metric and manometric abnormalities. This shows that esophageal monitoring during a treadmill stress test, although providing the possibility of diagnosing epGER in an additional 4% of patients and EPES in an extra 12% of subjects, did not increase the probability diagnosis of the origin of chest pain, mainly because of the low SI value. In addition, the outcome of epGER and EPES diagnosis was still obscure.

In the available papers, I did not find any analysis using EBM parameters of diagnostic test evaluation in patients with recurrent chest pain who were non-responders to PPI. However, it was reported that esophageal testing during exercise[9-16], dynamic position changing[17] and bending[18] made 24-h esophageal pH-metry more efficient in the detection of significant GER. Bovero *et al*[10] showed that the provocation of gastroesophageal acid reflux by exercise might improve the diagnostic efficiency of esophageal pH-metry. The clinically useful provocative effect of exercise on gastroesophageal reflux has also been reported by other authors[11-18]. Furthermore, Ravi *et al*[9], investigating the effect of treadmill use on esophageal motility, found that exercise decreased the esophageal wave amplitude in patients with GERD, nutcracker esophagus and diffuse esophageal spasm (DES). Unfortunately, the authors did not discuss the outcome of exercise on the effectiveness of esophageal motility, so they could reveal DES-like exertional motility disorders such as EPES. Some authors have shown one questionable role of esophageal motility disorders in noncardiac chest pain pathogenesis[35,36], mainly because of the personally-dependent overlapping of other noncardiac chest pain pathomechanisms, such as hypersensitivity or musculoskeletal disorders[4,33]. However, Adamek *et al*[20] have confirmed the role of esophageal spasm in noncardiac chest pain pathogenesis, reporting an increase in simultaneous contractions in patients with chest pain, both with and without significant coronary artery narrowing, in comparison to asymptomatic controls. Apart from the above mentioned discrepancies, in my opinion, my results might have a clinical importance. Firstly, they provide an analysis of the classic diagnostic procedures of noncardiac chest pain both in patients with and without significant coronary artery narrowing; unfortunately, in everyday praxis, the overlapping of causes of noncardiac chest pain in therapy-resistant patients with CAD is rarely recognized[3]. Secondly, they show the importance of angina-like chest pain analysis in the diagnosis, not only of cardiac but also noncardiac sources of chest pain. It is known that chest pain appearance during a treadmill stress test increases its clinical usefulness. My investigation showed that a lack of chest pain during a typical cardiological exercise test predicted the low diagnostic importance of 24-h esophageal pH-metry and manometry. This information may shorten the diagnostic process and prevent the performance of useless examinations and resource utilization because of the implied consideration of extraesophageal chest pain causes if a stress test during a cardiological work-up did not provoke chest pain. Thirdly, the tests showed that the newly-defined esophageal motility disorder of EPES with high specificity allowed the prediction of a lack of esophageal manometry usefulness in the diagnosis of non-GER-related chest pain. The influence of EPES diagnosis on the course of chest pain over a 2.7-year long follow-up will be discussed in other work.

This study, however, has certain limitations. The first results were from a small subject sample, but this was still greater than for the majority of works concerning diagnostic and therapeutic problems in patients with suspected noncardiac chest pain[4,9,10,20,21,36-50]. Secondly, diagnostic procedures were made "off-therapy", which was inconsistent with the majority of recommendations by Fass, Hirano and Sifrim suggesting chest pain investigation "on-PPI-therapy"[36,51,52]. However, a recent study considered the necessity of returning to such ("off-therapy") an esophageal function examination[7,53-55]. Thirdly, the reference points in the analysis of respective diagnostic test usefulness were subjective and susceptible to the effect of esophageal hypersensitivity, one being between the main noncardiac chest pain pathomechanisms[4,33,36]. On the other hand, the evaluation of relationships between symptoms and esophageal abnormalities is an acceptable method to increase the diagnostic yield of esophageal 24-h examinations[8]. However, a more reliable parameter for this purpose is SAP, not SI. Fourthly, esophageal pH-metry and 24-h manometry have recently been substituted by esophageal impedance with pH-metry and high resolution manometry[56,57] but, in particular, the latter does not seem to be useful in the diagnosis of esophageal function during a treadmill stress test.

In conclusion, the occurrence of angina-like chest pain, a decrease in esophageal pH to below 4, and an increase in simultaneous contraction percentage above 55% during a treadmill stress test has acceptable specificity and NPV to exclude an origin from the esophagus, both for GER-related and non-GER-related causes of recurrent chest pain, in comparison to the results obtained during 24-h esophageal function monitoring. However, less frequent chest pain appearance during a treadmill stress test than during 24-h esophageal function monitoring limits the clinical usefulness of this provocative examination to the diagnosis of previously unrecognized myocardial ischemia and exercise-provoked esophageal disorders such as epGER or EPES.

COMMENTS

Background

This article concerns a very important and still current problem in clinical praxis, which is the diagnosis of chest pain. The diagnostic procedures are often time-consuming, and their clinical yield is useful only 20%-60% of individuals. For this reason new diagnostic protocols are still being investigated, including provocative tests.

Research frontiers

The study was performed in a relatively small number of patients and needs confirmation. However, its results may be helpful both for cardiologists and gastroenterologists, especially as it was performed in patients who were unresponsive to proton pump inhibitors therapy, the first-line tool in the diagnosis

of gastroesophageal reflux-related chest pain. The shortcoming of it was in not using a more sensitive examination which might more securely differentiate between cardiac and esophageal exercise-provoked chest pain.

Innovations and breakthroughs

This study has shown that the asymptomatic course of the treadmill stress test predicted a low yield of esophageally-oriented diagnostic procedures for chest pain. In addition, this study showed a similar prevalence of esophageal abnormalities in patients with and without coronary artery disease.

Applications

The results of this study, after confirmation with a greater number of subjects, may change the strategy of chest pain diagnosis of suspected esophageal origin. The results imply a lack of usefulness of esophageal function monitoring in patients in whom a cardiological work-up did not provoke symptoms.

Peer review

The manuscript presents some interesting and novel results, however, the numbers are small. This is an original paper, which would be an asset to the journal.

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BRIEF ARTICLE

Effects of glutamine and curcumin on bacterial translocation in jaundiced rats

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Abstract

AIM: To investigate the effect of curcumin on bacterial translocation and oxidative damage in an obstructive jaundice model and compare the results to glutamine, an agent known to be effective and clinically used.

METHODS: Twenty-four female Wistar-Albino rats, weighing 200-250 g, were randomly divided into three groups (8 in each group). After ligation of the common bile duct in all animals, Group Ⅰ received oral normal saline, Group Ⅱ received oral glutamine and Group Ⅲ received oral curcumin for seven days. Blood samples via cardiac puncture, tissue samples (terminal ileum, liver and mesenteric lymph node) and peritoneal fluid were obtained from the animals at the time of death to investigate bacterial translocation and oxidative damage.

RESULTS: We observed that both glutamine and curcumin reduced bacterial translocation in blood, hepatocellular damage, plasma cytokine levels, oxidative tissue damage and apoptosis significantly compared to the control group. Additionally, glutamine showed protective effects on ileal epithelium and reduced villus atrophy.

CONCLUSION: On the basis of these findings, both curcumin and glutamine are thought to be effective in preventing or reducing bacterial translocation and oxidative damage in obstructive jaundice.

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Key words: Obstructive jaundice; Bacterial translocation; Oxidative damage; Glutamine; Curcumin

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Karatepe O, Acet E, Battal M, Adas G, Kemik A, Altiok M, Kamali G, Koculu S, Cagatay A, Kamali S, Karahan S. Effects of glutamine and curcumin on bacterial translocation in jaundiced rats. *World J Gastroenterol* 2010; 16(34): 4313-4320 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i34/4313.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i34.4313

INTRODUCTION

Obstructive jaundice is characterized by a disability in the secretion of bile into the intestinal system, accumulation of toxic bile salts and bilirubin in the tissues and signifi-

cant changes in systemic and hepatic functions[1]. Despite current modern diagnostic and therapeutic approaches, interventions in patients with biliary tract obstruction result in 10%-25% mortality and up to 56% major morbidity[2]. Biliary sepsis, wound infections, intra-abdominal abscess formation and renal failure are frequent complications in obstructive jaundice. Bacterial translocation and oxidative tissue damage have been emphasized as the leading causes of these complications in obstructive jaundice by numerous investigators[2-4]. Obstructive jaundice causes alterations leading to bacterial translocation both in the intestinal barrier and in the reticuloendothelial system. Some of these alterations may be listed as mucosal damage in the intestinal lumen due to lack of bile, apoptosis, bacterial overgrowth, motility disorder associated with oxidative stress and functional abnormalities in the tissue macrophages[2,5].

Glutamine, a non-essential amino acid, occupies a central role in numerous metabolic processes such as amino acid transport and nitrogen balance. It is the main energy source for rapidly proliferating cells such as enterocytes and lymphocytes. It has been reported not only to lower the rate of endotoxemia and translocation by preserving mucosal integrity but also to improve the immune system action against bacteria and endotoxins which succeed in passing the intestinal barrier[3,5-7].

Curcumin is a polyphenol derived from the herbal remedy and dietary spice turmeric. The antioxidant, anticancer, anti-inflammatory and cytoprotective effects of curcumin have been demonstrated by numerous experimental and clinical studies[8]. Gülçubuk *et al*[9] have declared that curcumin has positive effects on intestinal barrier function due to its anti-inflammatory properties and possibly can prevent bacterial translocation. We have previously shown positive effects of curcumin on oxidative damage and liver function in obstructive jaundice[10]. However, according to our knowledge, a comprehensive and comparative study regarding the effect of curcumin on bacterial translocation and oxidative damage in obstructive jaundice has not been performed yet.

The aim of this study was to compare the effects of curcumin with those of glutamine, a reliable control, on bacterial translocation and oxidative damage in an obstructive jaundice model and to evaluate the results with a review of the literature.

MATERIALS AND METHODS

Animals

Twenty-four healthy female rats weighing 200-250 g, housed in stainless steel cages under controlled temperature (22℃) and humidity and with 12-h dark/light cycles, were used in this study. Standard industrial rat feed containing 21% protein and fresh tap water were given *ad libitum* before and after operation. The experimental protocol was designed according to the ethical standards for animal use and approved by the local committee of animal use.

Surgical procedure and treatment

All procedures were performed under general anesthesia induced by intramuscular injections of ketamine hydrochloride 80 mg/kg (Ketalar flk; Pfizer, Istanbul, Turkey) plus 5 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey). The abdomen was shaved and soaked with Betadine solution. After a midline laparotomy of 1-2 cm, the common bile duct was identified, doubly ligated using 4/0 silk sutures and divided. Abdominal incisions were closed in two layers using 4/0 silk sutures. The animals were randomized into three groups (8 in each). Group Ⅰ was treated with normal saline 1 cc orally once daily after bile duct ligation. Group Ⅱ was treated with glutamine (Resource Glutamine powder 5 g; Nestle Health Care Nutrition, Germany) 200 mg/kg orally once daily after bile duct ligation. Group Ⅲ was treated with curcumin (Curcumin from *Curcuma longa*; Sigma Aldrich, Germany) 20 mg/kg orally once daily after bile duct ligation. Animals were regularly nourished and maintained for 7 d as described above. Glutamine was dissolved in distilled water and the solution was stirred immediately before use. Curcumin was suspended in distilled water and the suspension was stirred immediately before use. The re-laparotomy was performed through the old incision on postoperative 8th day under general anesthesia and subjects were sacrificed. Systemic blood via cardiac puncture, peritoneal fluid and tissue (terminal ileum, liver and mesenteric lymph node) samples were obtained to investigate bacterial translocation and oxidative damage. All procedures were performed aseptically using sterile instruments.

Microbiological examination

Systemic blood samples obtained via cardiac puncture and peritoneal fluid samples were cultured aerobically using BacTec Peds (BioMérieux, Durham, USA). Blood cultures were continuously monitored for 7 d. Positive cultures were plated out on appropriate media and species identified by Sceptor microdilution and standard bacteriological techniques. The mesenteric lymph node, liver and terminal ileum samples were removed and placed in pre-weighed sterile glass bottles containing sterile pre-reduced brain-heart infusion. The bottles were re-weighed and tissue homogenates were prepared in 2-mL brain-heart infusions using sterile mortars and pestles. A portion (0.1 mL) of homogenates was cultured on blood agar, eosin methylene blue agar. All the plates were examined after 24 and 48 h of incubation at 37℃.

Biochemical examination

Systemic blood *via* cardiac puncture and tissue (terminal ileum) samples were obtained from rats for biochemical evaluation. Tissues were washed with physiological serum for biochemical analysis, weighed and homogenized using the method of Sier *et al*[11]. Serum levels of cytokines tumor necrosis factor-α (TNF-α) (pg/mL) and interleukin-6 (IL-6) (pg/mL) were measured by immunoenzymatic enzyme-linked immunosorbent assay method (Quantikine High Sensitivity Human by R&D Systems, USA) according to the manufacturer's protocol. Minimum detectable concentrations were determined by the manufacturer to be 0.12 pg/mL and 0.03 pg/mL, respectively. Intra-assay

(2.6 for TNF-α and 1.6 for IL-6) and inter-assay (14 for TNF-α and 6.4 for IL-6) precision performances of the assay were determined on 20 replicates from the quality control data of the laboratory. Malondialdehyde (MDA) was determined spectrophotometrically by the thiobarbituric acid method. Aliquots of 0.2 mL of serum were mixed thoroughly with 0.8 mL of phosphate-buffered saline (pH 7.4) and 25 μL of butylated hydroxytoluene solution. The samples were placed on ice for 2 h after addition of 0.5 mL of 30% trichloroacetic acid. Then, samples were centrifuged at 2000 *g* at 25℃ for 15 min. After that, 1 mL of each supernatant was mixed with 0.075 mL of 0.1 mol/L ethylenediamine tetraacetic acid and 0.25 mL of 1% thiobarbituric acid in 0.05 mol/L sodium hydroxide (NaOH). Supernatant of each sample was kept in boiling water for 15 min and then cooled to room temperature. Finally, the absorbance of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm. The data regarding TBARS were expressed in mDA, using a molar extinction coefficient for MDA of 1.56×10^5/cm per mol/L and the results were expressed in nmol/L (range: 0.1-2.5). Serum nitric oxide (NO) levels were measured with Griess reagent as previously described[12]. The first step is the conversion of nitrate using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite to a purple azocompound. Protein interference was avoided by treatment of the reacted samples with zinc sulphate and centrifugation for 5 min at 10000 *g*, the azochromophore spectrophotometry was performed at 450 nm; sodium nitrate was used as the standard and results were expressed in mmol/L (range: 10-120 mmol/L). Myeloperoxidase (MPO) activity was measured as described previously[13]. In short, tissue homogenates were incubated with 0.5% hexadecyl-trimethylammonium bromide in 50 mol/L potassium phosphate buffer (pH 5.5), plus 0.026% orthodianisidine dihydrochloride substrate and 0.018% H_2O_2. The reaction kinetics were followed for 30 min at 450 nm in 96-well plates. The specificity of the reaction was checked with sodium azide (0.1 mmol/L). All samples were analyzed in duplicate and standardized using a homogenate of pooled human neutrophils, and MPO activity was expressed in arbitrary units (U/mg protein). The enzymatic activity of caspase-3 in tissue samples was measured as described previously[14]. Five 10 μm cryostat sections of tissues were suspended in a lysis buffer consisting of 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0), 40 mmol/L β-glycerophosphate, 50 mmol/L NaCl, 2 mmol/L $MgCl_2$, and 5 mmol/L ethylene glycol tetraacetic acid. After 10 min on ice, the cells were disrupted by 10 s of sonification followed by four cycles of freezing and thawing and stored at -80℃. Protein concentration was determined using the method described by Bradford[15]. For measurement of caspase-3 enzymatic activity, samples containing 15 μg protein were incubated with 2.5 nmol of the enzyme substrates DEVD-AMC (7-Amino-4-methylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-l-aspartic acid amide) in a 100 mmol/L HEPES buffer (pH 7.25) containing 10% (w/v) sucrose, 0.1% (v/v) NP40, and 10 mmol/L DL-Dithiothreitol. During incubation at 37℃, fluorescent AMC was cleaved off by active caspases, corresponding with the level of caspase activity in the sample. The fluorescent AMC was monitored at an excitation of 360 nm and emission of 460 nm using a FLUO star Optima plate reader. Calibration curves were constructed using free AMC. Caspase-3 activity was measured as pmolAMC/min per mg protein. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined according to Reitman and Frankel[16], whereas alkaline phosphatase (ALP) activity was estimated by the Belfield method[17]. Total bilirubin, and γ-glutamyl transferase (GGT) were determined using Diamond Diagnostic Kit as reported[18]. The results were reported as mean and standard deviation.

Histopathological examination

Liver and a 2-cm segment of terminal ileum samples were obtained at the final laparotomy. The bowel was stripped from its mesentery and the segment was opened along its length and rinsed in a cold solution. Specimens were fixed in 10% formalin in 0.15 mol/L phosphate buffer (pH 7.2), embedded in paraffin and then sections measuring 5 μm in thickness were cut. The specimens were stained with hematoxylin-eosin and examined under the light microscope (Olympus BX50, Japan). Histopathological examination was performed by an experienced pathologist who was not aware of the sample group. Ductular proliferation in liver samples was examined according to the modified scoring system used by Sheen-Chen *et al*[3]. This system, assessing ductular proliferation with seven different scores, was reduced to three scores since it was more appropriate for statistical assessment and number of subjects was fewer. Grade 1 (mild): Portal area involvement less than 50%; Grade 2 (moderate): Portal area involvement more than 50% or expansion of the portal tract; Grade 3 (severe): Presence of bridging in portal tracts. Apoptosis was also assessed in liver samples and reported as present or absent. Villus structures (villus length and width), lymphatic dilatation and sub-epithelial edema were examined to investigate mucosal damage in terminal ileum sections and reported as present or absent.

Statistical analysis

Findings obtained in the study were assessed using SPSS (Statistical Package for Social Sciences) for Windows 15.0 program. One-way Anova test was used in comparing parameters between groups and Tukey's HDS (Honestly Significant Difference) test was used in detecting the group causing variation when comparing qualitative data. Kruskal Wallis test was used in comparing parameters displaying abnormal distribution between groups and Mann Whitney *U* test was used in detecting the group causing variation. For comparison of qualitative data, χ^2 or Fisher exact test was used. Results were calculated as mean ± SD. $P < 0.05$ values were considered statistically significant.

RESULTS

Jaundice became apparent in all subjects on postoperative

Table 1 Biochemical results

	mean ± SD			*P*		
	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅰ-Ⅱ	Group Ⅰ-Ⅲ	Group Ⅱ-Ⅲ
T-Bil bilirubin	7.814 ± 0.855	7.303 ± 1.059	7.456 ± 1.037	> 0.05	> 0.05	> 0.05
ALT	285.714 ± 6.945	203.157 ± 5.777	230.5 ± 7.368	0.002	0.001	0.001
AST	277.571 ± 8.6	188.714 ± 5.908	227.25 ± 7.573	0.002	0.001	0.001
ALP	417.714 ± 13.865	373.714 ± 9.322	353.938 ± 141.502	0.002	0.042	0.015
GGT	410.429 ± 5.711	395.857 ± 4.337	402.375 ± 4.926	0.002	0.010	0.040
IL-6	1.087 ± 0.117	0.627 ± 0.147	0.984 ± 0.123	0.002	0.063	0.001
TNF-α	1.346 ± 0.21	0.53 ± 0.089	0.869 ± 0.104	0.002	0.002	0.001
MPO	0.537 ± 0.01	0.309 ± 0.038	0.468 ± 0.009	0.002	0.001	0.001
NO	149.286 ± 9.499	101.171 ± 3.396	135.625 ± 5.181	0.002	0.009	0.001
MDA	2.743 ± 0.196	1.753 ± 0.227	2.3 ± 0.379	0.002	0.011	0.015
CAS	35.741 ± 0.848	22.929 ± 1.533	30.445 ± 1.463	0.002	0.001	0.001

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; IL-6: interleukin-6; TNF-α: Tumor necrosis factor-α; MPO: Myeloperoxidase; NO: Nitric oxide; MDA: Malondialdehyde; CAS: Caspase 3 activity.

day 3. Two of the rats (one in Group Ⅰ and one in Group Ⅱ) died during the experiment. The experiment was completed with 7 rats in Group Ⅰ, 7 rats in Group Ⅱ and 8 rats in Group Ⅲ.

Microbiological findings

Significant microbial growth was investigated in blood obtained by intracardiac puncture, samples of mesenteric lymph nodes, peritoneal fluid, and terminal ileum tissue. *Escherichia coli* (*E. coli*) was the most common bacteria detected (34%) among all positive cultures. Other detected bacteria were identified as *Enterococci, Klebsiella oxytoca, Streptococcus* spp and *Klebsiella pneumoniae*. Positive blood cultures were detected in 6 of 7 animals (85%) in Group Ⅰ, in 1 of 7 animals (14%) in Group Ⅱ, and in 2 of 8 animals (25%) in Group Ⅲ. The rates in groups Ⅱ and Ⅲ were determined to be significantly less than that of group Ⅰ (P = 0.029 and P = 0.041, respectively). The difference between Groups Ⅱ and Ⅲ was not statistically significant (P > 0.05). Positive cultures of mesenteric lymph node samples were detected in all animals (100%) in Group Ⅰ, in 3 of 7 animals (42%) in Group Ⅱ and in 7 of 8 animals (87%) in Group Ⅲ. There was no statistically significant difference among groups although positive cultures were fewer in Group Ⅱ. Positive cultures of peritoneal fluids were detected in 4 of 7 animals (57%) in Group Ⅰ, none of 7 animals (0%) in Group Ⅱ and in 4 of 8 animals (50%) in Group Ⅲ. These results were not statistically significantly different between groups (P > 0.05). Significant pathogens in terminal ileum samples were detected in all animals (100%) in Group Ⅰ, in 5 of 7 animals (71%) in Group Ⅱ and in 7 of 8 animals (87%) in Group Ⅲ. These results were not statistically significant either (P > 0.05).

Biochemical findings

ALT, AST, ALP, GGT, total bilirubin, IL-6 and TNF-α levels were measured in blood samples. MPO, NO, MDA levels and caspase-3 activity were measured in terminal ileum samples (Table 1). There was no significant difference in terms of serum total bilirubin values among groups and obstructive jaundice was detected in all subjects. ALT and AST levels, markers of hepatocellular damage, in Group Ⅱ were found to be significantly reduced compared to Group Ⅰ and Group Ⅲ (P = 0.002 and P = 0.001, respectively). Furthermore, these enzyme levels were found to be significantly reduced in Group Ⅲ when compared with Group Ⅰ (P = 0.001). ALP and GGT levels, markers of cholestasis, were found to be significantly lower (P < 0.05) in Groups Ⅲ and Ⅱ than in Group Ⅰ. When the results of Groups Ⅱ and Ⅲ were compared, ALP levels were lower in Group Ⅱ; whereas, GGT was found to be lower in Group Ⅲ and these results were statistically significant (P < 0.05). TNF-α levels were detected to be significantly decreased in Group Ⅱ when compared with Group Ⅰ and Group Ⅱ (P = 0.001 and P = 0.002, respectively). TNF-α levels detected in Group Ⅲ were also significantly lower than that of Group Ⅰ (P = 0.002). IL-6 levels detected in Group Ⅱ were significantly less than those in Group Ⅰ and Group Ⅲ (P = 0.002 and P = 0.001, respectively). Although the results were lower in Group Ⅲ they were not statistically significant when compared with Group Ⅰ (P > 0.05). MPO levels, a marker of tissue inflammation and neutrophil sequestration, were found to be significantly lower in Group Ⅱ than the other groups (P = 0.002 for Group Ⅰ and P = 0.001 for Group Ⅲ). MPO levels detected in Group Ⅲ were also lower than that of Group Ⅰ, which was statistically significant (P = 0.001). NO levels, an indicator of oxidative damage and MDA, an end product of lipid peroxidation and an index of oxidative stress, were determined to be significantly lower in Group Ⅱ than the other groups (P < 0.05). Additionally, values detected in Group Ⅲ were lower than those of Group Ⅰ and this was also statistically significant (P < 0.05). Caspase-3 activity, an apoptosis marker, was found to be significantly lower in Group Ⅱ than the other groups (P = 0.002 for Group Ⅰ and P = 0.001 for Group Ⅲ). The value detected in Group Ⅲ was lower than that of Group Ⅰ and this was also statistically significant (P = 0.001).

Histopathological findings

Ductal proliferation and apoptosis rates were measured

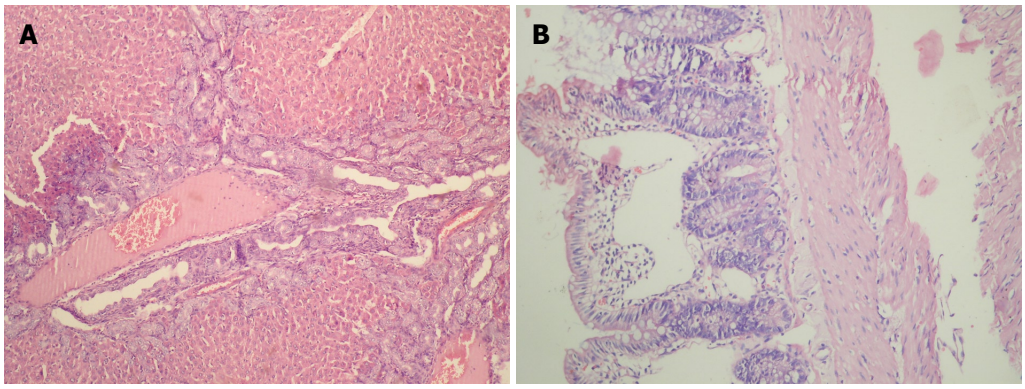

Figure 1 Histopathological view of liver and terminal ileum. A: Liver of a jaundiced rat, bridging of portal tracts, severe ductal proliferation, × 200; B: Terminal ileum of a jaundiced rat, shortening of villus length and severe lymphatic dilatation, × 200.

in the histopathological evaluation of liver sections (Figure 1A). Regarding the former, 14% mild, 57% moderate and 29% severe ductal proliferation was detected in Group Ⅰ, 43% mild, 14% moderate and 43% severe ductal proliferation was detected in Group Ⅱ and 38% mild and 62% severe ductal proliferation was detected in Group Ⅲ. Rates of apoptosis were determined to be 42% in Group Ⅰ and Group Ⅱ and 62% in Group Ⅲ. No significant difference in terms of either ductal proliferation or apoptosis was found when the results of histopathological examination of liver samples were statistically compared among groups ($P > 0.05$). Villus height and width, lymphatic dilatation and subepithelial edema were evaluated in terminal ileum sections. When shortening in villus height was compared among groups, shortening in Group Ⅱ was significantly less than Group Ⅰ ($P = 0.02$). Shortening observed in Group Ⅱ was less than that of Group Ⅲ and shortening determined in Group Ⅲ was less than that of Group Ⅰ but these differences were not statistically significant ($P > 0.05$). In the comparison of villus width, no significant difference was detected among groups, although it was found less frequently in Group Ⅱ ($P > 0.05$). Degree of lymphatic dilatation was observed to be significantly lower in Group Ⅱ than that of Group Ⅰ ($P = 0.02$). Lymphatic dilatation observed in Group Ⅱ was less than that of Group Ⅲ but this was not statistically significant. Lymphatic dilatation determined in Group Ⅲ was less than that of Group Ⅰ, but again this was not statistically significant ($P > 0.05$). No significant difference among groups in terms of subepithelial edema was detected ($P > 0.05$).

DISCUSSION

Bacterial translocation is the passage of bacteria or endotoxins from the gastrointestinal tract to extraintestinal sites, such as mesenteric lymph nodes, liver, spleen, and/or bloodstream. In a normal, healthy individual, gut-originated bacteremia and sepsis do not occur because the host has multiple defense mechanisms to prevent the bacteria and their products from crossing the mucosal barrier and spreading to systemic tissues. Under certain experimental and clinical circumstances, this intestinal barrier function becomes overwhelmed or impaired, resulting in bacterial translocation[19-21]. Current advances in the pathophysiology of intestinal failure in obstructive jaundice have shown that the breakdown of the gut barrier is multifactorial, involving disruption of immunologic, biological, mechanical, and biochemical barriers. Berg and Garlington[22] defined bacterial translocation as the passage of viable enteric bacteria through intestinal epithelial cells into the lamina propria and then to mesenteric lymph nodes, and possibly other tissues. Bacterial translocation was first defined as the passage of viable bacteria. However, at the present time either bacterial fragments or the translocation of bacterial products such as endotoxins from dead bacteria have been known to stimulate the immune system[23]. Under normal conditions, the gastrointestinal system keeps bacterial content in the intestinal lumen while absorbing nutrients. This is called "intestinal barrier function"[5]. Despite the number of bacteria present in the cecum, one cell beneath the mucosa is sterile. The intestinal barrier is both a functional and anatomical barrier to intestinal contents. The functional aspect is inherent in the modulation of tight junctional permeability and selective endocytosis of intestinal contents, while the anatomical barrier aspect is represented by the interconnected tight junctional/cell membrane system which effectively excludes large molecules and bacterial antigens. The first component of the intestinal barrier consists of intestinal microflora which have two functions, "bacterial antagonism" and "colonization resistance"[24,25]. The other components of the intestinal barrier are the physical barrier function of the mucosal epithelium, a mucus layer on the intestinal epithelium, blockade of epithelial adhesion sites by secreted IgA, and the preventive effects of intestinal peristalsis and intermittent desquamation of epithelial cells forming the mucosa. Despite these defense systems, translocated bacteria and bacterial products are neutralized in intestine-related lymphoid tissues (intraepithelial and lamina propria lymphocytes, lymphoid follicles, Peyer's patches and complexes of mesenteric lymph nodes), and the immune system (especially the reticuloendothelial system organs, such as the liver). In various studies bacterial translocation has been reported to develop in mechanical intestinal obstruction, hemorrhagic shock, sepsis,

endotoxemia, severe trauma, thermal injury, obstructive jaundice and cirrhosis[24,25]. The clinical importance of bacterial translocation was revealed in a study conducted by MacFie *et al*[26] in over 927 patients throughout 13 years. Bacterial translocation was detected in 130 patients (14%) in this study. Postoperative sepsis was seen more frequently (42.3% and 19.9%) in these patients. The authors reported ethical and methodological problems present in their study of bacterial translocation in humans and probably these problems are even bigger than described. According to our current knowledge, glutamine support, aggressive and targeted nutrition, adequate provision of visceral flow, appropriate use of antibiotics and selective intestinal decontamination are important objectives in restricting bacterial translocation[5].

Another important point in the pathophysiology of obstructive jaundice is the increased oxidative stress in the tissues. When the balance between the production of free oxygen radicals and antioxidant systems is impaired, oxidative stress leading to tissue damage occurs[26,27]. Increased intestinal oxidative stress which can cause intestinal damage and endotoxin translocation has been detected in rats with obstructive jaundice. In fact, obstructive jaundice causes oxidative stress in other organs such as the liver, kidneys, brain, heart and lungs. The development of tissue injury depends directly on the bile acids or occurs via macrophages; formation of oxygen free radicals as a result of systemic endotoxemia-induced xanthine oxidase, endotoxin-mediated systemic cytokine response, neutrophil chemotaxis of jaundice, increase of superoxide anion production and reduction of plasma levels of fat-soluble vitamins, particularly vitamin E, have been blamed for oxidative stress occurring in obstructive jaundice[28].

The effect of obstructive jaundice on bacterial translocation has been investigated in numerous clinical and experimental studies. Several changes have been shown to occur both in the intestinal barrier and in the reticuloendothelial system in obstructive jaundice. Bile and bile salts in the intestinal lumen are believed to have protective effects against bacterial translocation[23]. Luminal flow of bile salts has a regulatory effect on the intestinal flora and a direct detergent effect on endotoxins[25]. Furthermore, some trophic effects of pancreaticobiliary secretions on the intestinal mucosa have been identified[28]. Ogata *et al*[29] showed that oral administration of bile salts reduced absorption of endotoxin in rats with jaundice. Intestinal permeability increases with mucosal damage and as a consequence of changes in the intestinal flora a convenient media for bacterial translocation is prepared. Gatt *et al*[5] showed that reticuloendothelial system function was impaired subsequent to biliary tract obstruction and caused an increase in bacterial translocation. Reticuloendothelial system is defined as tissue macrophages. These are found chiefly in the liver, spleen, lung and bone marrow. They are responsible for cleaning up particulate materials such as bacteria, endotoxin, immune complexes and cell debris[3]. Kupffer cells in the liver are responsible for 80%-90% of the reticuloendothelial system activity. Assimakopoulos *et al*[28] detected increased intestinal oxidative stress which could cause intestinal damage and increased intestinal endotoxin translocation in an experimental model of rats with jaundice. Bacterial translocation and inflammatory responses in patients with obstructive jaundice were examined in a study performed by Ljungdahl *et al*[30] The results of this study which had few cases are as follows: bacterial translocation could not be detected in any of the patients. Elevated levels of preoperative endotoxin, TNF-α and IL-6 were measured in patients with jaundice. In these patients, the number of macrophages and apoptosis increased while T-lymphocyte count decreased in the mesenteric lymph nodes.

Treatment modalities and agents affecting the different pathophysiological steps described above have been investigated for many years, in order to reduce mortality and morbidity seen post-treatment or during the elapsed time until the definitive treatment of obstructive jaundice. Glutamine is one of the most important products surveyed for this purpose. Glutamine is the most common free amino acid in the body and has an important role in numerous metabolic events such as amino acid transport and nitrogen balance. It is the main food source of rapidly dividing cells such as enterocytes and lymphocytes. These cells have important roles in the intestinal mucosa barrier and the immune system. Numerous studies examining the effect of glutamine on bacterial translocation have been performed[6]. Glutamine reduces translocation not only by strengthening the intestinal barrier but also by reinforcing the immune system against bacteria and endotoxins successful in passing this barrier[3,6]. White *et al*[6] reported that glutamine regulated intestinal permeability, reduced bacterial translocation and even reinforced the immune system in rats with obstructive jaundice. Aldemir *et al*[7] showed that glutamine improved mucosal integrity and reduced bacterial translocation in the same model. In an experimental study performed by Margaritis *et al*[31], oral glutamine replacement was reported to reduce bacterial translocation, endotoxemia and apoptosis and to improve the ileal and liver histology in obstructive jaundice. Sheen-Chen *et al*[3] examined liver apoptosis in the obstructive jaundice rat model and determined that although glutamine replacement reduced liver apoptosis rate and ductal proliferation on day 3 of the experiment, the same effect could not be shown on day 7. In clinical studies, glutamine treatment was determined to have beneficial effects on bacterial translocation and sepsis. These effects can be listed as reduction in mucosal atrophy, rapid improvement in radiotherapy- and chemotherapy-induced mucosal damage, strengthening of intestinal and systemic immunity and decrease in length of hospital stay and infection rates in patients in intensive care. Use of glutamine before abdominal radiation has been shown to exert a protective effect on the intestinal mucosa, to increase intestinal glutamine metabolism and to decrease morbidity and mortality subsequent to total abdominal irradiation. Glutamine also shows this effect when administered after the radiation therapy. Glutamine also plays a critical role in synthesis of glutathione, a major antioxidant, which protects tis-

sues against free radical damage. The jejunal mucosal weight, DNA and nitrogen content increase and villus atrophy reduces significantly in glutamine-enriched total parenteral nutrition. Methotrexate-induced enterocolitis proceeds more slowly, and 5-fluorouracil-induced mucosal damage recovery occurs more rapidly, in patients fed on a glutamine-supplemented enteral diet[5,32]. In a study performed on rats by Kul *et al*[33] the positive effect of glutamine on oxidative stress both in a hypoxia-reoxygenation model and in healthy neonatal rats was reported. Glutamine supplementation has been suggested to prevent necrotizing enterocolitis in neonates.

In the present study, we examined the effects of glutamine and curcumin use on bacterial translocation and oxidative stress in rats with obstructive jaundice. Consistent with the literature, we detected the positive effects of glutamine use on these issues. Positive culture rates observed in the microbiological assays were less in all samples from the glutamine-treated group compared to those of the control and curcumin-treated groups. However, only the blood culture rates were statistically significant in comparison with the control group ($P = 0.029$). Moreover, shortening of villus height and lymphatic dilation were found to be significantly lower in the glutamine-treated group terminal ileum ($P < 0.05$).

Curcumin is a polyphenol derived from turmeric, which is used as a spice or herbal medicine. It is produced from the root of a plant, *Curcuma longa*. Dried roots of this plant have been used for thousands of years in Asian medicine[34]. Curcumin has been suggested to reduce inflammation which causes bacterial translocation by exhibiting an anti-inflammatory effect[9]. In the study performed by Shen *et al*[35], curcumin was shown to increase expression of antioxidant biomolecules and reduce neutrophil infiltration and reactive oxygen metabolites after ischemia-reperfusion injury in the liver. Treatment of rats with curcumin decreased total nitric oxide synthase activity after reperfusion. However, endothelial nitric oxide synthase activity was not affected. Moreover, curcumin has been shown to have positive effects on inflammatory damage and intestinal reperfusion injury in a recent experimental study by Karatepe *et al*[10].

In the present study, we examined the effects of curcumin on bacterial translocation and oxidative damage in an obstructive jaundice model and we compared it with glutamine, which is a reliable control and has been recently in clinical use. Microbiologically, positive culture rates were found to be less in all samples from the curcumin-treated group compared to those from the control group. However, only the rates of blood cultures were statistically significant ($P = 0.041$). No significant difference was observed when compared with the glutamine-treated group. All biochemical parameters (except ALP levels) of the glutamine-treated group were found to be lower in a statistically significant manner in comparison with those of the curcumin-treated group ($P < 0.05$). No statistically significant difference was detected in the histopathological examination of the samples obtained from the curcumin-treated group compared to the glutamine-treated and control groups ($P > 0.05$).

In conclusion, in the present study we detected positive effects of glutamine and curcumin on bacterial translocation and oxidative damage in rats with obstructive jaundice. Both glutamine and curcumin were observed to reduce bacterial translocation in blood, hepatocellular injury, serum cytokine levels, oxidative tissue damage and apoptosis rates significantly in comparison to the control group. However, more extensive comparative experimental and clinical studies are required before the clinical use of curcumin for this purpose and perhaps the combined use of glutamine with curcumin will be more effective.

COMMENTS

Background

Despite current modern diagnostic and therapeutic approaches, interventions in patients with biliary tract obstruction result in 10%-25% mortality and up to 56% major morbidity. Bacterial translocation and oxidative tissue damage have been emphasized as the leading cause of the complications in obstructive jaundice by numerous investigators.

Research frontiers

The antioxidant, anti-cancer, anti-inflammatory and cytoprotective effects of curcumin have been demonstrated by numerous experimental and clinical studies. Administration of glutamine has been shown to improve bacterial translocation and oxidative damage in obstructive jaundice but the exact role of curcumin in this issue is still unknown. In this study, the authors demonstrate that both curcumin and glutamine are effective in preventing or reducing bacterial translocation and oxidative damage in obstructive jaundice.

Innovations and breakthroughs

Obstructive jaundice causes alterations leading to bacterial translocation both in the intestinal barrier and in the reticuloendothelial system. Some of these alterations may be listed as mucosal damage in the intestinal lumen due to lack of bile, apoptosis, bacterial overgrowth, motility disorder associated with oxidative stress and functional abnormalities in the tissue macrophages. According to the authors' knowledge, a comprehensive and comparative study regarding the effect of curcumin on bacterial translocation and oxidative damage in obstructive jaundice has not been performed yet. Both glutamine and curcumin were observed to reduce bacterial translocation in blood, hepatocellular injury, serum cytokine levels, oxidative tissue damage and apoptosis rates significantly in comparison to the control group.

Applications

Curcumin can be used like glutamine in order to prevent bacterial translocation and oxidative damage observed in obstructive jaundice and to reduce mortality and morbidity observed in the elapsed time until definitive treatment or after treatment.

Terminology

Glutamine, a non-essential amino acid, has been reported not only to lower the rate of endotoxemia and translocation by preserving mucosal integrity but also to improve the action of the immune system against bacteria and endotoxins which succeed in passing the intestinal barrier. Curcumin is a polyphenol derived from the herbal remedy and dietary spice turmeric. The antioxidant, anti-cancer, anti-inflammatory and cytoprotective effects of curcumin have been demonstrated by numerous experimental and clinical studies.

Peer review

This is a well conceived and implemented experimental protocol which seeks to answer the question of the relative efficacy of glutamine and curcumin in the treatment or prevention of intestinal bacterial translocation in the setting of obstructive jaundice. The experimental protocol is well carried out and can be expected to extrapolate to human clinical situations in which obstructive jaundice is present.

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BRIEF ARTICLE

HCV genotype distribution and possible transmission risks in Lahore, Pakistan

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Abstract

AIM: To investigate the prevalence of hepatitis C virus (HCV) genotypes and their association with possible transmission routes in the general population of Lahore, as the data exclusively related to this city is limited.

METHODS: Complete data regarding patient's history, possible route of infection and biochemical tests was collected from the public hospital for 1364 patients. SPSS version 16 windows software was used for data analysis by univariate and multivariate techniques.

RESULTS: Age range ⩽ 40 years showed high prevalence of HCV infection. HCV genotype 3a was dominant (55.9%), followed by 1a (23.6%), 4a (12.5%), 3b (3.2%), untypable (2.5%), 4b (1.2%) and mixed type (1.2%). Blood transfusion, dental surgery and barber shops were the main risk factors for HCV transmission. Genotype prevalence was independent of age (P = 0.971) and gender (P = 0.122) while risk factors showed a significant association with age (P = 0.000) and genotypes (P = 0.000). We observed an independent association of risk factors and genotype 3a, while patients with genotype 1 and 4 were mostly infected due to dental surgery blood transfusion and barber shops. Risk factors of intravenous drug use and sexual exposure were exclusively found in ⩽ 40 years age group.

CONCLUSION: An increase in genotypes 1a and 4a suggest migration of people, possibly from Balochistan and the northern war-zone area. Government should focus on public education regarding infection routes.

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Key words: Hepatitis C virus; Prevalence; Genotypes; Risk factors; Lahore

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver asso-

ciated diseases all over the world. An estimated 3% of the world's populations are chronically infected by HCV which is the main cause of liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) in a substantial number of patients[1,2]. It is an enveloped virus with a single-stranded, positive sense non-segmented RNA genome of approximately 9.6 kb that encodes a poly-protein of approximately 3000 amino acids[3]. To date, at least six major genotypes of HCV, each having multiple subtypes, have been identified worldwide[4]. The different genotypes are relevant to epidemiology, vaccine development, and clinical management of chronic HCV infection[5].

Genotype distribution has been identified in three different patterns[6]. One pattern is genetic diversity, in geographically different areas like West Africa with 1 and 2[7], Central Africa with type 4[8], and Asia with type 3 and 6[9]. The second distribution pattern involves subtypes in specific risk groups e.g. intravenous drug use (IVDU), where the subtype 3a is more common[10]. The third pattern of genotype distribution is the circulation of a single subtype in particular areas such as in Egypt with 4a and South Africa with subtype 5a[11]. Recently, a shift in genotype distribution, mostly comprising an increase of the prevalence of the genotypes 3a, 1a and 4, and decrease of prevalence of other genotypes has been seen in many countries like Serbia, Germany, France and Greece[12-15].

Almost 10 million people in Pakistan are living with HCV. The most prevalent genotype in Pakistan is 3a followed by 3b and 1a[16]. Few studies are available on the distribution of various HCV genotypes in individual cities of Pakistan[17,18]. Unfortunately, there is no national data collection system for evaluation of infection routes and their correlation with genotypes or patients' demographic data[19]. Lahore is the second largest metropolitan city of Pakistan with more than 7 million population[20]. Data exclusively related to HCV genotype-specific prevalence and route of infection in this city is limited. Although, Ijaz *et al*[18] showed a high prevalence of HCV 3a genotype (n = 80, 51.61%) followed by 3b (n = 43, 27.74%), 1b (n = 21, 13.54%), untypable (n = 5, 3.22%), mix 3a1b (n = 4, 2.58%), 3a1a (n = 1, 0.64%) and 3b1a (n = 1, 0.64%) in the population of Lahore city, their study utilized a small population size (n = 155) without any association to the mode of transmission[18].

The aim of the present study was to determine the frequency distribution of HCV genotypes, various risk factors prevalence with genotypes and age for its transmission in Lahore.

MATERIALS AND METHODS

Patients

Patients in this study were from Jinnah Hospital, Lahore, which is the only public facility that has HCV patient's testing service and is the 2nd largest hospital in the area. Therefore, patients visiting at this hospital can be regarded as representative of general population of the Lahore city. The data and samples were collected from March 2007 to September 2009 from the Department of Pathology, Jinnah Hospital, Lahore, Pakistan and data analysis was performed in collaboration with National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. In total 1364 adult patients (18-75 years) who were HCV RNA-positive based on HCV antibody (anti-HCV)-positive results were included in this study. A written informed consent was obtained from patients. A complete history with possible route and estimated time of infection, standard biochemical liver function tests and patient's contact information were collected. This study was approved by the Institutional ethics committee.

Viral investigations

HCV detection and genotyping was performed at the Department of Pathology, Jinnah Hospital, Lahore, Pakistan. RNA was extracted from 140 μL of serum samples using QIAamp viral RNA extraction kit (Qiagen, USA) according to the manufacturer's protocol. cDNA was synthesized using Moloney murine leukemia virus (MmLV) followed by polymerase chain reaction (PCR) using primers derived from the 5'UTR non-coding region of HCV genome described by Chan *et al*[21]. For HCV RNA quantification, Qiagen HCV RG RT-PCR assay was used. Quantification was carried out with 10 μL of the extracted RNA on Rotor-gene Real-Time PCR machine (USA) using fluorescent probes to detect amplification after each replicating cycle as described by manufacturer protocol. The lower limit of detection for this assay is 1000 IU/mL HCV and genotyping was carried out using Invader HCV genotyping assay (Third wave technology, USA). Briefly, 100 ng of the HCV RNA was reverse transcribed to cDNA using 200 units of MmLV (Invitrogen, USA). From the amplified product, 2 μL were taken and the genotyping assay was performed for 12 different HCV types.

Statistical analysis

Data was analyzed using a statistical package SPSS version 16 for windows. The data is presented as mean and standard deviations, and categorical variables in absolute numbers and percentages. Student t-test and chi-square tests were applied to evaluate differences in proportions. A P value < 0.05 was considered significant. A multivariate analysis was used to identify variables associated within different genotypes. Bonferroni, Gabriel and LSD tests were performed to evaluate whether significant variables in the univariate analysis could predict differences among genotypes.

RESULTS

Age and gender specific prevalence of HCV

Of 1364 patients, 656 were male while 708 were female, with a median age of 36.8 ± 10.3 years (range 18-75 years). The age of infected patients was taken as a categorical as well as a continuous variable. Patients were divided in two age groups i.e. ≤ 40 years and > 40 years. Distribution of patients' gender in age groups is given in Table 1. It is clear from Table 1 that people of age group

Table 1 Patient data according to age and gender

Parameters	Age groups	
	⩽ 40 yr (*n* = 931, 68.3%)	> 40 yr (*n* = 433, 31.7%)
Gender		
Male (*n* = 656)	468 (50.2%)	188 (43.3%)
Female (*n* = 708)	463 (49.8%)	245 (56.7%)
Age (yr)		
mean ± SD (36.8 ± 10.3)	37.77 ± 10.65	46.8 ± 12.38
Age range (18-75)	18-40	41-75

Table 2 Univariate logistic analysis of genotypes involved in hepatitis C virus infection according to age and gender

Variables	Type Ⅲ sum of squares	υ	Mean square	*F* value	*P* value
Sex	150.641	1	150.641	2.396	0.122
Age groups	0.084	1	0.084	0.001	0.971

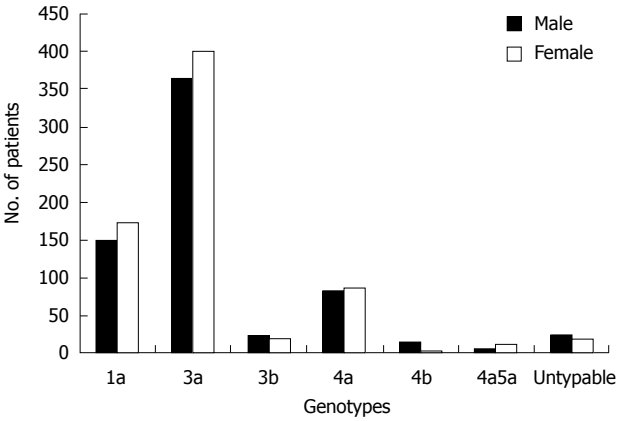

Figure 1 Frequency distribution of different genotypes in gender of patients. The prevalence of hepatitis C virus genotypes in gender was non-significant.

⩽ 40 years (*n* = 931, 68.3%) were more affected with HCV in comparison to those in the older age group (> 40 years, *n* = 433, 31.7%).

Genotype distribution

Based on weighted analysis of patients infected with HCV genotypes 1, 3, 4, mix (4a5a) and untypable genotypes, the most commonly detected genotype in the study was genotype 3 (*n* = 806, 59.1%), with predominant subtype 3a (*n* = 763, 55.9%) and 3b (*n* = 43, 3.2%). Genotype 1 (*n* = 322, 23.6%,) was exclusively consisted of the subtype 1a, while genotype 4 (*n* = 186, 13.7%) comprised of the subtypes a (*n* = 170, 12.5%) and b (*n* = 16, 1.2%). Mixed inter-genotype 4a5a (*n* = 16, 1.2%) and untypable (*n* = 34, 2.5%) genotypes were also detected in 50 (3.66%) patients. The frequency distribution of genotypes revealed that patients of the age group ⩽ 40 years were more affected with genotype 1a (71%) and 4a (68%) as compared to patients with the age group > 40 years. However, univariate analysis (Table 2) revealed

Table 3 Hepatitis C virus genotype subtypes prevalence in age groups

Genotype subtypes	Computation	Age groups	
		⩽ 40 yr	> 40 yr
1a	Count	230	92
	% within age groups	24.7%	21.2%
3a	Count	507	256
	% within age groups	54.5%	59.1%
3b	Count	32	11
	% within age groups	3.4%	2.5%
4a	Count	116	54
	% within age groups	12.5%	12.5%
4b	Count	13	3
	% within age groups	1.4%	0.7%
4&5	Count	11	5
	% within age groups	1.2%	1.2%
Untypable genotype	Count	22	12
	% within age groups	2.4%	2.8%

Table 4 Univariate logistic analysis of risk factors involved in hepatitis C virus infection according to age, gender and genotypes

Variables	Type Ⅲ sum of squares	υ	Mean square	*F* value	*P* value
Sex	0.014	1	0.014	0.004	0.950
Age	131.744	1	131.744	38.219	0.000
Genotype	457.878	6	76.313	22.139	0.000

that prevalence of genotype subtypes within age groups (*P* = 0.971) and gender (*P* = 0.122) of the patients were not statistically significant. Figure 1 illustrated the genotype distribution pattern in each gender of patients while Table 3 showed the prevalence of different genotype subtypes in different age groups.

Risk assessment for HCV infection

The possible routes of infection were determined by detailed questionnaire. Out of 1364 patients, the possible risk factors for 1183 (86.7%) patients were established, while 13.3% (*n* = 181) were unaware of their possible route of infection. The major route of HCV infection among patients resides within Lahore city was dental surgery (33.5%), followed by blood transfusion (22.6%), barber shops (12.4%), road accidents (8.4%), sexual exposure (5.9%) and IVDU (3.73%). Statistical analysis illustrated in Table 4 showed that risk factors were dependent on age (*P* = 0.000) and genotype (*P* = 0.000) and independent of gender (*P* = 0.950).

Age-specific distribution of the risk factors

The frequency distribution of different risk factors within age groups is illustrated in Table 5. Dental surgery was the cause of HCV infection in 458 of the 1364 patients (33.6%), and was found more frequently among patients ⩽ 40 years (71%) than patients with age > 40 years. Infection due to blood transfusion, road accidents and barber shops was also prevalent in patients ⩽ 40 years of age

Table 5 Distribution and multivariate analysis of risk factors in age groups

Risk factors	Computation	Age groups		95% CI		*P* value
		≤ 40 yr	> 40 yr	Lower	Upper	
Blood transfusion	Count	222	88	2.077	4.467	0.039
	% within age groups	23.8%	20.3%			
IVDU	Count	51	0	6.414×10^9	6.414×10^9	0.000
	% within age groups	5.5%	0.0%			
Sexual exposure	Count	79	0	9.014×10^9	9.014×10^9	0.000
	% within age groups	8.5%	0.0%			
Dental surgery	Count	325	133	2.068	4.209	0.610
	% within age groups	34.9%	30.7%			
Road accidents	Count	77	39	1.551	2.090	0.148
	% within age groups	8.3%	9.0%			
Barber shops	Count	95	74	1.016	2.363	0.042
	% within age groups	10.2%	17.1%			

IVDU: Intravenous drug use.

Table 6 Hepatitis C virus genotype prevalence within risk factors in Lahore - March 2007 - September 2009 (*n* = 1364) *n* (%)

Groups	Total No.	1a	3a	3b	4a	4b	Mix	Untypable
BT	310	51 (16.5)	202 (65.2)	13 (4.2)	23 (7.4)	4 (1.3)	4 (1.3)	13 (4.2)
≤ 40 yr	222	39 (17.6)	131 (59.0)	10 (4.5)	22 (9.9)	3 (1.4)	4 (1.8)	13 (5.9)
> 40 yr	88	12 (13.6)	71 (80.7)	3 (3.4)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)
IVDU	51	12 (23.5)	34 (66.7)	0 (0.0)	4 (7.8)	0 (0.0)	0 (0.0)	1 (2.0)
≤ 40 yr	51	12 (23.5)	34 (66.7)	0 (0.0)	4 (7.8)	0 (0.0)	0 (0.0)	1 (2.0)
> 40 yr	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
SE	79	14 (17.7)	53 (67.1)	0 (0.0)	10 (12.7)	1 (1.3)	0 (0.0)	1 (1.3)
≤ 40 yr	79	14 (17.7)	53 (67.1)	0 (0.0)	10 (12.7)	1 (1.3)	0 (0.0)	1 (1.3)
> 40 yr	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
DS	458	85 (18.6)	285 (62.2)	13 (2.8)	62 (13.5)	5 (1.1)	4 (0.9)	4 (0.9)
≤ 40 yr	325	69 (21.2)	173 (53.2)	13 (4.0)	58 (17.8)	5 (1.5)	3 (0.9)	4 (1.2)
> 40 yr	133	16 (12.0)	112 (84.2)	0 (0.0)	4 (3.0)	0 (0.0)	1 (0.8)	0 (0.0)
RA	116	30 (25.9)	68 (58.6)	4 (3.4)	11 (9.5)	1 (0.9)	1 (0.9)	1 (0.9)
≤ 40 yr	77	30 (39.0)	46 (59.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.3)
> 40 yr	39	0 (0.0)	22 (56.4)	4 (10.3)	11 (28.2)	1 (2.6)	1 (2.6)	0 (0.0)
BS	169	45 (26.6)	88 (52.1)	7 (4.1)	19 (11.2)	1 (0.6)	5 (3.0)	4 (2.4)
≤ 40 yr	95	19 (20.0)	64 (67.4)	7 (7.4)	0 (0.0)	0 (0.0)	3 (3.2)	2 (2.1)
> 40 yr	74	26 (35.1)	24 (32.4)	0 (0.0)	19 (25.7)	1 (1.4)	2 (2.7)	2 (2.7)
UN	181	85 (47.0)	33 (18.2)	6 (3.3)	41 (22.7)	4 (2.2)	2 (1.1)	10 (5.5)
≤ 40 yr	82	47 (57.3)	6 (7.3)	2 (2.4)	22 (26.8)	4 (4.9)	1 (1.2)	0 (0.0)
> 40 yr	99	38 (38.4)	27 (27.3)	4 (4.0)	19 (19.2)	0 (0.0)	1 (1.0)	10 (10.1)
Total	1364	322 (23.6)	763 (55.9)	43 (3.2)	170 (12.5)	16 (1.2)	16 (1.2)	34 (2.5)

BT: Blood transfusion; IVDU: Intravenous drug use; SE: Sexual exposure; DS: Dental surgery; RA: Road accidents; BS: Barber shops; UN: Unknown reasons.

(71.6%, 66.4% and 56.2%, respectively). Infection due to sexual exposure and IVDU was only observed among patients ≤ 40 years age. Our data revealed that infection due to dental surgery was more prevalent in both age groups (age group ≤ 40 years, 34.9%, *n* = 325; age group > 40 years, 30.7%, *n* = 133). The second most prevalent infection route in age groups was blood transfusion (age group ≤ 40 years, 23.8%, *n* = 222; age group > 40 years, 20.3%, *n* = 88), followed by barber shops (age group ≤ 40 years, 10.2%, *n* = 95; age group > 40 years, 17.1%, *n* = 74) and road accidents (age group ≤ 40 years, 8.3%, *n* = 77; age group > 40 years, 9.0%, *n* = 39). Multivariate analysis showed a significant association of IVDU (*P* = 0.000) and sexual exposure (*P* = 0.000) with age ≤ 40 years (Table 5) whereas route of infection due to dental surgery, blood transfusion, barber shops and road accidents was independent of age groups. Route of infection due to sexual exposure (*n* = 79, 8.5%) and IVDU (*n* = 51, 5.5%) was only observed in patients ≤ 40 years.

Prevalence of genotypes within risk factors

As risk factors showed a significant association with genotypes, the frequency distribution of various genotypes within risk groups (Table 6) revealed that subtypes 3a (65.2%) and 1a (16.5%) were found more frequently than subtypes 3b (4.2%), 4a (7.4%), 4b (1.3%), mixed (1.3%) and untypable (4.2%) in patients with a past history of blood transfusion. Moreover, subtypes 3a (62.2%), 1a (18.6%) and 4a (13.5%) were observed more frequently than subtypes 3b (2.8%), 4b (1.1%), mixed (0.9%) and un-

typable (0.9%) in patients infected due to dental surgery. In patients infected through barber shops and road accidents, genotype 3a (52.1% and 58.6%) and 1a (26.6% and 25.9%) were more prevalent than subtypes 3b (4.1% and 3.4%), 4a (11.2% and 9.5%), 4b (0.6% and 0.9%), mixed (3.0% and 0.9%) and untypable (2.4% and 0.9%) respectively. Patients with history of IVDU were mainly infected with genotype 3a (66.7%) and 1a (23.5%), while infection due to sexual exposure was predominantly genotype 3a (67.1%) followed by 1a (17.7%) and 4a (12.7%). These findings indicated that genotype subtype 3a is significantly dominant among all risk groups followed by 1a and 4a. Moreover our results revealed that the genotype with the highest frequency for risk factors is genotype 3a, the highest being in patients with a history of sexual exposure (53, 67.1%) followed by IVDU (34, 66.7%) and blood transfusion (65.2%). The second most frequent genotype was 1a with the highest frequency in patients possibly infected from barber shops (26.6%), subsequently; genotype 4a was the most frequent in patients experienced the dental surgery (13.5%). Patients (n = 5) with mixed (4a5a) and untypable genotypes (n = 13) were under risk due to barber shops and blood transfusion respectively.

Association of HCV risk factors with age and genotype

In patients infected due to blood transfusion, genotype 3a was more prevalent in age group > 40 years (80.7%) than ⩽ 40 years (59%), while genotype 4a was more prevalent in age group ⩽ 40 years (9.9%) than > 40 years (1.1%) (Table 6). The same prevalence order was observed in patients infected due to dental surgery where genotypes 1a (21.2%) and 4a (17.8%) were more prevalent in the age group ⩽ 40 years than the age group > 40 years (12.0% and 3.0%, respectively) and genotype 3a was predominant in the age group > 40 years (84.2%) than the age group ⩽ 40 years (53.2%). In patients infected due to barber shops, the prevalence of genotype 1a was higher in the age group > 40 years (35.1%) than the age group ⩽ 40 years (20.0%), while genotype 3a was most prevalent in age group ⩽ 40 years (67.4%) compared with age group > 40 years (32.4%).

DISCUSSION

HCV is an important cause of chronic liver disease and cirrhosis in Pakistan and accounts for significant morbidity and mortality. It is estimated that about 6% (10 million) of the Pakistani population is infected with HCV[19]. In the present study, we analyzed the relationship between distribution of HCV genotypes and mode of transmission of infection in different age groups and gender. Our results showed a non-significant prevalence of HCV distribution in gender and age which are in accordance to studies carried out by other groups who observed no relation between age, gender and HCV distribution[22,23]. When results were further analyzed keeping age as a categorical variable, people ⩽ 40 years of age were more affected with HCV in comparison to those > 40 years of age in Lahore. These results are in conflict with previous data from Muhammad *et al*[24] in 2005 showing that the prevalence of HCV in Pakistan is greater in old age, however, our results agreed with the findings of Ali *et al*[25] that HCV prevalence observed was highest among an age group of 13-50 years and similar findings were observed by Shah *et al*[26]. This could be due to an increasing awareness and early diagnosis of HCV in urban area communities in Pakistan.

HCV shows considerable sequence diversity and sequence comparisons in different parts of the genome. This has led to the classification of the virus into a series of genotypes that show distinct geographical distribution across the world[27]. Genotyping is important because it provides information as to strain variation and potential association with disease severity and a guide about treatment duration and outcomes[28]. Our finding indicating a high prevalence of genotype 3a (55.9%) in Lahore city is consistent with others describing HCV 3a prevalence in Pakistan[17,18], however, an increased prevalence of genotype 1a and 4a (23.6% and 12.5%) found in our study is significantly higher than the previously reported HCV prevalence for these genotypes. Idress *et al*[17] in 2008 showed that the prevalence of HCV infection due to genotype 4 and 1 is increasing without an increase in the frequency of genotype 3 in various areas of Pakistan mainly NWFP (1a, 6.56% and 4, 2.30%) and Balochistan (1a, 25.80% and 4, 4.03%). They claimed the appearance of genotype 4 in Pakistan for the first time with 1.15% prevalence in the Punjab area which includes Lahore city. A shift in genotype distribution, mostly comprising an increase in the prevalence of the genotypes 3a, 1a and 4, and decrease in prevalence of other genotypes, is also found in many countries largely due to migration[12,23]. Our results indicated an increased prevalence of genotype 4 and 1 in Lahore city may suggest a recent migration of people, possibly from Balochistan and northern areas (active terrorist war-zone area). Although there is evidence of increasing population size for Lahore, recent reports highlighting migration data to the city of Lahore are not available[29]. These results suggest a possible disaster in HCV management for Pakistan in coming years due to poor response against current therapy for genotypes 4 and 1. Furthermore, genotype 4 is reported to be associated with liver cirrhosis[30], therefore, it may potentially lead to an increased risk of liver cirrhosis in Pakistan. The absence of genotype 1b, 2a and 2b in our study indicates that these are rarely present in our population. A similar frequency distribution pattern of genotypes in neighboring countries like India and Iran has been observed where genotype 3 is most prevalent and genotype 2 is very rare[22,31].

As described earlier there is no national database for collection of risk factors involved in HCV transmission, however, studies from different areas of Pakistan revealed that IVDU due to excessive use of unnecessary injections and reuse of needles, dental surgery by using unhygienic practice and unsafe blood transfusion are the major causes of HCV infection[19,32-35], while HCV transmission due to

sexual exposure and barber shaving was also reported in Pakistan[36,37]. Kuo *et al*[38] reported 93% HCV prevalence among IVDUs in Lahore. Although IVDU has been identified as a route of infection in Lahore, it was not significant in our HCV population due to denial by a large number of people. Our results indicate dental surgery to be a leading cause of HCV transmission followed by unsafe blood transfusion. Our results are also in agreement with the results from other countries reporting blood transfusion, unsafe injection, barber shops and dental instruments as the main routes of HCV infection[39].

Data analysis for finding an association of various HCV genotypes with possible routes of transmission indicated blood transfusion and dental surgery to be the leading cause in all genotypes while infection due to IVDU and sexual exposure was predominant in genotype 1 and 3. In the present study, genotype 3 had a high prevalence in patients with a history of blood transfusion and IVDU, similar to USA and Europe[19,34] while genotype 1 was most prevalent in patients infected from barber shops. Genotype 4 has been reported to be related with different routes of infection such as dental surgery, dialysis, barber shops[19,40] and we observed dental surgery as a major route of infection in genotype 4 followed by sexual exposure. Barber shops and blood transfusion were observed as possible routes of infection in mixed and untypable genotype, respectively. We observed a notable variation in the distribution of HCV subtypes in different risk groups within age groups. As patients infected due to blood transfusion and dental surgery were more frequent (57%) than infection from other risk factors, genotype 3a was more prevalent in the age group > 40 years, while genotype 1a and 4a were observed in the age group ≤ 40 years. These findings conclude 3a is the original subtype present in Lahore while the others were introduced at a later date by an increase in population movements like migration and change in mode of risk factors. A high prevalence of genotype 1a and 4a in the age group ≤ 40 years can confirm this epidemiological shift in new generation.

Our results also revealed that the routes of HCV transmission were significantly associated with age groups while risk factors like dental surgery, blood transfusion and road accidents were evenly distributed in both age groups. Sexual exposure was reported in 79 patients and all of them belonged to the age group ≤ 40 years. The major reason for infection due to sexual exposure was non-awareness of people about sexually transmitted diseases and low use of condoms[41]. However, sexual activity cannot be applied as an independent risk factor as Tong *et al*[42] suggested low transmission of HCV among spouses. Blood transfusion, the most commonly recognized transmission mechanism of HCV, showed a high prevalence (71.6%) for patients ≤ 40 years. This could be due to many reasons - mainly concealing HCV status of donors from their relatives during blood donation and improper screening of blood donors[43]. HCV prevalence due to barber shops was also significantly higher (56.2%) in patients ≤ 40 years. The possible reason could be due to the use of inadequately sterilized instruments, reuse of razors and other shaving kits and transfusion of contaminated blood also seen by others in Pakistan[31,35-37]. The use of illegal drugs by injection is rising especially in young people in Pakistan as the effect of IDVU is more satisfying and intense[19,33]. Although the number of patients with IDVU was small, 100% IVDU patients belonged to the age group ≤ 40 years.

In conclusion, this study used a large HCV population for the first time and reported the HCV genotype-specific prevalence with age and possible risk factors associated with its transmission in Lahore city of Pakistan. Genotype 3 was predominant and patients infected with genotype 4 are increasing along with genotype 1 due to possible inland migration in Pakistan. We observed a maximum prevalence for HCV in the age group ≤ 40 years (68.3%) with involvement of multiple routes of infections. Infection due to sexual exposure and IVDU was exclusively linked to ≤ 40 years age group. Our study revealed that at least 66% of HCV infections are associated with the health-care sector as most of the patients infected with HCV developed the infection as a result of blood transfusion, dental surgery and IVDU. For genotype 3, blood transfusion was the major infection route, while infection attributed to barber shops was associated with genotype 1. The most prevalent risk factor for genotype 4 was found to be dental surgery. This study also highlights future possible HCV disease complications due to an increase in genotypes 4 and 1 and also provides a basis for the government of Pakistan to implement higher health standards.

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COMMENTS

Background

Chronic hepatitis C virus (HCV) is one of the major causes of liver fibrosis, with distortion of the hepatic architecture and ultimate progression to cirrhosis. To date, at least six major genotypes of HCV, each having multiple subtypes, have been identified worldwide. The different genotypes are relevant to epidemiology, route of infection, vaccine development, and clinical management of chronic HCV infection.

Research frontiers

In Pakistan, more than 10 million people are infected with chronic HCV. The exact data about the relationship between HCV genotypes, epidemiological factors and route of infection is limited.

Innovations and breakthroughs

The frequency distribution of different HCV genotypes and subtypes and their association with various risk factors in Lahore, the second largest city of Pakistan was investigated in 1364 patients with chronic HCV. The maximum prevalence of HCV was found in the age group ≤ 40 years (68.3%). Genotype 3a was the predominant genotype followed by an increase in 1a and 4a as compared to previous reports suggesting a recent migration of people possibly from Balochistan and northern areas (active terrorist war-zone area). The main risk factors observed in patients infected with HCV were blood transfusion, dental surgery and intravenous drug use. Blood transfusion and barber shops were the major infection route, in genotype 3a and 1a respectively.

Applications

This article reported the prevalence of HCV genotypes in Lahore city. Additionally the relationship between different genotypes and subtypes with age, gender and different possible routes of infection was studied. Shifts in HCV genotype distribution needs to be paid more attention as genotype 1a and 4a are associated with severe cirrhosis.

Peer review

This article describes the prevalence of HCV in Lahore population. It is a good work and should be accepted for publication.

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BRIEF ARTICLE

Associated factors for a hyperechogenic pancreas on endoscopic ultrasound

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Abstract

AIM: To identify the associated risk factors for hyperechogenic pancreas (HP) which may be observed on endoscopic ultrasound (EUS) and to assess the relationship between HP and obesity.

METHODS: From January 2007 to December 2007, we prospectively enrolled 524 consecutive adults who were scheduled to undergo EUS. Patients with a history of pancreatic disease or with hepatobiliary or advanced gastrointestinal cancer were excluded. Finally, 284 patients were included in the analyses. We further analyzed the risk of HP according to the categories of visceral adipose tissue (VAT) and subcutaneous adipose tissue in 132 patients who underwent abdominal computed tomography scans.

RESULTS: On univariate analysis, age older than 60 years, obesity (body mass index > 25 kg/m^2), fatty liver, diabetes mellitus, hypertension and hypercholesterolemia were identified as risk factors associated with HP (P < 0.05). On multivariate analysis, fatty liver [P = 0.008, odds ratio (OR) = 2.219], male gender (P = 0.013, OR = 2.636), age older than 60 years (P = 0.001, OR = 2.874) and hypertension (P = 0.044, OR = 2.037) were significantly associated with HP. In the subgroup analysis, VAT was a statistically significant risk factor for HP (P = 0.010, OR = 5.665, lowest quartile *vs* highest quartile).

CONCLUSION: HP observed on EUS was associated with fatty liver, male gender, age older than 60 years, hypertension and VAT.

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Key words: Endoscopic Ultrasound; Hyperechogenic pancreas; Obesity

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INTRODUCTION

Endoscopic ultrasound (EUS) has been an important tool for diagnosing gastrointestinal and pancreatobiliary disease since the 1980s[1]. EUS is particularly effective for evaluating patients with pancreatic disease, because EUS provides high resolution images of the pancreatic duct as well as the parenchyma. In recent years, EUS-guided fine-needle aspiration or trucut biopsy can be performed at the same time and this procedure enables tissue diagnosis.

Identification of hyperechogenic pancreas (HP) is not uncommon during EUS. However, the clinical significance of HP is still unclear. Fatty liver is associated with insulin resistance, dyslipidemia and obesity (especially central body fat distribution) and is considered a phenotype of metabolic syndrome[2-4]. Visceral fat is more important for metabolic syndrome and hepatic steatosis than subcutaneous fat owing to its steatogenesis and production of various cytokines[5,6]. The normal pancreatic echogenicity on ultrasound is equal to or slightly greater than that of the liver[7,8]. Pancreatic echogenicity is determined by fat deposited around the pancreas and within the septa transversing the normal pancreas[9]. However, the role of obesity as a risk factor for HP remains unclear. We hypothesized that HP is related to obesity in a similar way to its relationship with fatty liver. Many different methods have been used to quantify obesity, such as body mass index (BMI), waist circumference, waist-to-hip ratio, skin fold thickness and percentage of body fat. Among these methods, computed tomography (CT) is considered the gold standard not only for evaluating adipose tissue, but also for multi-compartment body measurements[10].

The aim of this study was to determine the incidence of HP in patients undergoing EUS and to identify the associated risk factors for HP on EUS.

MATERIALS AND METHODS

From January 2007 to December 2007, a total of 524 patients who underwent EUS were prospectively enrolled in the study. Pancreatic disease can alter the sonographic appearance of the pancreas, therefore patients with a history of or who showed the presence of pancreatic disease such as chronic pancreatitis were excluded (n = 156), and patients with hepatobiliary or advanced gastrointestinal cancer were also excluded from the study (n = 84). Finally, a total of 284 patients were included (Figure 1) and all EUS examinations were performed to evaluate subepithelial tumors. EUS examinations were performed using a radial echoendoscope (Olympus GF-UM2000 with 5 MHz and 7.5 MHz frequency transducers) by a single experienced endoscopist (Kim GH). Informed consent was obtained after the patients were given a complete description of the study. All the patients completed a questionnaire regarding their personal medical history, including alcohol intake and smoking. This study was approved by the Ethics Committee of Pusan National University Hospital.

During the study, we measured the levels of serum pancreatic enzymes, took clinical histories, conducted physical examinations, and performed blood analyses including blood sugar, total cholesterol and liver function tests. The degree of echogenicity of the pancreas was judged relative to the liver (or the kidney if the liver was hyperechogenic) (Figure 2). The patients' histories of alcohol consumption were obtained, and the term "nonalcoholic" was applied to men who consumed less than 30 g alcohol/d and to women who consumed less than 20 g alcohol/d. We further analyzed the risk of HP according to the categories of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) in 132 patients who underwent abdominal CT scanning for clinical purposes.

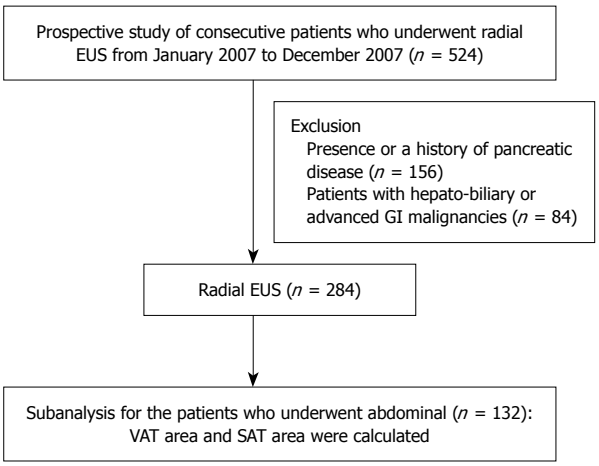

Figure 1 Flow chart indicating the progression from the initial assessment when first referred for endoscopic ultrasound to the final analysis. EUS: Endoscopic ultrasound; GI: Gastrointestinal; VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue.

Laboratory investigations and assessment of the abdominal visceral fat area

The clinical characteristics of all subjects were prospectively evaluated, including gender, age, systolic blood pressure, diastolic blood pressure, BMI and routine blood values. These parameters were measured within 30 d of EUS. Hypertension was defined as systolic BP $\geq$ 140 mmHg or diastolic BP $\geq$ 90 mmHg[11]. Type 2 diabetes mellitus (DM) was defined as a fasting plasma glucose level $\geq$ 126 mg/dL or if there were symptoms of hyperglycemia and the random venous plasma glucose level was $\geq$ 200 mg/dL[12]. Hypercholesterolemia was defined when the serum total cholesterol level was above the reference value (more than 240 mg/dL at our hospital). The body mass index (BMI) was calculated as the body weight (kg) divided by the square of the standing height (m). The BMI was categorized into three levels according to the WHO criteria for the Western Pacific region[13]: normal weight-BMI < 23 kg/m^2, overweight-BMI $\geq$ 23 kg/m^2 and $\leq$ 25 kg/m^2 and obese-BMI > 25 kg/m^2. To determine the VAT and SAT on CT scans, adipose tissue area was calculated at the level of the umbilicus, with an attenuation that ranged from -50 to -250 Hounsfield units[2,14]. The subjects were examined in the supine position. The VAT was defined as intra-abdominal fat bound by the pa-

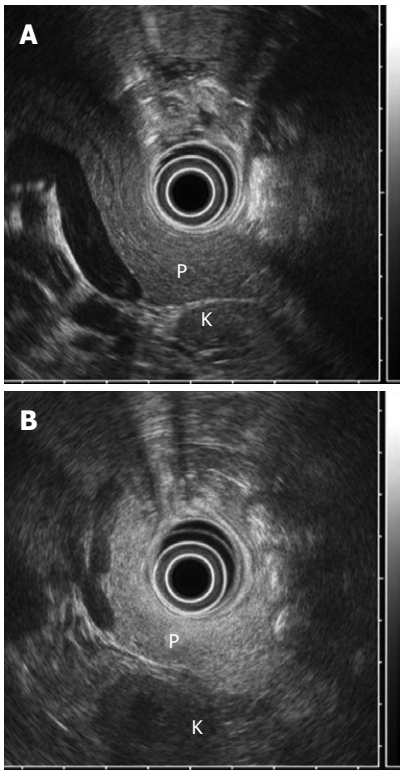

Figure 2 Echogenicity of the pancreas on endoscopic ultrasound. A: Normal echogenic pancreas; B: Hyperechogenic pancreas compared to kidney. P: Pancreas; K: Kidney.

rietal peritoneum or the transversalis fascia excluding the vertebral column and the para-spinal muscles, and the SAT was defined as fat superficial to the abdominal and back muscles. Using a cursor, the VAT was measured around the inner boundary of the abdominal wall muscles. A region of interest drawn around the external margin of the dermis was used to calculate the area of total adipose tissue (TAT). SAT was obtained by subtracting the VAT from the TAT (Figure 3)[2,15].

Statistical analysis

The data were expressed as means ± SD. The unpaired *t*-tests were used to compare the mean values of the characteristics between the HP group and the non-HP group. χ^2 tests were used for the nominal variables. Variables with a *P* value less than 0.25 on univariate analysis were included in a forward stepwise multiple logistic regression model. To detect the dose-response relationship, we used cut-off points to categorize the patients into quartiles for the SAT and VAT. Logistic regression analysis was used to estimate the crude and adjusted strength of the association between the positive and negative HP groups. All data analyses were performed using SPSS for Windows version 12.0 (SPSS, Chicago, IL, USA). A *P*-value less than 0.05 was considered significant.

RESULTS

Baseline characteristics of the study sample

The total number of subjects included was 284 (102 males and 182 females) and their mean age was 52.1 ± 12.2 years. Among the study subjects who underwent EUS, 11 were HP patients (38.7%). Thirty-four patients (33.1%) had fatty livers. There were 70 (24.6%) current smokers and 106 (37.3%) alcohol drinking patients. The mean BMI was 23.1 ± 2.9 kg/m^2. Among the 132 patients who underwent an abdominal CT scan, the mean SAT and VAT were 145.6 ± 49.3 and 71.7 ± 43.6 cm^2, respectively (Table 1).

Figure 3 Calculation of the abdominal adipose tissue distribution using computed tomography scans. The total adipose tissue (TAT) area was obtained by applying an adipose tissue threshold to a region of interest (ROI) that was traced around the dermis (1). An ROI was traced around the inner margin of the abdominal wall muscles, and an adipose tissue threshold was applied to determine the area of visceral adipose tissue (VAT) in the ROI (2). The subcutaneous adipose tissue area was then obtained by subtracting the VAT from the TAT.

Table 1 Baseline characteristics of the study population (mean ± SD) *n* (%)

	Total patients (*n* = 284)
Gender (M:F)	102:182
Age (yr)	52.1 ± 12.2
Hyperechogenic pancreas positive	110 (38.7)
Current cigarette smoking	70 (24.6)
Current alcohol drinking	106 (37.3)
Hypertension (≥ 140/90 mmHg)	61 (21.5)
BMI (kg/m^2)	23.1 ± 2.9
Fatty liver	94 (33.1)
Diabetes mellitus	46 (16.2)
Hypercholesterolemia	46 (16.1)
CT measurement (*n* = 132)	
SAT area (cm^2)	145.6 ± 49.3
VAT area (cm^2)	71.7 ± 43.6

BMI: Body mass index; CT: Computed tomography; SAT: Subcutaneous adipose tissue; VAT: Visceral adipose tissue.

Comparison of patients with and without hyperechogenic pancreas

We analyzed the potential risk factors for HP. On univariate analysis, age older than 60 years, obesity (BMI more than 25 kg/m^2), fatty liver, type 2 DM, hypertension and hypercholesterolemia were the associated risk factors for HP ($P < 0.05$) (Table 2). On multivariate analysis, fatty liver [$P = 0.008$, odds ratio (OR) = 2.219], male gender ($P = 0.013$, OR = 2.636), age older than 60 years ($P = 0.001$, OR = 2.874) and hypertension ($P = 0.044$, OR = 2.037) were the significant associated risk factors for HP (Table 3).

Table 2 Comparison between the patients with and without hyperechogenic pancreas (mean ± SD) *n* (%)

	HP negative (*n* = 174)	HP positive (n = 110)	*P* value
Age (yr)	48.9 ± 12.0	57.2 ± 10.7	< 0.001
Male gender	69 (39.9)	33 (30.0)	0.099
BMI (kg/m^2)	22.4 ± 2.8	24.0 ± 2.7	< 0.001
Smoking	47 (27.0)	23 (20.9)	0.245
Alcohol	71 (40.8)	35 (31.8)	0.127
Type 2 DM	7 (4.0)	12 (10.9)	0.024
Hypertension	25 (14.4)	36 (32.7)	< 0.001
Hypercholesterolemia	20 (11.5)	26 (23.6)	0.007
Fatty liver	46 (26.4)	48 (43.6)	0.003

HP: Hyperechogenic pancreas; BMI: Body mass index; DM: Diabetes mellitus.

Table 3 Multivariate analysis of the clinical risk factors for hyperechogenic pancreas

	P value	OR	95% CI
Male gender	0.013	2.636	1.224-5.678
Age older than 60 yr	0.001	2.874	1.537-5.372
Obesity (BMI > 25 kg/m^2)	0.439	1.296	0.673-2.496
Smoking	0.612	1.227	0.557-2.701
Alcohol	0.435	0.773	0.405-1.475
Type 2 DM	0.646	1.304	0.420-4.055
Hypertension	0.044	2.037	1.018-4.072
Hypercholesterolemia	0.099	1.821	0.893-3.713
Fatty liver	0.008	2.219	1.226-4.016

BMI: Body mass index; DM: Diabetes mellitus; OR: Odds ratio; CI: Confidence interval.

A subanalysis was performed in patients who underwent abdominal CT (*n* = 132). On univariate analysis, VAT and SAT were significantly different between the groups (89.5 ± 47.8 cm^2 *vs* 59.8 ± 36.3 cm^2, respectively, *P* < 0.001 for VAT and 162.2 ± 55.7 cm^2 *vs* 134.5 ± 59.3 cm^2, respectively, *P* = 0.008 for SAT) (Table 4). On multivariate analysis, for patients who underwent abdominal CT, VAT was a statistically significant risk factor for HP after adjusting for age, gender, alcohol, smoking and BMI (*P* = 0.010, OR = 5.665, the lowest quartile *vs* the highest quartile) (Table 5).

DISCUSSION

EUS represents a major advance in gastrointestinal imaging technology. EUS of the pancreas is particularly useful, because the pancreas can be visualized either from the duodenum or from the stomach. EUS is less risky than endoscopic retrograde pancreatography, which is the traditional imaging test of choice and the gold standard for diagnosing chronic pancreatitis[16,17]. Having an understanding of the normal variations in the pancreatic parenchyma is crucial when evaluating pancreatic abnormalities. HP is not an infrequent finding during EUS, but the clinical significance of hyperechogenicity of the normal pancreas is not known. In this study, pancreatic echogenicity was compared with liver echogenicity to evaluate fat deposition in the pancreas. However, in fatty liver, liver echogenicity is not a good reference value for HP. Therefore, we used kidney parenchymal echogenicity if fatty liver was present[18]. A very high echogenicity of the pancreas could be a sign of chronic pancreatitis, which is often accompanied by dilatation of the pancreatic duct. A previous study showed that body weight and fatty infiltration have a significant influence on pancreatic echogenicity[19]. Hepatic steatosis (or fatty liver) is associated with obesity, old age, hyperlipidemia, hyperglycemia and hypertension[2,3,5]. Visceral adiposity is known to be more important than BMI for predicting the presence of hepatic steatosis[5].

Table 4 Comparison of adipose tissue between patients with and without hyperechogenic pancreas *n* (%)

	HP negative (*n* = 79)	HP positive (*n* = 53)	*P* value
VAT area (cm^2)			< 0.001
Quartile Ⅰ (< 35.2)	23 (29.1)	9 (17.0)	
Quartile Ⅱ (35.2-65.9)	25 (31.6)	8 (15.1)	
Quartile Ⅲ (65.9-94.0)	19 (24.1)	14 (26.4)	
Quartile Ⅳ (> 94.0)	12 (15.2)	22 (41.5)	
SAT area (cm^2)			0.008
Quartile Ⅰ (< 109.3)	24 (30.4)	9 (17.0)	
Quartile Ⅱ (109.3-139.7)	22 (27.8)	11 (20.8)	
Quartile Ⅲ (139.7-179.2)	18 (22.8)	16 (30.2)	
Quartile Ⅳ (> 179.2)	15 (19.0)	17 (32.1)	

HP: Hyperechogenic pancreas; VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue.

At first, we assumed that HP in an otherwise normal pancreas would be associated with fatty liver and its associated risk factors, especially obesity. On multivariate analysis, fatty liver, age older than 60 years, male gender and hypertension were the significant risk factors for HP. The pancreas in older patients showed different changes, such as atrophy, fibrosis and fatty infiltration. A previous histopathologic study showed that after the age of 60 years, moderate to severe fat accumulation is typically evident in the acinar cells of the pancreas[20]. High echogenicity of the pancreas is a normal finding in older patients[21]. Another previous EUS study of the age-related changes of the pancreas showed that men had significantly greater odds for having abnormalities than did women (OR = 2.9, *P* = 0.01)[22]. Because the distribution of abdominal fat differs according to gender, the areas of subcutaneous fat are significantly greater in women than in men at the abdominal level[23-25]. Metabolic syndrome is more prevalent in men than in women up to the age of 60 and this is closely related to hepatic steatosis; our results may reflect such a profile[26,27].

Hepatic steatosis is usually prevalent in obese subjects and regional fat distribution associated with insulin resistance was found to be an important factor for hepatic steatosis in several studies[28]. The BMI reflects either the total body fat accumulation or the subcutaneous fat accumulation. Recent findings have shown that central obesity (visceral fat accumulation) may be a more important factor for hepatic steatosis than BMI[29]. CT is considered the gold standard not only for measuring adipose tissue, but also

Table 5 Multivariable analysis: Unadjusted and adjusted analyses for the relationships of the abdominal adipose tissue area with hyperechogenic pancreas, OR (95% CI)

	Unadjusted analysis	*P* value	Adjusted analysis[1]	*P* value
VAT area (cm^2)		0.015		0.010
Quartile Ⅰ (< 35.2)	Reference		Reference	
Quartile Ⅱ (35.2-65.9)	0.646 (0.202-2.059)		0.671 (0.214-2.365)	
Quartile Ⅲ (65.9-94.0)	1.311 (0.637-6.094)		1.997 (0.561-7.107)	
Quartile Ⅳ (> 94.0)	3.491(1.154-10.557)		5.665 (1.515-21.180)	
SAT area (cm^2)		0.414		0.960
Quartile Ⅰ (< 109.3)	Reference		Reference	
Quartile Ⅱ (109.3-139.7)	1.139 (0.374-3.471)		1.016 (0.316-3.271)	
Quartile Ⅲ (139.7-179.2)	1.97 (0.637-6.094)		1.353 (0.378-4.841)	
Quartile Ⅳ (> 179.2)	2.288 (0.720-7.268)		1.227 (0.301-4.992)	

[1]Adjusted for age, gender, smoking, alcohol, body mass index. VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue; OR: Odds ratio; CI: Confidence interval.

for performing multi-compartment body measurements[10]. Even so, there have been few studies on the relationship between HP and regional fat distribution as measured by CT. We performed a subgroup analysis on patients who underwent abdominal CT. This objective measure of visceral fat showed that VAT was an independent risk factor for HP after adjusting for age, gender, alcohol, smoking and BMI. Obesity is known to be accompanied by metabolic complications and is increasingly recognized as a risk factor for type 2 DM, dyslipidemia, atherosclerotic and cardiovascular disease. There is growing evidence that the regional distribution of adipose tissue appears to be an important indicator of metabolic and cardiovascular alterations, since only inconstant correlations between BMI and these disturbances have been found[30]. On multivariate analysis in the present study, although BMI was not a statistically significant risk factor, VAT was an independent, significant risk factor for HP. A possible reason for this is that VAT measured by CT scanning is more accurate for measuring visceral obesity than BMI[31].

Recently, two studies regarding fatty pancreas or hyperechogenic pancreas were reported[18,32]. In one study, the predictors of HP were found to be hepatic steatosis, alcohol use and increased BMI[32]. In the other study, fatty pancreas was associated with metabolic syndrome. In the latter study, visceral fat was also independently associated with fatty pancreas[18]. However, that study simply compared VAT area values between fatty pancreas and normal controls. In the present study, we subdivided SAT and VAT area into four quartiles to evaluate the relationship between adipose tissue area and HP.

In the present study, alcohol intake and cigarette smoking were not significant risk factors for HP. Another study has suggested that alcohol consumption and cigarette smoking affect the endosonographic appearance of the pancreas in a dose-dependent fashion[33]. Although an effort was made to gather precise information on drinking and smoking in our sample, underestimation of alcohol intake and cigarette smoking may explain this finding in our study.

Although we performed this study prospectively, there were some limitations. First, the control group (non-HP group) was not representative of the general healthy population because the sample was made up of patients who required EUS for the evaluation of a subepithelial tumor. Second, direct determination of the pancreatic fat and visceral fat in tissue was not conducted. Due to ethical considerations with regard to obtaining tissue specimens from disease-free subjects, we did not perform tissue biopsies. Third, quantitative analyses of the pancreatic parenchymal echogenicity were not performed. As we only compared pancreatic echogenicity with echogenicity of the liver or kidney, these comparisons might have been somewhat subjective. A future study that employs computer-assisted quantitative analysis may be warranted.

In conclusion, HP is correlated with hepatic steatosis, hypertension, male gender and age older than 60 years. VAT is positively correlated with HP regardless of BMI. Although it is unknown whether HP is a progressive condition, HP, and likewise fatty liver, may be one of the phenotypes of metabolic syndrome, which is characterized by obesity with visceral fat accumulation, DM, hyperlipidemia and hypertension. Further studies are needed to confirm this hypothesis.

COMMENTS

Background

A hyperechogenic pancreas (HP) is commonly found during endoscopic ultrasound (EUS). However, the clinical significance of HP is still unclear. Visceral fat is more important for metabolic syndrome and hepatic steatosis than subcutaneous fat owing to its steatogenesis and production of various cytokines. Pancreatic echogenicity is determined by fat deposited around the pancreas and within the septa transversing the normal pancreas. Yet the role of obesity as a risk factor for HP is unclear. The authors could assume that HP is related to obesity in a similar way to that of fatty liver.

Research frontiers

HP may is related to obesity in a similar way to that of fatty liver. Many different methods have been used to calculate a value for obesity, such as body mass index (BMI), waist circumference, waist-to-hip ratio, skin fold thickness and percentage of body fat. Among these methods, computed tomography is considered the gold standard not only for evaluating adipose tissue, but also for multi-compartment body measurements.

Innovations and breakthroughs

HP is correlated with hepatic steatosis, hypertension, male gender and age older than 60 years. Visceral adipose tissue is positively correlated with HP regardless of BMI.

Applications

Although it is unknown whether HP is a progressive condition, HP, and likewise fatty liver, may be one of the phenotypes for metabolic syndrome, which is characterized by obesity with visceral fat accumulation, diabetes mellitus, hyperlipidemia and hypertension.

Terminology

Hyperechogenic pancreas was observed when the degree of echogenicity of the pancreas relative to the liver (or the kidney if the liver was hyperechogenic) was higher.

Peer review

Choi *et al* have performed a prospective study in order to identify factors related to hyperechogenic pancreas on EUS. This is an interesting study as published data on "fatty" pancreas are not extensive.

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BRIEF ARTICLE

Limited endoscopic sphincterotomy plus large balloon dilation for choledocholithiasis with periampullary diverticula

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Abstract

AIM: To investigate the effectiveness and safety of limited endoscopic sphincterotomy (EST) plus large balloon dilation (LBD) for removing choledocholithiasis in patients with periampullary diverticula (PAD).

METHODS: A total of 139 patients with common bile duct (CBD) stones were treated with LBD (10-20 mm balloon diameter) after limited EST. Of this total, 73 patients had PAD and 66 patients did not have PAD (controls). The results of stone removal and complications were retrospectively evaluated.

RESULTS: There were no significant differences between the PAD and the control groups in overall successful stone removal (94.5% *vs* 93.9%), stone removal in first session (69.9% *vs* 81.8%), mechanical lithotripsy (12.3% *vs* 13.6%), and complications (11.0% *vs* 7.6%). Clinical outcomes were also similar between the types of PAD, but the rate of stone removal in first session and the number of sessions were significantly lower and more frequent, respectively, in type B PAD (papilla located near the diverticulum) than controls [23/38 (60.5%) *vs* 54/66 (81.8%), $P = 0.021$; and 1 (1-2) *vs* 1 (1-3), $P = 0.037$, respectively] and the frequency of pancreatitis was significantly higher in type A PAD (papilla located inside or in the margin of the diverticulum) than in controls (16.1% *vs* 3.0%, $P = 0.047$).

CONCLUSION: Limited EST plus LBD was an effective and safe procedure for removing choledocholithiasis in patients with PAD. However, some types of PAD should be managed with caution.

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Key words: Endoscopic sphincterotomy; Large balloon dilation; Choledocholithiasis; Periampullary diverticula

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INTRODUCTION

Although endoscopic sphincterotomy (EST) is the standard treatment for removing common bile duct (CBD) stones, it is associated with complications, including pancreatitis, bleeding, and perforation. Complications are primarily related to the indications for the procedure and applied endoscopic techniques, rather than age or general medical condition of the patients[1].

Staritz *et al*[2] introduced endoscopic papillary balloon dilation (EPBD) as an alternative method of removing bile duct stones. However, since Disario *et al*[3] reported a high frequency of pancreatitis and two deaths in an EPBD group, EPBD has seldom been used in removing bile duct stones. Recently, EPBD is primarily being used in patients with bleeding tendencies.

Since Ersoz *et al*[4] introduced large balloon dilation (LBD) after EST as an alternative technique in patients with bile duct stones that were difficult to remove with conventional methods, such as basket or balloon catheter extraction after EST, several studies have reported the safety and effectiveness of LBD after EST for removing bile duct stones[5-7].

Periampullary diverticula (PAD) are extraluminal mucosal outpouchings of the duodenum that arise within a radius of 2-3 cm from the ampulla of Vater[8]. PAD are found in 9% to 32.8% of patients who have undergone an endoscopic retrograde cholangiopancreatography (ERCP). The prevalence of PAD has a tendency to increase with age[8-15] and PAD occurred in up to 65% of elderly patients in some studies[16]. PAD are associated with an increased number of complications, which can be explained by a difficult technical approach during an ERCP; but many studies have reported conflicting results regarding the true impact of PAD on clinical outcomes[9-15].

Limited EST plus LBD can be a useful method for removing CBD stones in patients with PAD that are difficult to remove with conventional methods. However, the effectiveness and safety of limited EST plus LBD in these patients has not been evaluated. Therefore, we evaluated the clinical efficacy of limited EST plus LBD for removing CBD stones in patients with PAD.

MATERIALS AND METHODS

Patients

From August 2007 to August 2008, we enrolled consecutively 139 patients who had CBD stones ≥ 10 mm in diameter and who underwent limited EST followed by large-diameter (≥ 10 mm) balloon dilation for removal of bile duct stones. All patients were admitted to the hospital. Exclusion criteria included coagulopathy (international normalized ratio > 1.5), low platelet count (< 50 000/mL), anticoagulation or antiplatelet therapy, acute pancreatitis, septic shock, prior EST, Billroth Ⅱ anatomy or Roux-en-Y gastrojejunostomy, and combined intrahepatic bile-duct stones. Informed consent from all patients was obtained and the study was approved by the ethics committee of the internal review board of Pusan National University.

Methods

ERCPs were performed by experienced endoscopists who performed over 300 biliary interventions per year. Patients were placed under conscious sedation with midazolam and meperidine. After the side-viewing endoscope (JF-240 or TJF-240; Olympus Optical Co., Ltd., Tokyo, Japan) was advanced into the descending duodenum, 10 mg of cimetropium bromide was administered intravenously to reduce duodenal peristalsis. Selective cannulation of the bile duct was achieved by using a conventional catheter and a pull-type sphincterotome with or without a guidewire. If those attempts failed to yield deep bile duct cannulation after more than 10 min, and/or the pancreatic duct had been cannulated more than 3 times, a needle-knife fistulotomy (NKF) was used to gain access. The subsequent procedures were performed by Kang's methods[5]. Follow-up endoscopy was performed on the first or second day after the procedure to determine whether bleeding was present, if immediate bleeding after EST or balloon dilation was noted, and bleeding control therapy was administered. The results of stone removal and complications were retrospectively evaluated.

Definitions

Stone size, number, and diameter of CBD were documented on ultrasound, computed tomography, and ERCP. Stone size was estimated based on the relative diameters of the stone and the shaft of the endoscope, as measured on the cholangiogram.

Limited EST was defined as sphincterotomy performed until the upper margin of the cut portion was located at one third of major EST.

Two different types of PAD were classified according to the location of the major papilla with respect to the diverticulum: type A: papilla located inside or in the margin of the diverticulum; type B: papilla located near the diverticulum.

Serum amylase (reference range, 36-128 IU/L) and lipase (22-51 IU/L) concentrations were measured before and after (4 and 24 h, respectively) the procedure. A complete blood cell count and a liver function test were checked the next morning after the procedure.

Post-ERCP pancreatitis was defined as sustained abdominal pain for 24 h after the procedure and a serum amylase level increased by three-fold or more[1,17,18]. Hemorrhage was considered to be clinically significant only when there was clinical evidence of bleeding, such as me-

Table 1 Baseline characteristics of patients with common bile duct stones who also had periampullary diverticula (periampullary diverticula group) or did not have periampullary diverticula (control group), median (range)

Characteristics	PAD group	PAD subtypes			Control group	P^2 value
		Type A	Type B	P^1 value		
No. of patients	73	35 (47.9)	38 (52.1)		66	
M:F	36:37	19:16	17:21	NS	39:27	NS
Age (yr)	70 (40-89)	74 (54-88)	66.5 (40-89)	NS	64 (23-89)	< 0.001
CBD diameter (mm)	15 (10-30)	16 (10-26)	15 (10-30)	NS	15 (11-38)	NS
Size of stones (mm)	14 (10-33)	14 (10-30)	14 (10-33)	NS	12 (10-35)	NS
No. of stones	2 (1-20)	2 (1-9)	2 (1-20)	NS	2 (1-7)	NS
Distal CBD stricture	4	3	1	NS	1	NS
Needle-knife fistulotomy	7	3	4	NS	11	NS

[1]Comparing between subgroups of periampullary diverticula (PAD); [2]Comparing PAD group with control group. CBD: Common bile duct; NS: Not significant.

Table 2 Outcome of limited endoscopic sphincterotomy plus large balloon dilation in patients with common bile duct stones who also had periampullary diverticula (periampullary diverticula group) or did not have periampullary diverticula (control group)

Characteristics	PAD group	Control group	P^2 value
Overall stone removal, *n* (%)	69/73 (94.5)	62/66 (93.9)	NS
Type A	33/35 (94.3)		NS
Type B	36/38 (94.7)		NS
P^1 value	NS		
Stone removal in first session, *n* (%)	51/73 (69.9)	54/66 (81.8)	NS
Type A	28/35 (80)		NS
Type B	23/38 (60.5)		0.021
P^1 value	NS		
No. of sessions, median (range)	1 (1-3)	1 (1-2)	NS
Type A	1 (1-3)		NS
Type B	1 (1-3)		0.037
P^1 value	NS		
Diameter of balloon dilation (mm), median (range)	13.5 (10-20)	12.5 (10-20)	NS
Type A	13.5 (10-20)		NS
Type B	13.8 (10-20)		NS
P^1 value	NS		
Mechanical lithotripsy required, *n* (%)	9/73 (12.3)	9/66 (13.6)	NS
Type A	5/35 (14.3)		NS
Type B	4/38 (10.5)		NS
P^1 value	NS		

[1]Comparing between subgroups of periampullary diverticula (PAD); [2]Comparing PAD group with control group. NS: Not significant.

lena or hematemesis, together with a decrease of at least 2 g/dL in the hemoglobin level, or the need for blood transfusion for stabilization of vital signs[1,17,18].

Statistical analysis

For the statistical analysis, the χ^2 test and Fisher's exact test was used for categorical variables and the Student *t* test or ANOVA test for continuous variables. Analyses were performed with SPSS 12.0 (SPSS Inc., Chicago, IL). A *P* value < 0.05 was considered statistically significant. Continuous variables are expressed as the median (range).

RESULTS

A total of 139 patients (median age, 68 years old; 76 men, 63 women) with CBD stones underwent limited EST plus LBD. Seventy-three patients (median age, 70 years old; 36 men, 37 women) had PAD (PAD group) and 66 patients (median age, 64 years old; 39 male, 27 female) did not have PAD (control group). There were no differences between the two groups regarding baseline characteristics, except age (70 years *vs* 64 years, $P < 0.001$) (Table 1).

In the PAD group, type A PAD comprised 35 patients (47.9%) and type B PAD comprised 38 patients (52.1%). There were no differences between the two types regarding baseline characteristics (Table 1).

The rate of overall stone removal and stone removal in first session did not differ significantly between the PAD and the control groups [overall, 69/73 (94.5%) *vs* 62/66 (93.9%); and first session, 51/73 (69.9%) *vs* 54/66 (81.8%), respectively] (Table 2). Failure of complete stone clearance occurred in 8 patients, 4 from each group. The major causes in 7 patients were capture failure with mechanical basket due to multiple, impacted, or large stones (3 patients in the PAD group, 4 patients in the control group) and one patient (PAD group) had large stones above

Table 3 Complications of limited endoscopic sphincterotomy plus large balloon dilation in patients with common bile duct stones who also had periampullary diverticula (periampullary diverticula group) or did not have periampullary diverticula (control group) *n* (%)

Complications	PAD group	Control group	P^2 value
Pancreatitis	7/73 (9.6)	2/66 (3.0)	NS
Type A	5/35 (14.3)		0.047
Type B	2/38 (5.3)		NS
P^1 value	NS		
Hemorrhage	0/73 (0)	1/66 (1.5)	NS
All complications	7/73 (9.6)	3/66 (4.5)	NS
Type A	5/35 (14.3)		NS
Type B	2/38 (5.3)		NS
P^1 value	NS		

[1]Comparing between subgroups of periampullary diverticula (PAD); [2]Comparing PAD group with control group. NS: Not significant.

the stricture. These patients had a biliary stent placed to ensure biliary drainage and were treated by percutaneous transhepatic cholangioscopy with electrohydraulic lithotripsy (7 patients) or open surgery (1 patient).

The groups had a similar frequency of mechanical lithotripsy [9/73 (12.3%) *vs* 9/66 (13.6%)]. Large-sized stones (> 15 mm) were the main indication for mechanical lithotripsy.

Between the types of PAD, there were no significant differences in the overall stone clearance, the stone removal in the first session, or the use of mechanical lithotripsy. However, when comparing each type of PAD with the controls, the rate of stone removal in first session and the number of sessions in type B PAD were significantly lower and more frequent, respectively, than controls [23/38 (60.5%) *vs* 54/66 (81.8%), $P = 0.021$ and 1 (1-2) *vs* 1 (1-3), $P = 0.037$, respectively] (Table 2).

The overall frequency of short-term complications was similar between the PAD and control groups [7/73 (9.6%) *vs* 3/66 (4.5%)] (Table 3). The frequency of pancreatitis did not differ significantly between the PAD and control groups [7/73 (9.6%) *vs* 2/66 (3.0%)]. Pancreatitis occurred in 5 and 2 patients with type A and B PAD, respectively. Pancreatitis related to NKF only occurred in one control patient. In comparing each type of PAD with controls, pancreatitis was significantly higher in type A PAD than controls [5/31 (14.3%) *vs* 2/66 (3.0%), $P = 0.047$]. All pancreatitis cases were clinically mild and they were treated conservatively. Clinically significant hemorrhage occurred in one patient in the control group. Immediate bleeding occurred after 10 mm balloon dilation and was controlled by endoscopic treatment. The next day, melena occurred and a blood transfusion was performed. Active bleeding was not found in a follow-up endoscopy, but blood clots appeared in the major papilla. Hemorrhage did not occur in any patient in the PAD group. Perforation and cholangitis did not occur in any patient.

DISCUSSION

The majority of CBD stones are removed by EST and conventional methods, but 10%-15% may be difficult to remove by conventional methods. The main reasons for failure are a difficult approach to the bile duct (PAD, Billroth Ⅱ anatomy, Roux-en-Y gastrojejunostomy), large (> 15 mm) stones, and impacted stones[19,20].

Conflicting results have been reported regarding the true impact of PAD on the technical success and complications of ERCP[9-15]. Many studies have reported recently that PAD does not make a difference to the success and complication rates of ERCP[14,15,21]. However, clinical outcomes associated with the technical success of selective cannulation of the bile duct or EST may be influenced by PAD. Boix *et al*[15] classified PAD into three types, according to the position of the major papilla. That study concluded that the presence or type of PAD did not significantly influence the difficulty of deep cannulation, but they did not evaluate the association between the types of PAD and the technical difficulties in removing CBD stones.

After the first study from Ersoz *et al*[4] demonstrating the technique of EST plus LBD, several studies established this procedure as an effective and safe treatment for removing CBD stones[5-7]. However, there have been few studies about the effectiveness and safety of EST plus LBD in patients with PAD. Three recent studies reported clinical outcomes of EST plus LBD in patients with PAD[22-24]. Two studies reported similar results for stone removal (84% *vs* 87.5%, 93.8% *vs* 89.2%) and complications (8.3% *vs* 18.8%, 3.1% *vs* 10.8%) between the PAD and control groups[22,23]. These studies suggested that minor EST with LBD in patients with PAD was a safe treatment modality for removing CBD stones. Another study compared minor EST plus EPBD with EST alone in patients with PAD and found similar outcomes in terms of overall stone clearance (100% *vs* 100%), stone clearance at first attempt (78% *vs* 72%), and the use of mechanical lithotripter (12% *vs* 21%)[24]. However, complications were rare in the EST plus EPBD group compared to EST alone (4% *vs* 21%, $P < 0.005$). The authors suggested that minor EST plus EPBD was safer than EST alone for removing bile duct stones in patients with PAD. However, these studies had limitations due to being published in abstract form and having a small number of patients.

In the current study, the rates of overall stone removal and the stone removal in first session did not differ significantly between the PAD and control groups (94.5% *vs* 93.9% and 69.9% *vs* 81.8%, respectively). The overall success rate of stone removal was similar to those of previous studies (84%-100%)[5,23,24]. The stone removal in first session rate in the PAD group was lower than that of the control group and of previous studies (81.8%-95%)[5,7,22], although this was not statistically different. This finding might be attributed to an older age group (median ages, 70 years old *vs* 62 years old, $P < 0.001$); elderly patients tend to have cardiopulmonary instability or poor general condition, thus they are less able to tolerate the procedure for long. The high prevalence of multiple stones in the PAD group might also have influenced the poor result, though this was not significantly different from controls.

Finally, the PAD condition might reduce the potential aggressiveness of the procedure by the endoscopist due to the consideration of possible complications.

The outcomes of stone removal were not different between the types of PAD. However, the rate of stone removal in the first session in type B PAD was lower compared to type A PAD, although the difference was not statistically significant. In comparing each type of PAD with controls, the rate of stone removal in first session and the number of sessions in type B PAD was significantly lower and more frequent, respectively, than controls, [23/38 (60.5%) *vs* 54/66 (81.8%), $P = 0.021$; and 1 (1-2) *vs* 1 (1-3), $P = 0.037$, respectively]. This finding might be attributed to a higher prevalence of multiple stones in type B PAD compared to controls and type A PAD, although this difference was not statistically significant.

The frequency of mechanical lithotripsy was similar between the PAD and control groups (12.3% *vs* 13.6%) and was similar to results with other studies (8.0%-12%)[5,6,24]. Again, no differences were found between the types of PAD in the frequency of mechanical lithotripsy.

These results suggest that the success rate for the clearance of bile duct stones and the use of mechanical lithotripsy were influenced by the number and size of the stones rather than the presence or type of PAD.

Generally, the length of EST is shorter in patients with PAD than in those without PAD due to the weakness of the sphincter of choledochus and risk of perforation in patients with PAD. For similar reasons, the diameter of the balloon may be influenced by the position of the major papilla in PAD; there was a tendency to use a smaller sized balloon in PAD compared to controls. However, in the current study, there was no difference in balloon diameters between the PAD and control groups or between the types of PAD. Although the precise reasons are not clear, PAD itself had no influence on the diameter of balloon.

In the current study, the overall rates of complication were not significantly different between the PAD and control groups (9.6% *vs* 4.5%). However, pancreatitis in the PAD group occurred more frequently than in other studies (4%-8.3%)[5-7,23,24]. Nevertheless, all pancreatitis cases were clinically mild and they were treated conservatively. The rate of pancreatitis was not statistically different between the types of PAD. However, the frequency of pancreatitis in type A PAD was significantly higher than in controls (14.3% *vs* 3.0%, $P = 0.047$). In the current study, the cause of more frequent pancreatitis in type A PAD is not clear, but it may be related to the presence of type A PAD. Firstly, the cannulation of the bile duct in type A PAD is generally more difficult than in type B PAD or in controls due to more frequent cases of poorly detectable papilla or more difficult prediction of the direction of bile duct in type A PAD. These features may lead to induction of pancreatitis because of the unnecessary injection of contrast medium or manipulation of the pancreatic duct, but the frequency of NKF due to difficult cannulation was not significantly different among the groups in our study. Secondly, because EST before LBD was performed to prevent post-ERCP pancreatitis by induction of separation between the pancreatic and biliary orifices, the more frequent pancreatitis in type A PAD may be related to injury of the pancreatic duct during balloon dilation due to less separation between the pancreatic and biliary orifices after EST compared to the control group and type B PAD group. Clinically significant hemorrhage did not occur in any patient in the PAD group.

In conclusion, limited EST plus LBD was equally successful and had similar complication rates in the PAD and control groups for the clearance of CBD stones. Therefore, this procedure is effective and safe for removing CBD stones in patients with PAD. Nevertheless, this procedure requires caution in some types of PAD for successful stone removal and prevention of complications. Larger and prospective studies are needed to evaluate clinical outcome in the presence of different types of PAD due to the retrospective nature and relatively small sample sizes in this study.

COMMENTS

Background

Endoscopic sphincterotomy (EST) plus large balloon dilation (LBD) is a useful method to remove common bile duct (CBD) stones, but the effectiveness and safety of this procedure is not well known in patients with periampullary diverticula (PAD) which are reportedly associated with difficulties and complications during associated procedures. We conducted this trial to evaluate the effectiveness and safety of limited EST plus LBD for removing CBD stones in patients with PAD.

Research frontiers

The majority of CBD stones are removed by EST and conventional methods, but 10%-15% may be difficult to remove by conventional methods. The main reasons for failure are a difficult approach to the bile duct (PAD, Billroth II anatomy, Roux-en-Y gastrojejunostomy), large (> 15 mm) stones, and impacted stones. In addition, PAD are associated with an increased number of complications, which can be explained by a difficult technical approach during an ERCP. However, conflicting results have been reported regarding the true impact of PAD on the technical success and complications of ERCP. Therefore, LBD after EST in some patients with PAD may be ineffective and complicated.

Innovations and breakthroughs

After the first study by Ersoz *et al* demonstrating the technique of EST plus LBD, several studies established this procedure as an effective and safe treatment for the removal of bile duct stones; but there have been few studies about the effectiveness and safety of EST plus LBD in patients with PAD. Three recent studies reported clinical outcomes of EST plus LBD in patients with PAD, but these studies had limitations due to being published in abstract form and having a small number of patients.

Applications

Limited EST plus LBD was equally successful and had similar complication rates in the PAD and control groups for the clearance of CBD stones. Therefore, this procedure is effective and safe for removing CBD stones in patients with PAD.

Terminology

Limited EST was defined as sphincterotomy performed until the upper margin of the cut portion was located at one third of major EST. Two different types of PAD were classified according to the location of the major papilla with respect to the diverticulum: type A: papilla located inside or in the margin of the diverticulum; type B: papilla located near the diverticulum. LBD was defined as the diameter of the balloon used for dilation being 10 to 20 mm.

Peer review

Kim *et al* have performed a retrospective study in order to evaluate the effectiveness and safety of limited EST plus LBD for removing CBD stones in patients with PAD. This paper is interesting and it could be valuable for other researchers.

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BRIEF ARTICLE

Pathophysiological significance of gallbladder volume changes in gallstone diseases

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Abstract

AIM: To study the pathophysiological significance of gallbladder volume (GBV) and ejection fraction changes in gallstone patients.

METHODS: The fasting GBV of gallstone patients with acute cholecystitis (n = 99), chronic cholecystitis (n = 85) and non-gallstone disease (n = 240) were measured by preoperative computed tomography. Direct saline injection measurements of GBV after cholecystectomy were also performed. The fasting and postprandial GBV of 65 patients with gallstones and chronic cholecystitis and 53 healthy subjects who received health examinations were measured by abdominal ultrasonography. Proper adjustments were made after the correction factors were calculated by comparing the preoperative and postoperative measurements. Pathological correlations between gallbladder changes in patients with acute calculous cholecystitis and the stages defined by the Tokyo International Consensus Meeting in 2007 were made. Unpaired Student's t tests were used. $P < 0.05$ was deemed statistically significant.

RESULTS: The fasting GBV was larger in late stage than in early/second stage acute cholecystitis gallbladders (84.66 ± 26.32 cm^3, n = 12, *vs* 53.19 ± 33.80 cm^3, n = 87, P = 0.002). The fasting volume/ejection fraction of gallbladders in chronic cholecystitis were larger/lower than those of normal subjects (28.77 ± 15.00 cm^3 *vs* 6.77 ± 15.75 cm^3, $P < 0.0001$)/(34.6% ± 10.6%, n = 65, *vs* 53.3% ± 24.9%, n = 53, $P < 0.0001$).

CONCLUSION: GBV increases as acute cholecystitis progresses to gangrene and/or empyema. Gallstone formation is associated with poorer contractility and larger volume in gallbladders that contain stones.

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Key words: Gallbladder volume; Pathophysiology; Gallbladder ejection fraction; Gallstone formation; Acute cholecystitis

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INTRODUCTION

Gallbladder volume (GBV) can reflect clinical and therapeutic implications, physiological and functional status, and possibly pathophysiological mechanisms of gallstone diseases. In 1880, Courvoisier and Terrier's law stated that large painless, palpable gallbladder was mostly due to malignant obstruction of the bile duct[1]. In 1963, Maingot found that tensely swollen, tenderly palpable gallbladder in acute cholecystitis was due to stone impaction in the cystic duct[2]. In 1989, Masclee noted, in one third of patients with cholesterol gallstones, increased GBV and delayed gallbladder emptying were present at the postprandial phase[3-5]. In 2000, Portincasa demonstrated in a comparative study that patients with cholesterol gallstones showed significantly larger fasting volume and postprandial volume of gallbladders than did controls and patients with pigment stones at each time point during a 2-h study[6]. He proposed that larger GBV predisposes to bile stasis.

However, the differences and changes in GBV among different age groups in non-gallstone subjects, and their correlations with the formation of gallstones have not been reported previously. Also, the correlations between GBV changes at different stages in the pathophysiological development of acute cholecystitis in gallstone patients are not clear. Furthermore, we wished to investigate the possible reciprocal correlation between GBV and gallbladder contractibility.

The aims of this study were to study the pathophysiological significance of gallbladder volume and ejection fraction changes in gallstone patients by comparing the GBV among gallstone patients with acute cholecystitis, chronic cholecystitis, contracted gallbladder and non-gallstone subjects, and by comparison between gallstone patients with chronic cholecystitis and non-gallstone subjects with different ages and sex. Furthermore, to investigate the correlations between gallbladder physiological changes and the development of gallstones, we further investigated the differences in gallbladder contractility between gallstone patients with chronic cholecystitis and normal subjects.

MATERIALS AND METHODS

Between October 1, 2007 and September 30, 2008, 99 gallstone patients with acute cholecystitis, 85 gallstone patients with chronic cholecystitis, and five patients with gallbladder contraction were admitted to Chung Shan Medical University Hospital to undergo cholecystectomy.

Diagnostic criteria

Acute cholecystitis: The clinical imaging findings of acute cholecystitis in abdominal ultrasonography include thick gallbladder wall (> 3 mm), stones in the gallbladder, distended gallbladder with pericholecystic fluid, and sonographic Murphy's sign with tenderness over the gallbladder from the ultrasound transducer. The pathological features of acute cholecystitis include leukocyte infiltration throughout the tissues, local flattening and denuded mucosal folds, and tissue edema. If untreated or treated late, localized regions of necrosis (gangrene) or abscess formation (empyema) can be found.

Chronic cholecystitis: The pathological features of chronic cholecystitis include subserosal fibrous tissues, lymphocytes, plasma cells, macrophages found beneath the columnar epithelium, and Rokitansky-Aschoff sinus in the muscular layer.

Gallbladder contraction: Gallbladder contraction is known to result from long-standing chronic cholecystitis. Anatomical characteristics include severe adhesions and complete coverage by greater omentum, severe pericholecystic fibrosis, thickening of gallbladder walls (usually thicker than 5 mm), loss of elasticity, obscure anatomy at triangle of Calot, dense adhesions to the duodenum, transverse colon and common hepatic duct, and small GBV (usually < 10 mL).

Non-gallstone controls: We randomly chose 240 patients who received abdominal computed tomography (CT) for non-gallstone reasons between July 1, 2008 and July 31, 2008.

Demographic data: The age, sex, associated diseases and previous operations for all patients were recorded. The procedures included preoperative CT measurement of gallbladder diameter, and directly measured gallbladder diameter. Directly measured GBV by the saline-filling method in patients with gallstones and acute and chronic cholecystitis were also recorded. The CT measurements of non-gallstone controls were also recorded. The GBV was calculated from the gallbladder lengths and short axis diameters by the equation shown below.

GBV measurements and calculations: We measured the lengths and short axis diameters of the fasting gallbladder of patients from their preoperative CT films, measured the lengths and short axis diameters directly after their removal in the operating room, and recorded their lengths and short axis diameters measured by the pathologist postoperatively. We then calculated the GBV according to the equation: GBV (cm^3) = $4/3 \times \pi \times r1 \times r2 \times r3/\cos\theta$, where r1, r2 and r3 represent the three greatest radii of the gallbladder ellipsoid and θ is the angle between the z axis of the gallbladder and the z axis of the transverse cross sectional CT imaging.

We also measured directly the GBV after the gallbladders were removed. After withdrawing the gallbladder bile with a syringe, the volume of normal saline injected

into the intact gallbladders was added to the volume of gallbladder stones measured after opening the GB walls. Comparing the GBV obtained by direct measurement and calculations using the various techniques, correction factors for different measuring methods were obtained to modify the calculated GBV, thus minimizing the measurement bias.

Rationale for GBV equation[7]

The gallbladder is a pear-shaped organ located under the liver, which stores bile. It can be approximated to an ellipsoid shape. The volume of an ellipsoid can be obtained as follows[8]: Claim area for ellipse is π r1 r2: given an ellipse $x^2/r1^2 + x^2/r2^2 = 1$. Since an ellipse is symmetric to the x and y axes, Area $= 4\int^{r1}_0 r2\ (1 - x^2/r1^2)^{1/2}$ dx $= 4r2/r1\int^{r1}_0 (r1^2 - x^2)^{1/2}$ dx. (Let x = r1sinθ, then dx = r1cosθdθ, where $-\pi/2 < \theta < \pi/2$) $= 4r2/r1\int^{\pi/2}_0 (r1^2 - r1^2\sin^2\theta)^{1/2}$ r1cosθ dθ $= 4r1r2\int^{\pi/2}_0 \cos^2\theta d\theta = 4r1r2\int^{\pi/2}_0 1/2\ (1 + \cos2\theta)$ dθ $= 2$ r1r2 $[\theta + 1/2\sin^2\theta]^{\pi/2}_0 = \pi$ r1r2 = > For a slice of ellipse of the ellipsoid parallel to the yz plane, the radius parallel to the z axis is g (z) = r1 $(1 - z^2/r3^2)^{1/2}$. Similarly, its radius parallel to the y axis is h (z) = r2 $(1 - z^2/r3^2)^{1/2}$. Thus, the volume V of the ellipsoid $= \int^{r3}_{-r3}\pi$ g (z) h(z)dz $= \int^{r3}_{-r3}\pi$ r1 $(1 - z^2/r3^2)^{1/2}$ r2 $(1 - z^2/r3^2)^{1/2}$ dz $=\pi$ r1 r2$\int^{r3}_{-r3} (1 - z^2/r3^2)$ dz $=\pi$ r1 r2 $[z - z^3/3r3^2]^{r3}_{-r3} = 4/3\pi$ r1r2r3 #.

Correction factors for GBV

θ: The gallbladder does not always lie in the same plane, therefore, the measured GBV is corrected by multiplying a correction factor 1/cos θ, where θ is the angle between the z axis of the gallbladder and the z axis of the transverse cross sectional CT imaging and $0° \leqslant \theta < 90°$.

c = 0.82 ± 0.40 (n = 65): CT measurement correction factor = (Direct measurement calculated GBV)/(CT measurement calculated GBV).

p = 0.85 ± 0.42 (n = 65): Pathology measurement correction factor = (Pathology measurement calculated GBV)/(Direct measurement calculated GBV).

s = 0.88 ± 0.36 (n = 65): Shueh-Ding correction factor = (Direct injection measurement GBV)/(Direct measurement calculated GBV).

Direct measurement GBV = Direct measurement calculated GBV × 0.88 (s, Shueh-Ding correction factor).

u = 0.90 (n = 65): Ultrasound (US) measurement correction factor = (Direct measurement calculated GBV)/(US measurement calculated GBV).

Direct injection measurement GBV = Direct measurement calculated GBV × s = CT measurement calculated GBV × cs (cs = 0.72 = 0.82 × 0.88) = US measurement calculated GBV × us (us = 0.79 = 0.9 × 0.88).

Substudy on gallbladder contractility: To investigate and compare the gallbladder contractility between patients with gallstones and chronic cholecystitis and normal subjects, and the possible reciprocal correlation between GBV and gallbladder contractility, we designed and conducted a subsequent second study. Between October 1, 2008 and June 30, 2009, we measured the fasting and postprandial GBV of 65 patients with gallstones and chronic cholecystitis and 53 healthy subjects, by abdominal ultrasonography.

Study protocol: Subjects came to the hospital after an overnight fast.

They were reminded the night before the study not to eat or drink after supper. Their adherence to this instruction was confirmed by performing a history taking to make sure that the subjects did adhere to the investigator's instructions, including nothing by mouth after the previous supper. Gallbladder size was determined in the fasting state. The subjects then drank a standard liquid meal with a total 420 kcal: 30% as fat, 15% as protein, and the rest as carbohydrate. The postprandial gallbladder size was measured and GBV was calculated 90 min after the meal[9].

Ultrasound technique: The abdominal sonography was performed by the same investigator (Huang SM) with techniques modified from the literature[9]. The hand-held transducer was placed in a sagittal longitudinal projection in the right-upper quadrant and rotated until the largest longitudinal dimension of the gallbladder was obtained on the oscilloscope screen. The image was frozen, measured by electronic calipers, and photographed. The transducer was then rotated 90° and the largest short axis through the gallbladder was obtained. Occasionally, deep inspiration was required, but all patients were successfully examined. We used three dimensions - length, lateral and antero-posterior diameters - to calculate the GBV, which was modified empirically as described above.

The ejection fraction (EF) of the gallbladder was calculated by the following equation: EF (%) = [Fasting GBV (mL) - Postprandial GBV (mL)]/[Fasting GBV (mL)] × 100.

Statistics: We compared the GBV measurements and patient age using Student's unpaired t test, and the χ^2 test to compare the sex differences. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data

We found a male predominance in the acute cholecystitis group and a female predominance in the chronic cholecystitis group: male/female (M/F) = 53/46 and 32/53, respectively ($P = 0.04$ by χ^2 test). There was no sex difference between patients in the chronic cholecystitis group and the non-gallstone group (M/F = 32/53 *vs* 117/123, $P = 0.10$). There was no age difference among patients in the acute cholecystitis group, the chronic cholecystitis group and the non-gallstone group (57 ± 17 years, 53 ± 15 years and 54 ± 18 years, $P = 0.09$ and 0.64, respectively). Associated diseases and previous operations undergone for all patients and normal subjects included cerebral vascular accident (3), diabetes mellitus

Table 1 Demographic data and gallbladder volume in gallstone and non-gallstone subjects

Variables diagnosis	M/F	Mean age (yr)	Mean GBV (cm^3) (*n*)	SD (cm^3)	*P*-value (95% CI[1])
Acute Cholecystitis	[D]53/46[2]	57 ± 17	[A]57.00 (99)	41.20	0.0004[3] (7.73-26.35)
Chronic Cholecystitis	32/53	53 ± 15	[B]28.77 (85)	15.00	< 0.0001[4] (8.14-15.86)
Contracted Gallbladder	1/4	44 ± 21	6.15 (5)	2.52	
Non-gallstone	[E]39/41	54 ± 18	[C]16.77 (240)	15.75 -	

[1]95% CI of gallbladder volume (GBV) difference; [2]*P* value D *vs* E = 0.04, between gender of acute and chronic cholecystitis; [3]*P* value A *vs* B: between GBV of acute and chronic cholecystitis; [4]*P* value B *vs* C: between chronic cholecystitis and non-gallstone subjects. SD: Standard deviation.

Table 2 Incidence of gallstones and gallbladder volume of non-gallstone subjects at different ages

Patients age periods (*n* = 85)	Incidence of gallstone[1] (%)	Non-gallstone		*P* value
		Mean GBV (cm^3) (*n*)	SD (cm^3)	
Age ≤ 20	0	[A]6.33 (9)	2.75	0.007[2]
20 < Age ≤ 40	15	[B]16.84 (35)	11.04	0.30
40 < Age ≤ 60	42.5	[C]15.12 (106)	7.55	0.0003[3]
60 < Age ≤ 80	42.5	[D]19.83 (81)	9.99	0.76
80 < Age	0	18.73 (9)	10.32	

[1]*P* = 0.0027 by Fisher's exact test; [2]*P* value A *vs* B: between Age ≤ 20 and 20 < Age ≤ 40; [3]*P* value C *vs* D: between 40 < Age ≤ 60 and 60 < Age ≤ 80. GBV: Gallbladder volume; SD: Standard deviation.

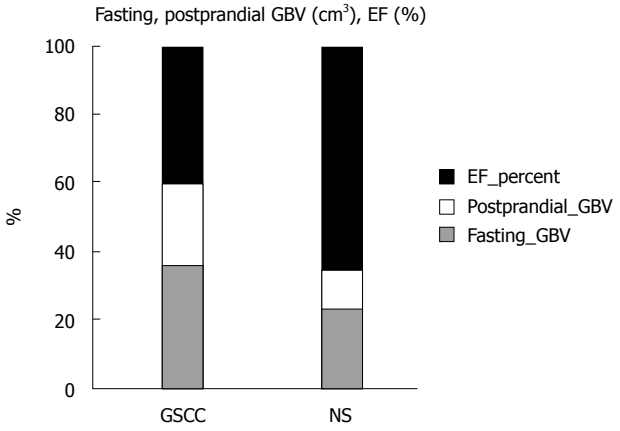

Figure 1 Histogram of the mean fasting, postprandial gallbladder volume and ejection fraction in gallstone patients with chronic cholecystitis and normal subjects, measured by abdominal ultrasonography. GBV: Gallbladder volume; EF: Ejection fraction; GSCC: Gallstones with chronic cholecystitis; NS: Non-stone.

(4), pneumonia (1), hyperlipidemia (1), hypertensive cardiovascular diseases (5), status/post exploratory laparotomy (2) and coronary artery disease (5) in the gallstones patients with acute cholecystitis; diabetes mellitus (5) and manic-depressive psychosis (1) and status/post gastrectomy/vagotomy (2) in the gallstones patients with chronic cholecystitis; and liver tumor (2), liver cysts (5), diabetes mellitus (9), hypertensive cardiovascular diseases (10) and status/post exploratory laparotomy (5) in the non-gallstone group. The GBV in the diabetic and non-diabetic non-gallstone patients did not differ significantly (16.18 ± 2.79 cm^3, *n* = 9 *vs* 16.79 ± 9.43 cm^3, *n* = 231, *P* = 0.85). The GBV in the s/p gastrectomy/vagotomy and non-s/p gastrectomy/vagotomy, non-gallstone patients did not differ significantly either (19.85 ± 3.41 cm^3, *n* = 2 *vs* 16.74 ± 9.30 cm^3, *n* = 238, *P* = 0.64). Thirty patients in the acute cholecystitis group underwent laparoscopic cholecystectomy. They comprised 30.3% (30/99) of the acute cholecystitis patients. Seventy-seven patients in the chronic cholecystitis group underwent laparoscopic cholecystectomy. They comprised 90.6% (77/85) of the chronic cholecystitis patients. The remaining 77 patients underwent open cholecystectomy.

The fasting GBV in gallstone patients with acute cholecystitis (*n* = 99), chronic cholecystitis (*n* = 85), gallbladder contraction (*n* = 5) and non-gallstone subjects (*n* = 240) was 57.00 ± 41.20 cm^3, 28.77 ± 15.00 cm^3, 6.15 ± 2.52 cm^3 and 16.77 ± 15.75 cm^3, respectively (Table 1). The average fasting GBV was larger in gallstone patients with acute cholecystitis than that with chronic cholecystitis (*P* < 0.0001). In turn, the average fasting GBV was larger in gallstone patients with chronic cholecystitis than in non-gallstone subjects (*P* < 0.0001, Table 1).

In non-gallstone subjects, there was a significant difference in the fasting GBV between two age groups, i.e. age ≤ 20 years *vs* 20-40 years (6.33 ± 2.75 cm^3, *n* = 9 *vs* 16.84 ± 4.04 cm^3, *n* = 35, *P* = 0.0070) and age 40-60 years *vs* 60-80 years (15.12 ± 7.55 cm^3, *n* = 106 *vs* 19.83 ± 9.99 cm^3, *n* = 81, *P* < 0.0003) (Table 2). The fasting GBV had a tendency to increase with age. In accordance with this tendency, the occurrence of gallstones with chronic cholecystitis also increased with age (*P* = 0.0027 by Fisher's exact test, Table 2).

In the second substudy, the average fasting/postprandial GBV in patients with gallstones and chronic cholecystitis was 31.44 ± 16.46 cm^3/20.50 ± 6.20 cm^3. The average gallbladder EF of patients with gallstones and chronic cholecystitis was 34.6% ± 10.6% (*n* = 65). The average fasting/postprandial GBV in normal subjects was 19.22 ± 11.30 cm^3/9.03 ± 3.63 cm^3. The average EF of normal gallbladder was 53.3% ± 24.9% (*n* = 53). The differences in the average fasting, postprandial GBV and EF were statistically significant (all three *P* < 0.0001, Figure 1, Table 3). Also, the differences in GBV between fasting and postprandial states in patients with gallstones and chronic cholecystitis and normal subjects were statistically significant (both *P* < 0.0001).

The fasting GBV was larger in late stage than early/second stage acute cholecystitis in gallstone patients (84.66 ± 26.32 cm^3, *n* = 12, *vs* 53.19 ± 33.80 cm^3, *n* = 87, *P* = 0.002, Table 4). The average fasting GBV was larger (28.77 ± 15.00 cm^3 *vs* 6.77 ± 15.75 cm^3, *P* < 0.0001) and the average EF was lower (34.6% ± 10.6%, *n* = 65, *vs*

Table 3 Mean fasting, postprandial gallbladder volume and ejection fraction in gallstones patients and normal subjects

	Fasting mean GBV ± SD (cm^3) (*n*)	Postprandial mean GBV ± SD (cm^3) (*n*)	EF ± SD (%) (*n*)
GS	[A]31.34 ± 16.46 (65)	[B]20.50 ± 6.20[1] (65)	[C]34.6 ± 10.6 (65)
Normal subjects	[D]19.24 ± 11.30[2] (53)	[E]9.03 ± 3.63[2] (53)	[F]53.3 ± 24.9 (53)
P-value	< 0.0001[3]	< 0.0001[4]	< 0.0001[5]
(95% CI)	(A-D, 6.93-17.47)	(B-E, 9.56-13.48)	(C-F, 11.9-25.5)

[1]*P* value A *vs* B < 0.0001, A-B 95% CI: 6.62–15.26; [2]*P* value D *vs* E < 0.0001; D-E 95% CI: 6.98-13.44; [3]*P* values A *vs* D: between fasting gallbladder volume (GBV) of chronic cholecystitis and normal subjects; [4]*P* values B *vs* E: between postprandial GBV of chronic cholecystitis and normal subjects; [5]*P* values C *vs* F: between ejection fraction (EF) of chronic cholecystitis and normal subjects. SD: Standard deviation; GS: Gallstones.

53.3% ± 24.9%, $n = 53$, $P < 0.0001$, Table 3) in gallstone patients with chronic cholecystitis, compared with those in non-gallstone and normal subjects.

DISCUSSION

From clinical observations, male patients with gallstone disease seem to have a higher threshold for pain caused by biliary stones than their female counterparts. Perhaps this is one of the reasons that underlay the male preponderance seen in the acute calculous cholecystitis group in this study.

Our study showed that the fasting GBV increased in two age groups in non-gallstone subjects, i.e. from age 20 to 40 years and 60 to 80 years; especially the former period. In accordance with this differential increase in GBV in specific periods, the occurrence of gallstones reached its plateau between 40 and 80 years of age (from 15% before 40 years old to 42.5% after 40 years, $P = 0.0027$, Table 2).

The gallbladder is a hollow visceral elastic organ. Its actual maximal fasting volume under *in vivo* physiological conditions is difficult to measure. Measuring methods include *in vivo* ultrasonography, nuclear scintigraphy, CT and *in vitro* post-cholecystectomy saline-filling measurement. hepatobiliary iminodiacetic acid scintigraphy is seldom used clinically except to demonstrate cystic duct obstruction in acute cholecystitis patients, due to its isotope radioactivity. On the other hand, *in vitro* post-cholecystectomy saline-filling measurement is the most accurate gold standard for measuring GBV. In contrast, abdominal ultrasonography is the most convenient examination for measuring GBV. However, the accuracy and precision depend greatly on the operators' individual experiences. CT represent the more uniform, constantly used and easier method for diagnosis of gallstone diseases and for measuring GBV. These are the reasons why we adopted abdominal ultrasonography, CT and *in vitro* post-cholecystectomy saline-filling measurement in the present study.

Table 4 Mean gallbladder volume in acute calculous cholecystitis group: simple *vs* with gangrene/abscess

Variable GS patients	Mean GBV ± SD (cm^3) (*n*)	[1]*P*-value A *vs* B (A-B, 95% CI)
Simple ACC	[A]53.19 ± 33.80 (87)	0.002 (12.28-52.66)
With gangrene/abscess	[B]84.66 ± 26.32 (12)	

[1]*P* values, A *vs* B: mean gallbladder volume (GBV) of patients with simple acute calculous cholecystitis (ACC) *vs* ACC with gangrene/abscess. SD: Standard deviation; GS: Gallstones.

GBV can have clinical and therapeutic implications and reflect pathophysiological mechanisms of gallstone diseases. The clinical and therapeutic implications of increased GBV lie in the cystic duct obstruction in acute calculous cholecystitis. The dilatation of the gallbladder is due to stones being trapped in the spiral valve of Heist. The onset of painful dilatation of the gallbladder is acute and is caused by the stones irritating the gallbladder mucosa, and the pressure exerted by the dilated gallbladder on the visceral nerve endings distributed over the entire gallbladder wall. Early dilatation of the gallbladder due to undrained mucus secretions also predisposes to compromised local blood circulation to the mucosa and the gallbladder wall, which leads to impaired absorption of water and electrolytes, which further aggravates the dilatation[10-12]. GBV can usually reach up to 3.4 times the normal size. The vicious cycle of undrained mucus secretions and impaired absorption of water and electrolytes, coupled with chemical irritation by lysozyme, cytokines and chemokines, sometimes causes gangrene and/or perforation in 15% and 1.5%, respectively, of patients with acute calculous cholecystitis[2].

In our study, the fasting GBV was larger in gallstone patients with acute cholecystitis than with chronic cholecystitis (Table 1). Furthermore, local gallbladder wall necrosis/abscess was associated with further increases in GBV (Table 4). It is possible that gallbladder decompression might prevent gangrene/empyema and perforation and make it easier to manipulate the gallbladder during cholecystectomy.

Gallbladder contraction is known to result from long-standing chronic cholecystitis and has the smallest GBV. The anatomical characteristics make it a difficult challenge to perform laparoscopic cholecystectomy safely.

The fasting GBV was larger in gallstone patients with chronic cholecystitis than that in non-gallstone subjects (28.77 ± 15.00 cm^3, $n = 85$, *vs* 16.77 ± 15.75, $n = 240$, $P < 0.0001$), which indicated possible pathophysiological mechanisms of weaker gallbladder contractility in gallstone patients with chronic cholecystitis, which was confirmed in our second study. In 2000, Portincasa proposed that larger GBV predisposes to bile stasis[6].

Weaker gallbladder contractility in gallstone patients with chronic cholecystitis might provide a suitable micro-milieu for cholesterol monohydrate micro-crystals

to grow into larger gallbladder stones, by preventing them from expulsion from the gallbladder lumen, when the gallbladder contracts in response to cholecystokinin (CCK) secreted from duodenum I cells stimulated by proteins and lipids in ingested food[8].

In our second study, we measured fasting and postprandial GBV of 65 patients with gallstones and chronic cholecystitis and 53 healthy subjects, by abdominal ultrasonography and calculated the gallbladder EF. The average EF of patients with gallstones and chronic cholecystitis was lower than that of the healthy subjects. The difference in EF was statistically significant (Table 3).

Developmental stages of acute cholecystitis

From our observations on the GBV changes during the development of acute cholecystitis from a chronic inflammatory state, we hypothesize that there are three stages based on GBV and pathological changes. These correspond to the three stages of severity of acute cholecystitis proposed by Miura *et al*[13] at the Tokyo International Consensus Meeting in 2007.

Early (clinical) stage

In the early (clinical) stage of acute cholecystitis, which corresponds to mild (grade I) severity in the Tokyo consensus meeting, the mechanical effect of cystic duct obstruction causes an increase in GBV (from 28.77 ± 15.00 cm^3, n = 85, to 54.16 ± 35.17 cm^3, n = 44, P < 0.0001). No acute inflammatory leukocyte infiltration is observed, but chronic inflammatory reactions, such as subserosal fibrous tissues, and infiltration of lymphocytes, plasma cells, and macrophages beneath the columnar epithelium and Rokitansky-Aschoff sinus in the muscular layer are present, as revealed by pathological examinations. The early (clinical) stage of acute cholecystitis comprises about 44.4% (44/99). Early laparoscopic cholecystectomy is suggested by the Tokyo guidelines.

Second (pathological) stage

In the second (pathological) stage of development, which corresponds to the moderate severity (grade II) stage of the Tokyo guidelines, the GBV does not change significantly. However, pathological findings of acute immunoreactive and inflammatory responses appear in the gallbladder. These include leukocyte infiltration throughout the tissues, local flattening and denuded mucosal folds, and tissue edema. It comprises about 43.4% (43/99). Early laparoscopic or open cholecystectomy is suggested by the Tokyo guidelines. If a patient has serious local inflammation that makes early cholecystectomy difficult, then percutaneous or operative drainage of the gallbladder is recommended. Elective cholecystectomy can be performed after improvement of the acute inflammatory process.

Late (complicated) stage

If the condition goes untreated or is unresolved by treatment, the late (complicated) stage ensues, which corresponds to the severe (grade III) organ dysfunction stage of the Tokyo guidelines. In this stage, GBV further increases (Table 4). Besides the pathological findings of acute immunoreactive and inflammatory responses, local gallbladder wall necrosis (gangrene), abscess and/or even perforations occur. Organ dysfunctions appear. It comprises about 12.2% (12/99). Appropriate organ support in addition to medical treatment for patients with organ dysfunction is suggested by the Tokyo guidelines. Management of severe local inflammation by percutaneous gallbladder drainage and/or cholecystectomy is needed. Biliary peritonitis due to perforation of the gallbladder is an indication for urgent cholecystectomy and drainage. Elective cholecystectomy may be performed after improvement of the acute illness by gallbladder drainage[13].

Zhu *et al*[14] have found that the amount of gallbladder CCK receptor is lower in patients with gallstones who have poor gallbladder contraction. Choi has found that FGF15 knockout mice have a gallbladder that is completely devoid of bile, and administration of recombinant FGF15 or FGF19 restores the GBV[8,15]. Whether gallstone patients with poor gallbladder contraction and increased GBV have lower levels of CCK receptor and/or lower FGF19 gene expression is worth further investigation.

Different kinds of diet predispose humans to different gallstone diseases. At present, we have no data concerning how the diet consumed by the gallstone patients was different from that consumed by the normal population. If the diet differs, it is worthwhile investigating whether the diet predisposes the patients to gallstone diseases through its effect on GBV.

There is a limitation to this study. The gallstone patients were not subgrouped into those with cholesterol and pigment stones. Therefore, the findings of Masclee[3-5] and Portincasa[6] could not be investigated in this study.

In summary, we found that the fasting GBV increased in two periods in non-gallstone subjects, i.e. from age 20 to 40 years and 60 to 80 years. In accordance with these age preferences, the occurrence of gallstones reached its peak between 40 and 80 years of age. Moreover, we found that gallstone formation was associated with poorer gallbladder contractility and larger fasting and postprandial GBV Also, GBV increased as acute cholecystitis progressed. Therefore, gallbladder decompression is mandatory to prevent gangrene and/or empyema of gallbladders.

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COMMENTS

Background

Gallbladder volume (GBV) changes can have clinical and therapeutic implications, and reflect physiological and functional status, and possibly pathophysiological mechanisms of gallstone diseases. However, the differences and changes in GBV among different age groups in non-gallstone subjects and their correlations with the formation of gallstones have not been reported previously. Also, the correlations between GBV changes in different stages in the pathophysiological development of acute cholecystitis in gallstone patients are not

clear. Furthermore, the authors wished to investigate the possible reciprocal correlation between GBV and gallbladder contractility. The methodology used to measure *in vivo* GBV is hardly reported in the literature.

Research frontiers

The methods the authors developed in this study can provide a reliable method to measure the *in vivo* GBV in a way that can be adjusted to approximate the direct measurements by the saline injection method. In this study, the authors found that GBV increased as acute cholecystitis progressed to gangrene and/or empyema. The fasting volume of gallbladders was larger in late stage than in early/second stage acute cholecystitis. This forms the pathophysiological basis for the guidelines suggested by the Tokyo International Consensus Meeting in 2007.

Innovations and breakthroughs

This is believed to be the first study to report the differential increase in GBV in specific age groups, which coincides with and partly explains the high occurrence rate of gallstones, which reached their plateau between 40 and 80 years of age. This is also believed to be the first study to demonstrate one of the pathophysiological mechanisms that underlie the development of acute calculous cholecystitis, which form the basis for the severity staging system in the Tokyo International Consensus Meeting.

Applications

An interesting area of future investigation would be to apply the authors' measurements and gallbladder emptying assessment to patients without gallstone disease but with right upper quadrant pain. By contributing to the authors' understanding of how acute calculous cholecystitis develops, this study could represent a rationale for therapeutic decompression intervention, by drainage or straight cholecystectomy, in the treatment of patients with acute calculous cholecystitis.

Peer review

The reviewer found that this was an interesting study that was performed well. Also, the reviewer wonders what the diet was like in the normal and diseased populations, because that would have an effect on GBV. This is a good contribution to a topic that has not been described previously.

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BRIEF ARTICLE

Association of NOD1 (CARD4) insertion/deletion polymorphism with susceptibility to IBD: A meta-analysis

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Abstract

AIM: To find evidences about whether NOD1/CARD4 insertion/deletion polymorphism is associated with inflammatory bowel disease by meta-analysis.

METHODS: We surveyed the studies on the association of NOD1/CARD4 insertion/deletion polymorphism with inflammatory bowel disease in PubMed. Meta-analysis was performed for genotypes GG/T *vs* T/T, GG/GG *vs* T/T, GG/T + GG/GG *vs* T/T, GG/GG *vs* T/T + GG/T, and GG allele *vs* T allele in a fixed/random effect model.

RESULTS: We identified 8 studies (6439 cases and 4798 controls) in Caucasian populations using PubMed search. We found no association between NOD1/CARD4 insertion/deletion polymorphism and inflammatory bowel disease, Crohn's disease, and ulcerative colitis. Stratification of cases by age showed that NOD1/CARD4 insertion/deletion polymorphism was associated with inflammatory bowel disease in younger age group at onset (< 40 years) (GG *vs* T: OR = 0.68, 95% CI: 0.50-0.93, P = 0.02; GG/T + GG/GG *vs* T/T: OR = 0.71, 95% CI: 0.59-0.85, P = 0.0003).

CONCLUSION: This meta-analysis demonstrates an association between NOD1/CARD4 insertion/deletion polymorphism and inflammatory bowel disease in the younger age group at onset (< 40 years) in Caucasian populations.

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Key words: NOD1; CARD4; Genetic polymorphisms; Inflammatory bowel disease; Meta-analysis

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Lu WG, Zou YF, Feng XL, Yuan FL, Gu YL, Li X, Li CW, Jin C, Li JP. Association of NOD1 (CARD4) insertion/deletion polymorphism with susceptibility to IBD: A meta-analysis. *World J Gastroenterol* 2010; 16(34): 4348-4356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i34/4348.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i34.4348

INTRODUCTION

The inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a com-

mon relapsing condition characterized by both gastrointestinal and systemic manifestations and is responsible for a significant morbidity in both adults and children. Clinical symptoms of IBD include weight loss, abdominal pain, as well as diarrhea accompanied by blood, and disease progression is often accompanied by an increase in granulomas and activated monocytes, which produce significant amounts of eicosanoids and cytokines[1,2]. The etiology of IBD most likely involves a complex interaction of genetic, environmental and immunoregulatory factors[3].

The normal gut consists of an epithelial barrier, the mucosal immune system, and a number of stromal/supportive cells. The external environment comprises native mucosal microbiota, potential pathogenic microorganisms, abundant food antigens and allergens, all of which are encountered mainly at the vast surface areas of mucosal membranes, and forms the most important source of stimulation of the entire immune system. The induction of preventive and protective immune responses to mucosal infectious agents and to inert food antigens and environmental allergens that would limit their absorption, is usually the most emphasized functional aspect of the mucosal immune system[4]. Dysfunctional innate immune response seems important in the pathogenesis of IBD[5]. By means of genome-wide scans, numerous IBD susceptibility loci have been identified[6]. Specific single gene defects have been discovered, including mutations in the leucine rich region (LRR) of the nucleotide-binding oligomerization domain 2 (NOD-2) gene, also known as CARD-15 (caspase activation and recruitment domain 15)[7,8]. The identification of the NOD2 is a breakthrough in IBD genetics, which heralded extensive analyses of signaling pathways of the innate immune system implicated in the pathogenesis of IBD[9,10].

Innate immunity depends on the specific recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs). The NOD protein is a family of intracellular PRRs. After the intracellular PRRs recognized PAMPs, the pro-inflammatory pathways would be activated[11-13]. NOD1 (also known as CARD-4) is a host cytosolic signaling PRR, and acts as a cytosolic receptor for the diaminopimelate (DAP)-containing GlcNAc-tripeptide muropeptide found mostly in Gram-negative bacterial peptidoglycans[14]. NOD1/CARD4 signaling leads to activation of NF-κB, and plays an important role in innate immunity[15]. In 1903, Sutton[16] explained that dominance and recessiveness were features of "chromatin entities" rather than morphological characters. In other words, dominance and recessiveness are properties of genetic information resulting in a certain function rather than the function itself. This means that certain polymorphisms and mutations in NOD1/CARD4 may result in dysfunctional innate immune response during bacterial recognition with direct implications for IBD pathogenesis. Genome-wide scans for IBD linkage demonstrated a susceptibility locus on chromosome 7p14, and the same locus where the NOD1/CARD4 gene is located[17]. Therefore, NOD1/CARD4 gene is a perfect candidate for predisposition to IBD.

NOD1/CARD4 insertion/deletion polymorphism (rs6958571) was identified by Hysi *et al*[18]. Since its discovery in 2005, this polymorphism has attracted widespread attention. A number of case-control studies were conducted to investigate the association of this polymorphism with human IBD[19-25]. However, these studies reported conflicting results. There are several possible explanations for this, such as small sample size, ethnic background, uncorrected multiple hypothesis test, and publication bias.

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis for the estimation of genetic effects[26]. The aim of the present study was to investigate the association of NOD1/CARD4 insertion/deletion polymorphism with human IBD, using a meta-analysis.

MATERIALS AND METHODS

Identification of eligible studies

Available articles were identified through a literature search using the keywords "nucleotide-binding oligomerization domain 1" or "NOD1" or "caspase activation and recruitment domain 4" or "CARD4" and "polymorphism" in the PubMed database. Additional literature was collected from cross-references within both original and review articles. We only recruited data from the wholly published paper, but not from meeting or conference abstracts. No language restrictions were applied. A study was included in the current meta-analysis if (1) it was published up to December 2009; and (2) it was a case-control study. We excluded the studies containing overlapping data and the family-based studies because our analysis was based on linkage considerations. When there were multiple publications from the same population, only the largest study was included. When a study reported the results on different subpopulations, we took it as a separate study.

Additionally, an independent PubMed search was done (by Lu WG and Feng XL) by the same method. The abstracts were reviewed independently by two investigators (Yuan FL and Gu YL) to determine if they met the eligibility criteria for inclusion. References in the studies were reviewed (by Jin C, Li X and Li CW) to identify additional studies. If discrepancies occurred, a third investigator (Li JP) did an additional assessment.

Data extraction

If original genotype frequency data was unavailable in relevant articles, a request for additional data was sent to the corresponding authors. In addition, two investigators (Feng XL and Li JP) independently extracted the data with the standard protocol and the result was reviewed by a third investigator (Zou YF). Discrepancies were resolved

Table 1 Characteristics of the studies included in the meta-analysis

ID	Study	Yr	Ethnic group	Diseases	Sample size (frequency of GG allele, %)		OR (95%CI) for GG *vs* T allele	Hardy-Weinberg equilibrium of genotype control
					Case	Control		
1	Hancock *et al*[19]	2008	Caucasian	CD	594 (27.1)	1024 (24.6)	1.130 (0.960-1.330)	0.010
2	Cantó *et al*[20]	2007	Caucasian	CD	97 (21.7)	50 (31.0)	0.615 (0.357-1.060)	0.147
3	Henckaerts *et al*[21]	2007	Caucasian	IBD	1052 (24.5)	280 (25.4)	0.952 (0.768-1.180)	0.751
				CD	809 (24.8)		0.973 (0.780-1.214)	
				UC	222 (22.9)		0.878 (0.656-1.175)	
4	Van Limbergen *et al*[22]	2007	Caucasian (Scottish)	IBD	1079 (26.1)	1233 (26.4)	0.984 (0.863-1.089)	0.261
				CD	515 (26.4)		1.003 (0.850-1.182)	
				UC	537 (26.0)		0.985 (0.837-1.160)	
5	Van Limbergen *et al*[22]	2007	Caucasian (Swedish)	IBD	632 (25.3)	277 (23.3)	1.112 (0.880-1.406)	0.741
				CD	244 (24.7)		1.086 (0.817-1.444)	
				UC	388 (25.5)		1.129 (0.875-1.456)	
6	Franke *et al*[23]	2006	Caucasian	IBD	961 (22.4)	841 (21.5)	1.055 (0.900-1.235)	0.958
				CD	633 (21.1)		0.983 (0.822-1.174)	
				UC	332 (24.4)		1.181 (0.955-1.460)	
7	Tremelling *et al*[24]	2006	Caucasian	IBD	1360 (25.2)	758 (25.7)	0.975 (0.844-1.126)	0.580
				CD	641 (24.9)		0.964 (0.812-1.144)	
				UC	665 (25.4)		0.991 (0.837-1.173)	
8	McGovern *et al*[25]	2005	Caucasian	IBD	664 (26.6)	335 (31.8)	0.777 (0.634-0.952)	0.195
				CD	358 (24.4)		0.694 (0.548-0.878)	
				UC	306 (29.1)		0.880 (0.693-1.117)	

OR: Odds ratio; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

through discussion among our research team. From each study, we extracted the first author's name, year of publication, source of publication, racial ancestry, type of diseases, the number of cases and controls, and the available genotype and allele frequency information from the NOD1/CARD4 insertion/deletion polymorphism.

Meta-analysis methods

Allele frequencies at the NOD1/CARD4 insertion/deletion polymorphism from the respective studies were determined by the allele counting method. A χ^2 test was used to determine if the observed frequencies of genotypes conformed to Hardy-Weinberg equilibrium expectations.

We examined the relationship between the allele and susceptibility to IBD (GG *vs* T), and the genotypes. The following genotype contrasts were included: GG/T + GG/GG *vs* T/T, GG/GG *vs* T/T + GG/T, GG/GG *vs* T/T, and GG/T *vs* T/T. The contrast of GG/T + GG/GG *vs* T/T genotypes corresponds to a dominant genetics effect of the GG allele. The contrast of GG/GG *vs* T/T + GG/T genotypes corresponds to a recessive genetics effect of the GG allele. The odds ratio (OR) and its 95% confidence interval (95% CI) were estimated for each study. The heterogeneity between studies was assessed by the Chi-square test based Q-statistics[27]. A significant Q-statistics ($P < 0.10$) indicated the heterogeneity among studies, and then the result of the random effect model was selected. Otherwise, the result of fixed effect model was selected. We also measured the effect of heterogeneity using the formula: $I^2 = 100\% \times (Q\text{-}df)/Q$[28].

Finally, the pooled OR was obtained by Mantel-Haenszel method in the fixed effect model and by DerSimonian and Laird method in the random effect model[29,30]. The pooled OR was performed, weighting the individual OR by the inverse of their variance. The significance of the pooled OR was determined by the *Z* test.

Evaluation of publication bias

Publication bias was investigated with the funnel plot. Funnel plot asymmetry was further assessed by the method of Egger's linear regression test[26]. Analyses were performed using the software Review Manager 4.2 (Cochrane Collaboration, http://www.cc-ims.net/RevMan/relnotes.htm/) and Stata version 10 (StataCorp LP, College Station, Texas, USA). A *P* value less than 0.05 was considered statistically significant, and all the *P* values were two sided.

RESULTS

Characteristics of eligible studies

Characteristics of studies included in the current meta-analysis are presented in Table 1[19-25]. There were 46 papers relevant to the searching words. Through the screening of the abstract, 19 of these articles were excluded (5 were reviews; 4 were not conducted in humans; 10 did not explore NOD1/CARD4 gene polymorphisms), leaving 27 studies for full publication review. Of the 27 studies, 13 without focusing on IBD, were excluded, leaving 14 studies[19-25,31-37] for more detailed assessment. Seven of them were excluded (one was a family-based study; one was a duplicate report; and 5 did not study the NOD1/CARD4 insertion/deletion polymorphism)[31-37]. As a result, 7 studies were included in the current meta-analysis (Figure 1). One of the eligible studies contained data on two dif-

Table 2 Meta-analysis of association between NOD1/CARD4 insertion/deletion polymorphism and inflammatory bowel disease

Polymorphism	Disease	Sample size		*n*	Test of association				Test of heterogeneity		
		Case	Control		OR (95%CI)	*Z*	*P* value	Model	χ^2	*P* value	I^2 (%)
GG *vs* T	Overall	12878	9596	8	0.98 (0.90-1.07)	0.39	0.70	R	12.60	0.08	44.4
	CD	7782	9596	8	0.96 (0.86-1.07)	0.78	0.43	R	14.49	0.04	51.7
	UC	4900	7448	6	1.01 (0.92-1.09)	0.14	0.89	F	5.12	0.40	2.3
	IBD onset < 40	1486	4752	4	0.68 (0.50-0.93)	2.38	0.02	R	8.30	0.04	63.9
GG/T + GG/GG *vs* T/T	Overall	6439	4798	8	1.00 (0.98-1.08)	0.01	0.99	F	10.69	0.15	34.5
	CD	3891	4798	8	0.97 (0.86-1.10)	0.42	0.67	R	12.36	0.09	43.4
	UC	2450	3724	6	1.00 (0.90-1.11)	0.06	0.95	F	4.64	0.46	0.0
	IBD onset < 40	743	2376	4	0.71 (0.59-0.85)	3.65	0.0003	F	4.83	0.18	37.8
GG/GG *vs* T/T + GG/T	Overall	6439	4798	8	0.95 (0.81-1.11)	0.62	0.53	F	12.02	0.10	41.8
	CD	3891	4798	8	0.91 (0.76-1.09)	0.99	0.32	F	11.74	0.11	40.4
	UC	2450	3724	6	1.03 (0.83-1.27)	0.23	0.81	F	4.89	0.43	0.0
	IBD onset < 40	743	2376	4	0.61 (0.28-1.35)	1.22	0.22	R	7.98	0.05	62.3
GG/GG *vs* T/T	Overall	4033	3020	8	0.93 (0.74-1.17)	0.63	0.53	R	12.59	0.08	44.4
	CD	2442	3020	8	0.88 (0.68-1.14)	0.96	0.34	R	12.96	0.07	46.0
	UC	1623	2312	6	0.97 (0.78-1.20)	0.32	0.75	F	3.04	0.69	0.0
	IBD onset < 40	500	1435	4	0.52 (0.22-1.21)	1.52	0.13	R	8.84	0.03	66.0
GG/T *vs* T/T	Overall	2816	2098	8	0.95 (0.80-1.12)	0.64	0.53	F	10.85	0.15	35.5
	CD	1685	2098	8	0.92 (0.76-1.11)	0.92	0.36	F	9.94	0.19	29.6
	UC	1093	1648	6	1.03 (0.83-1.28)	0.27	0.79	F	4.67	0.46	0.0
	IBD onset < 40	290	1112	4	0.94 (0.64-1.37)	0.33	0.74	F	5.95	0.11	49.6

R: Random effect model; F: Fixed effect model; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

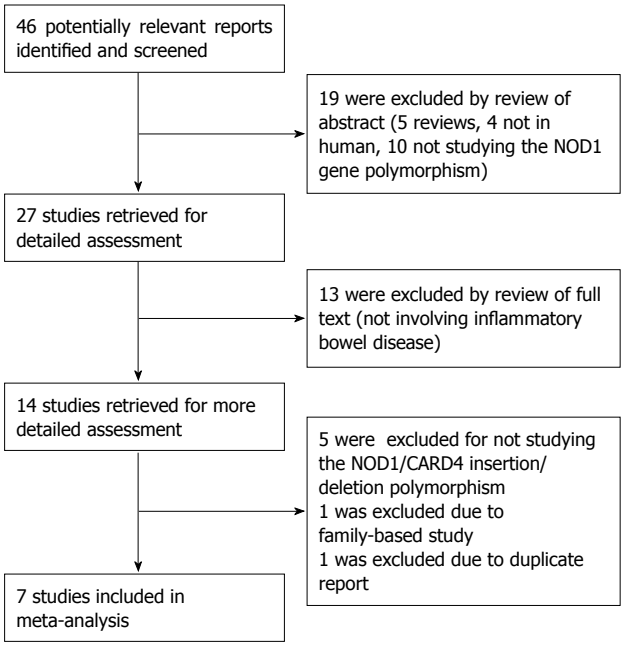

Figure 1 Study selection in Medline.

ferent subpopulations and we treated it independently[22]. Finally, a total of 8 separate studies were considered in the current meta-analysis (Table 1).

We got the data from the corresponding author of the study by Franke *et al*[23]. Thus, the allele and the genotype frequencies of the NOD1/CARD4 insertion/deletion polymorphism were extracted from all the eligible studies. The 8 separate studies were conducted in Caucasian populations. Of these, 8[19-25] were conducted in patients with CD and 6[21-25] were conducted in patients with UC. Meanwhile, 4 studies[20,22,24,25] showed stratified data of cases by age (IBD onset at < 40 years). The results of Hardy-Weinberg equilibrium test for the distribution of the genotype in control populations are shown in Table 1. Only one study belonged to Hardy-Weinberg equilibrium among the eligible studies[19]. The distribution of the genotype in the overall control population was consistent with Hardy-Weinberg equilibrium ($P = 0.240$).

Meta-analysis

The summary of the meta-analysis for the NOD1/CARD4 insertion/deletion polymorphism and IBD is shown in Table 2.

Overall effects

The *Q*-test of heterogeneity was not significant and we conducted analyses using fixed effect models except in the contrasts of GG *vs* T and GG/GG *vs* T/T. We found no association between NOD1/CARD4 insertion/deletion polymorphism and IBD in the overall population (GG *vs* T: OR = 0.98, 95% CI: 0.90-1.07, $P = 0.70$; GG/T + GG/GG *vs* T/T: OR = 1.00, 95% CI: 0.98-1.08, $P = 0.99$; GG/GG *vs* T/T + GG/T: OR = 0.95, 95% CI: 0.81-1.11, $P = 0.53$; GG/GG *vs* T/T: OR = 0.93, 95% CI: 0.74-1.17, $P = 0.53$; GG/T *vs* T/T: OR = 0.95, 95% CI: 0.80-1.12, $P = 0.53$).

Subgroup analyses

We performed group-specific meta-analysis of CD, UC and IBD onset in the populations aged < 40 years.

Analysis of CD population: The *Q*-test of heterogeneity was significant and we conducted analyses using random effect models except in the contrasts of GG/GG *vs* T/T + GG/T and GG/T *vs* T/T. No association of

Table 3 Egger's linear regression test to measure the funnel plot asymmetry[1]

Comparisons	Y axle intercept: a (95%CI)				
	GG *vs* T	GG/T + GG/GG *vs* T/T	GG/GG *vs* T/T + GG/T	GG/GG *vs* T/T	GG/T *vs* T/T
Overall	-1.92 (-5.57-1.72)	0.96 (-2.65-4.58)	2.38 (-0.87-5.64)	2.38 (-1.00-5.77)	2.32 (-0.71-5.36)
CD	-2.62 (-6.54-1.29)	1.60 (-2.38-5.59)	2.79 (-0.20-5.78)	2.89 (-0.31-6.09)	2.51 (-0.29-5.33)
UC	-0.39 (-6.54-5.75)	0.57 (-5.25-6.39)	-0.57 (-7.13-5.97)	-0.43 (-7.00-6.13)	-0.57 (-6.89-5.74)
IBD onset at < 40 yr of age	-2.16 (-8.80-4.47)	1.49 (-4.16-7.15)	1.86 (-4.04-7.77)	1.94 (-4.40-8.29)	1.63 (-3.48-6.75)

[1]All $P > 0.05$. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

NOD1/CARD4 insertion/deletion polymorphism was found with CD (GG *vs* T: OR = 0.96, 95% CI: 0.86-1.07, $P = 0.43$; GG/T + GG/GG *vs* T/T: OR = 0.97, 95% CI: 0.86-1.10, $P = 0.67$; GG/GG *vs* T/T + GG/T: OR = 0.91, 95% CI: 0.76-1.09, $P = 0.32$; GG/GG *vs* T/T: OR = 0.88, 95% CI: 0.68-1.14, $P = 0.34$; GG/T *vs* T/T: OR = 0.92, 95% CI: 0.76-1.11, $P = 0.36$).

Analysis of UC population: The *Q*-test of heterogeneity was not significant and we conducted analyses using fixed effect models in the UC population. No association of NOD1/CARD4 insertion/deletion polymorphism with UC was discovered (GG *vs* T: OR = 1.01, 95% CI: 0.92-1.09, $P = 0.89$; GG/T + GG/GG *vs* T/T: OR = 1.00, 95% CI: 0.90-1.11, $P = 0.95$; GG/GG *vs* T/T + GG/T: OR = 1.03, 95% CI: 0.83-1.27, $P = 0.81$; GG/GG *vs* T/T: OR = 0.97, 95% CI: 0.78-1.20, $P = 0.75$; GG/T *vs* T/T: OR = 1.03, 95% CI: 0.83-1.28, $P = 0.79$).

Analysis of IBD onset in a population aged < 40 years: The *Q*-test of heterogeneity was significant and we conducted analyses using random effect models except in the contrasts of GG/T + GG/GG *vs* T/T, and GG/T *vs* T/T. We found an association between NOD1/CARD4 insertion/deletion polymorphism and IBD in a younger age group at onset (< 40 years) when examining the contrasts of GG *vs* T, and GG/T + GG/GG *vs* T/T (GG *vs* T: OR = 0.68, 95% CI: 0.50-0.93, $P = 0.02$; GG/T + GG/GG *vs* T/T: OR = 0.71, 95% CI: 0.59-0.85, $P = 0.0003$), and the forest plots are shown in Figure 2. However, the association was not found when the contrasts of GG/GG *vs* T/T + GG/T, GG/GG *vs* T/T and GG/T *vs* T/T were examined (GG/GG *vs* T/T + GG/T: OR = 0.61, 95% CI: 0.28-1.35, $P = 0.22$; GG/GG *vs* T/T: OR = 0.52, 95% CI: 0.22-1.21, $P = 0.13$; GG/T *vs* T/T: OR = 0.94, 95% CI: 0.64-1.37, $P = 0.74$).

Evaluation of publication bias

Funnel plot asymmetry was assessed by the method of Egger's linear regression test. If there was asymmetry, the regression line would not run through the origin. The larger its deviation from zero, the more pronounced the asymmetry. The results of Egger's linear regression test are shown in Table 3. It was shown that there was no publication bias (all $P > 0.05$). For the association of NOD1/CARD4 insertion/deletion polymorphism with IBD in the group of younger age at onset (< 40 years), the Egger's linear regression test provided no evidence of publication bias (GG *vs* T: $t = -1.40$, $P = 0.296$; GG/T + GG/GG *vs* T/T: $t = 1.14$, $P = 0.373$) (Figure 3A). Figure 3B shows that the distribution of the ORs from individual studies in relation to their respective standard deviation was symmetric in funnel plot.

DISCUSSION

The identification of NOD2/CARD15 as a CD susceptibility gene makes its homologous gene NOD1/CARD4 a potential candidate gene for predisposition to IBD[8,38,39]. NOD1/CARD4 is the founding member of the Nod-like receptors (NLRs) family, and is expressed in large and small bowel[18]. It plays an important role in colonic epithelial defenses against intracellular organisms, such as enteroinvasive *E. coli* and *Shigella flexneri*[40,41]. The presence of bacterial flora is essential for IBD to develop in animal models[42]. Antibiotics and fecal diversion are effective therapies for CD[43,44]. NOD1/CARD4 has been mapped to chromosome bands 7p14-p15 (UniGene Cluster Hs 19405), a region which was previously reported to contain an IBD susceptibility locus[17]. Thus, NOD1/CARD4 appeared to be a good candidate for IBD. Recently, many studies have been conducted to test the association of NOD1/CARD4 insertion/deletion polymorphism with IBD, but the association trends observed have been variable with several studies showing an association while others do not[19-25]. It is, therefore, necessary to perform a comprehensive meta-analysis to assess the importance of the NOD1/CARD4 insertion/deletion polymorphism for IBD pathogenesis.

In the present study, we retrieved 8 studies, including 6439 cases and 4798 controls, to evaluate the association of NOD1/CARD4 insertion/deletion polymorphism with IBD in Caucasian populations. Our meta-analysis did not detect the association of NOD1/CARD4 insertion/deletion polymorphism with IBD, CD, and UC in the overall population. However, we did find a significant genetic association between NOD1/CARD4 insertion/deletion polymorphism and IBD in the group of younger age at onset (< 40 years), and GG allele was a protective allele for IBD pathogenesis. As far as we know, this is the first meta-analysis carried out so far which aimed at investigating the association of NOD1/CARD4 insertion/deletion polymorphism with IBD.

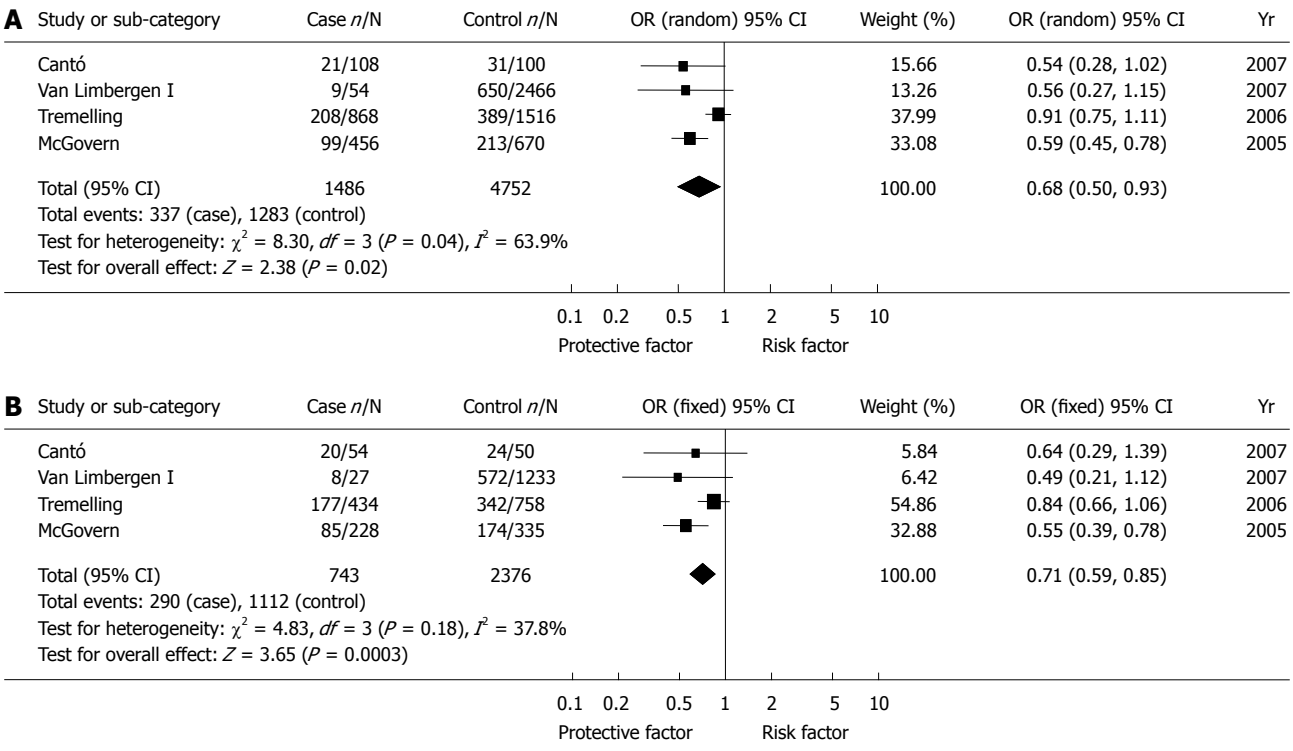

Figure 2 Forest plots for meta-analysis of positive results. Inflammatory bowel disease onset at < 40 years of age. A: GG *vs* T; B: GG/T + GG/GG *vs* T/T.

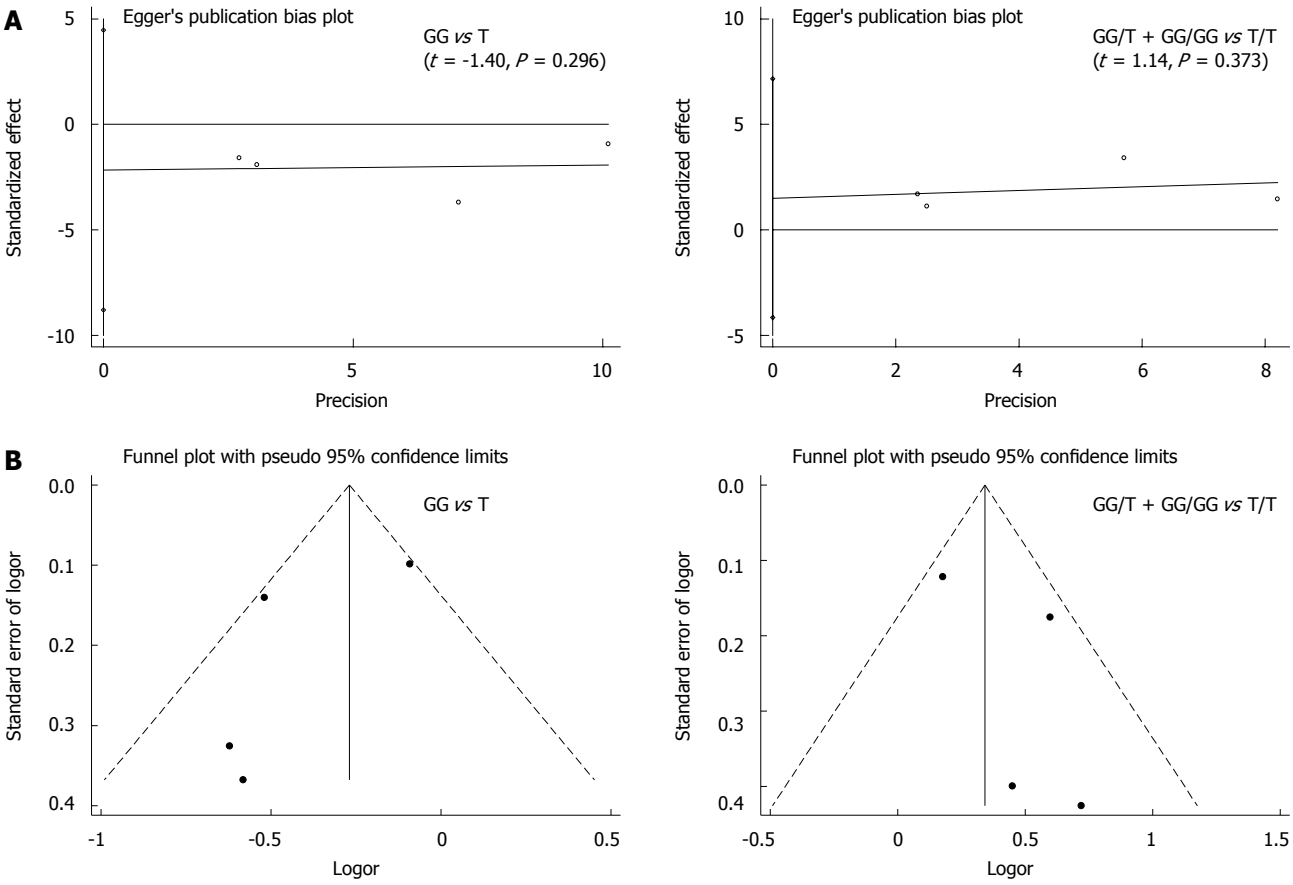

Figure 3 Egger's linear regression test for publication bias of positive results (A), funnel plots for meta-analysis of positive results; inflammatory bowel disease onset at < 40 yeas of age (B).

In our study, we found that the NOD1/CARD4 GG allele decreased the risk of IBD in the group of younger

age at onset (< 40 years), indicating that this locus is important in determining the susceptibility to IBD. The result is not surprising, since NOD1, similar to NOD2, is involved in the recognition of intracellular bacterial PAMPs[45]. The two molecules share structure and functional similarities. NOD1/CARD4 insertion/deletion polymorphism is located at the beginning of intron IX[18]. Hysi *et al*[18] firstly demonstrated an effect of this polymorphism on the binding of an unidentified nuclear protein. Hysi *et al*[18] demonstrated the presence of different isoforms of NOD1 transcripts. A recent study showed that some of these isoforms resulted in disruption of the LRR region critical for NOD1 mediated bacterial sensing[46]. Therefore, although noncoding, this polymorphism may affect immune response with direct implications for IBD pathogenesis either by altered binding of a cis/trans activating protein, resulting in abnormal gene expression, or by the generation of functionally significant splice variants. However, to date, the detailed functions of this polymorphism are still unclear. Further studies on the function of NOD1/CARD4 insertion/deletion polymorphism are required. Of course, the association may result from the direct effect of the polymorphism itself, or through linkage disequilibrium with another functional polymorphism in the structural part of the gene or in regulatory regions. Additionally, the association of NOD1/CARD4 insertion/deletion polymorphism with IBD was only detected in the contrasts of GG *vs* T and GG/T + GG/GG *vs* T/T, indicating that the GG allele of this polymorphism may have a dominant effect on risk for IBD.

Some limitations of this study should be discussed. Firstly, the current meta-analysis only included the wholly-published studies, not the meeting or conference abstracts. Thus, publication bias may have occurred, even though the use of a statistical test did not show it. Secondly, significant heterogeneity between studies was detected in the current meta-analysis, which may distort the analysis. However, it is not a major problem because IBD itself is heterogeneous, and different populations may contribute to the heterogeneity. Thirdly, these results should be interpreted with caution because the population from six countries was not uniform. Fourthly, the analysis in IBD onset in the population aged < 40 years only included four studies (743 cases and 2376 controls), and more studies based on a larger sample size, case-control design and stratification by age are still needed in the future research. Finally, meta-analysis remains retrospective that is subject to the methodological deficiencies of the included studies. Therefore, we minimized the likelihood of bias by developing a detailed protocol before initiating the study by performing a meticulous search for published studies and by using explicit methods for study selection, data extraction and data analysis.

In conclusion, our study demonstrates the association of NOD1/CARD4 insertion/deletion polymorphism with inflammatory bowel disease in the younger age group at onset (< 40 years) in Caucasian populations.

ACKNOWLEDGMENTS

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COMMENTS

Background

Recently, many studies have been conducted to prove the association of NOD1/CARD4 insertion/deletion polymorphism with inflammatory bowel disease (IBD), but the association trends observed have been variable with several studies showing an association while others do not. It is, therefore, necessary to perform a comprehensive meta-analysis to assess the importance of the NOD1/CARD4 insertion/deletion polymorphism for IBD pathogenesis.

Research frontiers

The etiology of IBD most likely involves a complex interaction of genetic, environmental and immunoregulatory factors. The identification of the NOD2 is a breakthrough in IBD genetics, which heralded extensive analyses of signaling pathways of the innate immune system implicated in the pathogenesis of IBD. NOD1/CARD4 signaling leads to activation of nuclear factor-κB, and plays an important role in innate immunity. Certain polymorphisms and mutations in NOD1/CARD4 may result in dysfunctional innate immune response during bacterial recognition with direct implications for IBD pathogenesis.

Innovations and breakthroughs

The authors collected 8 studies (6439 cases and 4798 controls) in Caucasian populations to evaluate whether NOD1/CARD4 insertion/deletion polymorphism is associated with IBD by meta-analysis. They found the association of NOD1/CARD4 insertion/deletion polymorphism with IBD in the younger age group at onset (< 40 years) in Caucasian populations.

Applications

The authors found that the NOD1/CARD4 GG allele decreased the risk of IBD in the younger age group at onset (< 40 years), indicating that this locus is important in determining the susceptibility to IBD. The association of NOD1/CARD4 insertion/deletion polymorphism with IBD was only detected in the contrasts of GG *vs* T and GG/T + GG/GG *vs* T/T, indicating that the GG allele of this polymorphism may have a dominant effect on risk for IBD.

Terminology

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis.

Peer review

This is a very interesting meta-analytic study dealing with an important topic in IBD.

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BRIEF ARTICLE

Standard triple, bismuth pectin quadruple and sequential therapies for *Helicobacter pylori* eradication

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Abstract

AIM: To compare the effectiveness of standard triple, bismuth pectin quadruple and sequential therapies for *Helicobacter pylori* (*H. pylori*) eradication in a randomized, double-blinded, comparative clinical trial in China.

METHODS: A total of 215 *H. pylori*-positive patients were enrolled in the study and randomly allocated into three groups: group A (n = 72) received a 10-d bismuth pectin quadruple therapy (20 mg rabeprazole *bid*, 1000 mg amoxicillin *bid*, 100 mg bismuth pectin *qid*, and 500 mg levofloxacin *qd*); group B (n = 72) received the sequential therapy (20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, in 5 d, followed by 20 mg omeprazole *bid*, 500 mg tinidazole *bid*, 500 mg clarithromycin *bid*, for another 5 d); group C (n = 71) received a standard 1-wk triple therapy (20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, 500 mg clarithromycin *bid*). After all these treatments, 20 mg omeprazole *bid* was administrated for 3 wk. *H. pylori* status was assessed by histology, 13C-urea breath test and rapid urease test at baseline and 4-6 wk after completion of treatment. Ulcer cicatrization was assessed by gastroscopy. χ^2 test ($P < 0.05$) was used to compare the eradication rates and ulcer cicatrisation rates among the three groups.

RESULTS: The eradication rate was 83.33% (60/72) in group A, 88.89% (64/72) in group B, and 80.56% (58/71) in group C. The ulcer cicatrisation rate was 86.44% (51/59) in group A, 90.16% (55/61) in group B, and 84.91% (45/53) in group C. The sequential therapy yielded a higher eradication rate and ulcer cicatrisation rate than the standard triple and bismuth pectin quadruple therapies. Statistically, the eradication rate of group B was significantly different from groups A and C ($P < 0.05$), but the difference of ulcer cicatrisation rate and side effects was not statistically significant among the three groups ($P > 0.05$). The three protocols were generally well tolerated.

CONCLUSION: The sequential therapy has achieved a significantly higher eradication rate, and is a more suitable first-line alternative protocol for anti-*H. pylori* infection compared with the standard triple and bismuth pectin quadruple therapies.

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Key words: *Helicobacter pylori*; Sequential therapy; Triple therapy; Bismuth pectin quadruple therapy; Eradication rate

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INTRODUCTION

Helicobacter pylori (H. pylori) infection habitually causes chronic active gastritis, which significantly enhances the risk for intestinal metaplasia in the stomach, and it is undoubtedly involved in gastric carcinogenesis. Moreover, *H. pylori* also play a crucial role in the pathogenesis of peptic ulcer and mucosa-associated lymphoid tissue lymphoma, including peptic ulcer complications, such as bleeding or stenosis[1-4]. According to the Maastricht 2 guidelines, the first-line treatment for *H. plori* eradication is the triple therapy using a proton-pump inhibitor (PPI) *bid*, 1 g amoxicillin *bid*, and 500 mg clarithromycin *bid*. In the case of penicillin allergy, 500 mg metronidazole *bid* is substituted for amoxicillin. After a decade of clarithromycin-based treatment and continued widespread use of long-acting macrolides in general practice, 10%-15% of *H. pylori* strains are resistant *de novo* to clarithromycin[3]. As a result, the failure rate is around 20% for the triple therapy (PPI plus amoxicillin plus clarithromycin)[4,5]. When the first-line *H. pylori* eradication treatment fails, a second-line treatment of quadruple therapy, with a PPI *bid*, colloidal bismuth subcitrate *qid*, 500 mg metronidazole *tid*, and 500 mg tetracycline *qid*, is recommended. Some recent studies have compared the efficacy of the triple *vs* quadruple therapy, and a meta-analysis has assessed these studies[6]. Eradication rates were not significantly different among patients receiving triple or quadruple therapy. The eradication rates in the patients receiving either triple or quadruple therapy in this study were almost similar to those obtained previously[4,7,8].

Clarithromycin and metronidazole resistance has increased substantially in recent years, and there has been a corresponding decrease in the eradication rate for *H. pylori* infection in most Western countries[4]. In China, recent nationwide multi-center studies have demonstrated that clarithromycin resistance increased to 27.6%, and metronidazole resistance is extremely common, the average resistance rate being 75.6%. Furthermore, combined clarithromycin-metronidazole cross-resistance was found in 85.1% of clarithromycin-resistance *H. pylori* strains. Eradication rates in most Western countries and China have declined to unacceptable levels. Therefore, Antibiotic resistance is the main cause of failure in *H. pylori* eradication and bata-lactamase produced by resistant *H. pylori* strains is a possible mechanism underlying the ineffectiveness of an amoxicillin-based triple or quadruple therapy[1].

Sequential therapy is a latest protocol for *H. pylori* eradication suggested by De Francesco *et al*[9]. Sequential therapy refers to the idea of adding more antibiotics to the treatment regimen but giving them in sequence rather than giving all 4 drugs together. Typically, this involves an initial 5-d therapy with a benign combination (e.g. 40 mg pantoprazole with 1 g amoxicillin *bid*) followed by 5 d of two more antibiotics plus a PPI (e.g. 500 mg clarithromycin and 500 mg tinidazole plus 40 mg pantoprazole *bid*). A subgroup data-analysis in a large, prospective, controlled multi-center study showed that the eradication rate of this "new" treatment was significantly higher than that of the clarithromycin-based treatment (82% *vs* 44%, $P < 0.0155$). Child, adult and elderly patients receiving this "new" treatment achieved a high eradication rate and had less adverse reactions[10,11].

To our knowledge, no data are available about the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy in China. The present study aimed to compare the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy; to further test whether the 10-d sequential therapy is able to increase the eradication rate compared with the 7-d standard triple therapy and 10-d bismuth pectin quadruple therapy; to observe the adverse reactions; and to evaluate the reliability, safety and efficacy of this treatment in China.

MATERIALS AND METHODS

Patients

This is a prospective, parallel, open-label, randomized study. The study population consisted of patients with dyspepsia defined as having pain or discomfort in the upper abdomen.

A total of 215 patients infected with *H. pylori* were enrolled. The patients were screened from 15 322 patients who underwent gastroscopy at the Endoscopy Center of Weihai Municipal Hospital from January 1, 2005 to December 31, 2009. Patients enrolled in the present study had not been previously treated for *H. pylori* infection. Patients were excluded if they were taking PPI, H2-receptor antagonists, bismuth preparations or antibiotics 4 wk before the study. Pregnant women, patients with antibiotic allergy or severe diseases of organs, neoplasm, serious complications of ulcers, and hepatic impairment or kidney failure were not enrolled. All the participants signed informed consent form.

H. pylori infection was assessed at entry. All patients underwent endoscopy with biopsies for histology (two samples from the antrum and two from the corpus) and a rapid urease test (one sample from the antrum) (CP-test, China). Patients were diagnosed to be *H. pylori*-positive if both tests were positive. Biopsy specimens were histologically detected for *H. pylori* by hematoxylin and eosin stain. In patients diagnosed with ulcers by gastroscopy, the diameter of ulcers must be between 5 mm and 3 cm, and number of ulcers must be no more than 2 in stomach and/or duodenum, except for those with a history of peptic ulcer before present illness. The post-eradication assessment was undertaken 4-6 wk after completion of the treatment (after the subsequent 3-wk course of PPI) using a 13C-urea breath test (Infai, Sofar, Italy). Citric acid (1.5 g) as a test meal and 13C-urea (75 mg) as a water solution were given to the patients after collection of a baseline sample by blowing through a disposable plastic straw into a 20-mL container; an additional breath sample was collected 30 min later. The breath samples were considered positive if there was a greater than five per 1000 of $13CO_2$ difference over baseline, according to the manufacturer's recommendations. Meanwhile, all patients

underwent endoscopy with biopsies for histology (two samples from the antrum and two from the corpus) and a rapid urease test (one sample from the antrum), and the healing of ulcers *vs* pre-therapy was observed.

Therapeutic regimens

In the center, patients were randomly assigned using a computer generated list to one of the following treatments: Group A (n = 72): A 10-d triple therapy with 20 mg rabeprazole *bid*, 1000 mg amoxicillin *bid*, 100 mg bismuth pectin *qid*, and 500 mg levofloxacin *qd*; Group B (n = 72): A sequential therapy with 20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, for 5 d, followed by 20 mg omeprazole *bid*, 500 mg tinidazole *bid*, 500 mg clarithromycin *bid*, for another 5 d; and Group C (n = 71): A standard triple therapy with 20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, 500 mg clarithromycin *bid*.

For each regimen, the PPI was prescribed at 30 min before meals, but all antibiotics were given after meals. Patients were asked to return to assess the compliance and estimate the adverse reactions at the end of the treatment. Side effects were evaluated using a structured questionnaire by personal interview.

Statistical analysis

The sample size was calculated based on available data in the literature. By hypothesizing a 95% eradication rate for the sequential regimen[12] and 80% for either the 7-d standard triple, 10-d sequential therapy or 10-d bismuth pectin quadruple therapy[13], it was calculated that all patients per treatment arm were needed to find a statistically significant difference with a level of $P < 0.05$ and a power of 0.85. The eradication rates and their 95% CIs were calculated for each treatment regimen. For all other variables, χ^2, Fisher's exact test and Student's t test were used as appropriate, and $P < 0.05$ was considered significant. The difference in the eradication rates among the three treatments was estimated. Before pooling those estimates, a Fisher's exact test was applied to investigate the heterogeneity between the differences.

RESULTS

Eradication rates

Two hundred and thirteen patients with *H. pylori* were enrolled in the study. As shown in Table 1, the three patient groups did not differ in age, sex, gastritis distribution and location and number of peptic ulcers in gastric mucosa. All patients completed the treatment. *H. pylori* infection was successfully cured in 60/72 (83.33%) with a 10-d bismuth pectin quadruple therapy, in 64/72 (88.89%) with the sequential therapy, and in 58/71 (80.56%) with the 7-d standard triple therapy, respectively. As shown in Table 2, the eradication rates achieved by the sequential therapy were significantly higher than that by both the 10-d bismuth pectin quadruple therapy and 7-d standard triple therapy, with significant differences ($P < 0.05$). The ulcer cicatrisation was successfully cured in 86.44% by the 10-d bismuth pectin quadruple therapy, in 90.16% by the sequential therapy, and in 84.91% by the 7-d standard triple therapy. As shown in Table 1, although the sequential therapy tended to give better results in eradication rates when compared with the 10-d bismuth pectin quadruple therapy and 7-d standard triple therapy, no statistically significant difference was found ($P > 0.05$).

Table 1 Demographic and clinical characteristics of patients at entry into each treatment group

Patient characteristics (n)	Group A	Group B	Group C
Number of patients	72	72	71
Sex (M/F)	31/41	35/37	34/37
Age (yr), mean ± SD	45 ± 10	47 ± 13	43 ± 15
Antral gastritis	58	61	57
Pangastritis	17	15	19
Intestinal metaplasia	19	21	17
Duodenitis	11	13	10
Gastric ulcer	40	42	39
Duodenal bulb ulcer	13	12	10
Compound ulcers	6	7	4

Table 2 Eradication and ulcer cicatrisation rates in each treatment group

	Group A	Group B	Group C
Eradication rate (%)[a]	83.33 (60/72)	88.89 (64/72)	80.56 (58/71)
Ulcer cicatrisation rate (%)[b]	86.44 (51/59)	90.16 (55/61)	84.91 (45/53)

[a]$P < 0.05$, Group B *vs* Group C, Group A; [b]$P > 0.05$, Group B *vs* Group C, Group A.

Compliance and side effects

Compliance with the therapy was good (greater than 95% of prescribed drugs). Six patients (16.67%) treated with the 10-d bismuth pectin quadruple therapy complained of side effects (three with abdominal discomfort, two with abdominal pain, four with nausea/vomiting, two with parageusia and one with glossitis). Fourteen patients (19.44%) receiving the sequential therapy reported side effects (five with abdominal pain, one with constipation, two with parageusia, three with nausea/vomiting and three with pruritus). Eleven patients (15.49%) receiving the 7-d standard triple therapy complained of side effects (one with diarrhea, four with abdominal pain, one with parageusia, one with glossitis and three with nausea/vomiting). No statistically significant difference in the incidence of side effects was found among the three groups ($P > 0.05$). All side effects were self-limiting after the therapy was ended.

DISCUSSION

Since antibacterial activity of the majority of antibacterials decreases under intragastric low pH and the slime layer may prevent the drugs penetrating fully into the depth of the biofilm, *H. pylori* is not easily eliminated and can develop resistance to antimicrobial drugs. It is extremely important that a protocol with a high eradication rate should

be selected to ensure a successful eradication of *H. plori* in the treatment of peptic ulcers. At present, triple therapies suggested by either Canadian or European guidelines are the most preferred first-line protocols in clinical practice[2,14]. The proposal is being used by 85%, 84% and 67% of primary-care physicians in Italy, Israel and the United States, respectively[7,15,16]. However, the eradication rates substantially decreased by the triple therapy in several countries. Indeed, a success rate of less than 80% has been found in several European and Asian countries, the United States and Canada[17-25]. Eradication rate was extremely low (25%) in a recent study[26]. The resistance to clarithromycin and/or metronidazole is the primary cause of the descending *H. pylori* eradication rate[8,27,28]. In order to reinforce the curative effect of the standard triple therapy, some scholars suggest that the duration of the treatment may be extended to 14 d. One meta-analysis suggests that the 14-d triple therapy can increase the *H. pylori* eradication rate by 12% compared with the 7-d therapy, but the expenditures increase simultaneously. Therefore, it is imminent to seek a new eradication strategy.

Sequential therapy is a recent proposal for *H. pylori* eradication suggested by Zullo *et al*[29]. De Francesco found that double drugs administration for 14 d and subsequent triple drugs for 7 d significantly increased the eradication rate (97.3%) compared with the proposal of converse administration (81.6%, triple drugs administration for 7 d and subsequent double drugs for 14 d). It suggests that the sequence of antibiotic administration affects the *H. pylori* eradication. Zullo *et al*[30], Sánchez-Delgado *et al*[31] and Zullo *et al*[32] further simplified this proposal, and named it sequential therapy. Sequential therapy refers to the idea of adding more antibiotics to the treatment regimen but giving them in sequence rather than giving all 4 drugs together. Typically, this involves an initial 5-d therapy with a benign combination (40 mg pantoprazole and 1 g amoxicillin *bid*) followed by 5 d of two more antibiotics plus a PPI (500 mg clarithromycin and 500 mg tinidazole plus 40 mg pantoprazole *bid*). Subgroup data analysis in a large, prospective, controlled multi-center study showed that the eradication rate of this "new" treatment was significantly higher compared with the clarithromycin-based treatment (82% *vs* 44%, $P < 0.0155$). Child, adult and elderly patients receiving this "new" protocol all achieved a high eradication rate and had less adverse reactions[10,11].

This study compared the effectiveness among sequential therapy, triple therapy, and Bismuth Pectin quadruple therapy for *H. pylori* eradication. *H. pylori* was eradicated effectively in all groups, with a success rate of over 80% that was consistent with the standards of Maastricht and other guidelines. Sequential therapy reached an eradication rate of 88.89%, with significant differences compared with other therapies ($P < 0.05$), but the healing rate of ulcers was not significantly different ($P > 0.05$) among the three groups. It was basically the same as the previous publications. Sequential therapy (omeprazole, clarithromycin, amoxicillin plus tinidazole are administrated in sequence for 10 d) has several advantages: the treatment duration is appropriately increased. Amoxicillin acts on the cell wall of bacteria in the first 5-d treatment to prevent clarithromycin pathway formation, thus increasing the sensitivity of the bacteria to clarithromycin, and effectively avoiding the *collateral resistance to* clarithromycin. Omeprazole, clarithromycin plus tinidazole are administrated for the remaining 5-d treatment. Clarithromycin acts on bacterial nucleic acid, restrains protein synthesis, stabilizes in acid environment, and increases the synergetic effects of the drugs and the cure rate of *H. pylori* infection.

Resistance to metronidazole and clarithromycin is the main reason for treatment failure of eradicating *H pylori*[33].

Early documents generally demonstrated that *H. pylori* primary resistance to clarithromycin is very low and usually not more than 10%. But for the past a few years, following the wide use of clarithromycin, *H. pylori* resistance to clarithromycin has gradually increased, so did the nitroimidazoles, and cross-resistance has also appeared. In China, the recent nationwide multi-center studies[34] have demonstrated that the resistance to clarithromycin increased to 27.6%, and resistance to metronidazole reached 75.6%. Furthermore, cross-resistance to metronidazoles appeared in 85.1% of clarithromycin-resistant *H. pylori* strains. It suggests that *H. pylori* resistance to clarithromycin and metronidazoles is an extremely serious problem. As resistance to clarithromycin also increased in Western countries, there has been a corresponding decrease in the eradication rate for *H. pylori* infection[4]. A study in Italy[35] presented that in the past 15 years, resistance to clarithromycin doubled from 10.2% in 1989-1990 to 21.3% in 2004-2005. But the resistance rate to metronidazole in adult is 10%-50% in Western countries and 77%-95% in developing countries[36]. The eradication rate of the sequential therapy in this study (88.89%) is lower than in Western countries (over 90%). According to the known mechanism of the proposal, it only improves the *H. pylori* sensitivity and prevents collateral resistance to clarithromycin. As resistance to metronidazole is extremely common in China, it has decreased the *H. pylori* eradication rate of the protocol.

Therefore, antibiotic resistance is the main cause of failure in *H pylori* eradication and beta-lactamase produced by resistant *H. pylori* strains is a possible mechanism underlying the ineffectiveness of an amoxicillin-based triple or quadruple therapy[1].

In short, the 10-d sequential therapy is significantly dominant compared with standard triple and bismuth pectin quadruple therapy, and adverse effects are not significantly different ($P > 0.05$). Therefore, the sequential therapy is a better choice of treatment for *H. pylori* eradication. But further researches are needed to formulate the strategies of sequential therapy and probe into the exact mechanism of eradicating *H. pylori*.

COMMENTS

Background

Clarithromycin and metronidazole resistance has increased substantially in recent years, and there has been a corresponding decrease in the eradication

rate for *Helicobacter pylori* (*H. pylori*) infection in most Western countries and China. Sequential therapy is a recent protocol for *H. pylori* eradication.

Research frontiers

To the authors' knowledge, no data are available about the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy in China. The present study aimed to compare the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy in China.

Innovations and breakthroughs

The results denote that the 10-d sequential therapy is significantly dominant compared with standard triple and bismuth pectin quadruple therapy, and adverse effects are not significant different. The eradication rate of the sequential therapy in this study (88.89%) is lower than in Western countries (over 90%). As resistance to metronidazole and clarithromycin is extremely more common in China than in Western countries, it has decreased the *H. pylori* eradication rate of the therapy.

Applications

Sequential therapy is a better choice of treatment for *H. pylori* eradication in China. It may be suggested as the first-line protocol for eradicating *H. pylori*. Therefore, it may increase the eradicate rate, decrease the resistance to antibiotics, then decrease the prevalence of *H. pylori*-related diseases. However, the strategies of sequential therapy need further studies to fit for the situation of China.

Terminology

Sequential therapy refers to the idea of adding more antibiotics to the treatment regimen, but giving them in sequence rather than giving all 4 drugs together. Typically, this involves an initial 5-d therapy with a benign combination of drugs, followed by 5 d of two more antibiotics plus a proton-pump inhibitor.

Peer review

This is an interesting study that provides further strong support for sequential therapy being superior to other regimens. The study appears to have been designed well, but some details of the design and study structure are absent.

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CASE REPORT

Therapy-refractory gastrointestinal motility disorder in a child with c-kit mutations

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Abstract

Constipation and fecal impaction are frequent and distressing complaints in pediatric gastroenterology. Especially in neurologically handicapped children, treatment of severe forms of slow-transit constipation (STC) can be difficult. In the majority of cases, STC is of unknown etiology. However, in recent years, there is growing evidence that interstitial cells of Cajal (ICCs), which serve as electrical pacemakers and generate spontaneous electrical slow waves in the gastrointestinal tract, might play an important role in the pathophysiology of STC. It remains unclear whether morphological ICC alterations seen in affected patients are based on congenital developmental anomalies, or whether they are a consequence of long-term constipation with secondary damage of the gastrointestinal nervous system. To the best of our knowledge, we present the first case of a patient with histological alterations in ICC morphology who displayed multiple alterations of c-kit at the level of mRNA. The protein encoded by c-kit is the receptor tyrosine kinase Kit (CD117), which is crucial for development and function of ICCs. Therefore, these findings provide a new explanation for congenital alterations of ICC development that result in gastrointestinal motility disorders.

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Key words: Slow-transit constipation; Interstitial cells of Cajal; c-kit

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INTRODUCTION

Chronic constipation is one of the most common complaints of patients in pediatric gastroenterology. Most cases can be classified as a functional disorder and are usually managed by dietary modifications and administration of oral laxatives.

Nevertheless, during childhood, some patients develop severe forms of slow-transit constipation (STC), associated with prolonged bowel passage and resistance

to medical treatment. Clinical symptoms can include low stool frequency, lack of urge to defecate, abdominal distention, bloating, and abdominal discomfort[1]. Although the pathophysiology has not yet been fully understood, visceral neuropathies[2], dysregulation of neurotransmitters[3], degeneration of enteric neurons[4], and alterations of interstitial cells of Cajal (ICCs)[5] are possible etiological mechanisms that have been discussed in the literature[6].

ICCs form a network that is widely distributed in all layers of the gastrointestinal tract. Myenteric ICCs are located to the myenteric plexus and integrate between the enteric nervous system and the muscle components of the bowel. In recent years, there has been growing evidence that ICCs serve as electrical pacemakers and generate spontaneous electrical slow waves in the gastrointestinal tract[7]. Slow waves organize gut contractions into phasic contractions that are the basis for peristalsis and segmentation. Important new insights have been gathered by studies on animals with loss-of function mutations in the c-kit signaling pathway[8,9]. The protein encoded by c-kit is the receptor tyrosine kinase Kit (CD117). The activation of Kit is crucial for development and function of the ICC phenotype in the gastrointestinal tract. Functional studies in c-kit-knock-out mice and in animals with specific mutations that modify the binding of the receptor ligand stem cell factor (SCF) have shown grossly underdeveloped networks of ICCs[10] with significantly reduced gastrointestinal motility[9]. Recognition of these physiological properties in animals has led to studies of ICCs in several human gastrointestinal motility disorders, especially in cases of idiopathic megacolon and STC. Nowadays, there is no doubt that damage or dysfunction of ICCs results in gut dysmotility.

Here, we present a new case of a patient with specific genetic alterations of the receptor tyrosine kinase Kit that led to abnormal ICC architecture and function in the gut, with severe chronic constipation.

CASE REPORT

The male patient was born to a healthy woman with an inconspicuous family history at the 36th gestational week. His birth weight and height were below the 3rd percentile. Vital signs were normal at birth (Apgar score 9/10/10). Physical examination showed cleft palate, low-set deformed ears, a flat nasal bridge, brachycephalus, strabismus, and oblique eye fissures with epicanthic skin folds. Laboratory investigations at birth revealed no abnormal findings. Chromosomal analysis did not detect any abnormalities. Developmental delay became evident by the end of the first year, accompanied by a significant failure to thrive. The patient neither learned to speak nor to walk unassisted. Spoon-feeding was possible, but was supported by a percutaneous gastrostomy.

At the age of 14 years, a malignant melanoma on the left lower leg was diagnosed and subsequently excised. In recent years, the patient developed recurring episodes of abdominal pain and chronic constipation, without evidence of stool-withholding behavior. Laboratory tests demonstrated no electrolyte abnormalities, anemia, hypothyroidism, or evidence of celiac disease or colitis. We used different laxatives without much benefit. By the age of 16 years, the patient had developed a severe megacolon and mechanical assistance for defecation was needed daily. Hinton's test showed considerably extended intestinal transit time. Although usually not the first choice in treating patients with slow transit, double-barreled ileostomy construction was performed to avoid further complications. During the procedure, we took intestinal biopsies from the ileum and sigmoid colon for histopathological analysis. After surgery, no laxative therapy was needed. Colonic diameter and the patient's complaints were significantly reduced.

Histological examination of the intestinal biopsies revealed abnormal morphology and an extremely low density of c-kit-positive ICCs in the colon (Figure 1). Immunohistochemistry showed normal staining of mast cells and neurons in the gut mucosa and submucosa. To investigate whether the lack of ICCs might be due to functionally relevant genetic alterations in the synthesis of the tyrosine kinase Kit, we used mast cells of the patient as easy-to-obtain Kit-expressing cells. Multiple genetic alterations of Kit were detected at the level of mRNA in mast cell progenitors, which resulted in changes of the deduced amino acid sequence (Figure 2): E270K, insertion of intron 20 between exons 20 and 21, which created a stop codon at amino acid position 935; and six isoforms of transcripts of c-kit, which are due to alternative pre-mRNA splicing (ins Q252; del GNNK510-513; del S715).

However, the examination of genomic mast cell DNA for the presence of another well known mutation of c-kit, the gain-of-function mutation D816V, which is found in > 80% of cases of systemic mastocytosis, was negative. In bone marrow examination, normal hematopoietic tissue was found, and in laboratory work-up, normal values for tryptase in serum and histamine metabolites in urine were detected. The absence of any hematopoietic abnormalities in our patient suggests appropriate secretion of SCF.

DISCUSSION

In patients with idiopathic megacolon and STC, it has been proposed that colonic dysmotility might result from alterations of neuronal cells, smooth muscle cells and/or ICCs in the gastrointestinal tissue. By now, histopathological and functional abnormalities of all three final effectors of gastrointestinal sensomotoric function have been reported in the literature. However, it still remains unclear whether these changes are primary, secondary or merely epigenomic[11]. Only a few specific histopathological abnormalities have been found and described to date[12]. Concerning ICCs, there have been two studies of patients with idiopathic megabowl[5,13] and four studies of patients with idiopathic constipation with decreased ICC density[6,14-16]. By contrast, another study of 63 patients with megacolon has shown no consistent alterations in colonic ICC histology[17].

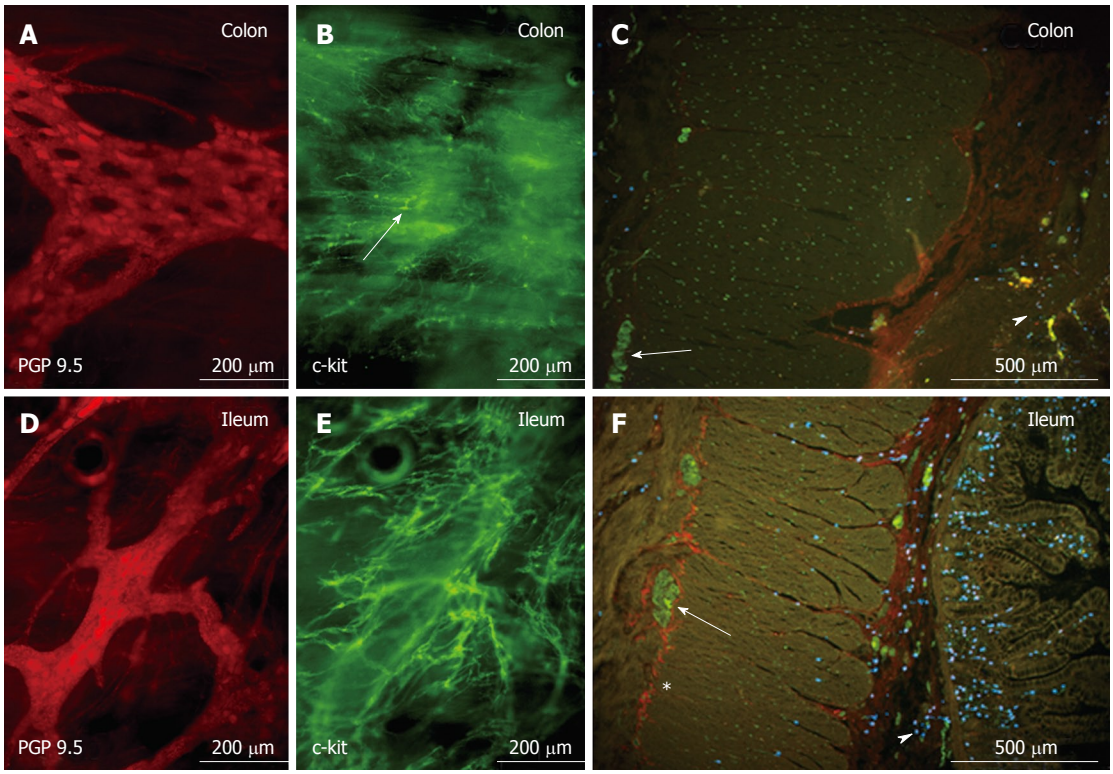

Figure 1 Whole mount preparation of the myenteric plexus of the patient's colon (A and B) and ileum (D and E), which shows abnormal morphology (arrow in B) and extreme low density of c-kit-positive interstitial cells of Cajal in the colonic biopsy specimen (B), while a dense interstitial cells of Cajal network with normal cell morphology was present in the ileum (E). Also remarkable are the holes in the colonic ganglia (A), which are very uncommon in young patients. D shows normal morphology and density of ganglia and protein gene product (PGP)-positive myenteric neurons in the ileum. Cross-sections of colon (C) and ileum (F) illustrate the normal appearance of myenteric interstitial cells of Cajal (ICCs) (*c-kit, red) in the ileum, but the complete absence of myenteric ICCs in the colon. (A and D: staining for the neuronal marker PGP 9.5, red; PH164, The Binding Site, Birmingham, UK; B and E: staining for c-kit as ICC marker, green; PC34, Oncogene, Boston, MA, USA; C and F: PGP-positive neurons (green) in a ganglion (arrow), mast cells in mucosa/submucosa (arrowhead), stained by a tryptase antibody, blue; MAB1222, Chemicon, Schwalbach, Germany.

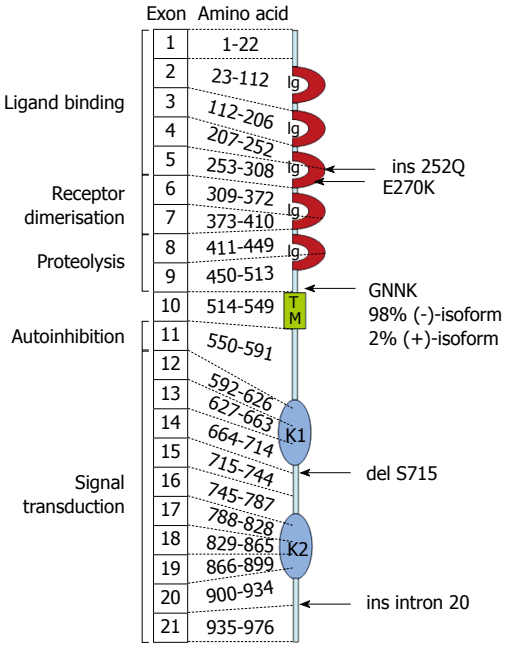

Figure 2 Schematic representation of the molecular and functional structure of the tyrosine kinase Kit (CD117), which indicated the patient's mutations in the deduced amino acid sequence. Details are discussed in the text (Ig, immunoglobulin-like domain; TM, transmembranous domain; K1, ATP-binding region of kinase domain; KI, kinase-insert-region; K2, region of phosphorylation of kinase domain).

In our patient, the biopsy specimen showed only a few ICCs in the ileum and complete absence of ICCs in the sigmoid colon, which is similar to single patients reported by Sabri *et al*[18] and Kenny *et al*[19]. However, in our patient, mutational analysis of Kit revealed multiple specific genetic alterations at the level of mRNA, which could have resulted in a loss of function of the Kit protein. In particular, mRNA that contains the premature stop codon at amino acid position 935 can be rapidly degraded via nonsense-mediated mRNA decay[20]. Accordingly, synthesis of the corresponding Kit protein would be reduced. The functional relevance of the six isoforms of Kit (Figure 2) cannot be assessed on the basis of the present data. In particular, the two GNNK-isoforms differ markedly in their functional activities, with the GNNK(-) isoform showing tumorigenic potential[21,22], which was almost exclusively detected in our patient. The point mutation E270K is probably only of minor relevance for the functional activity of Kit, because E270 is not highly conserved between species.

To date, animal studies have already shown that point mutations in the tyrosine kinase Kit correlate with abnormal intestinal contractions *in vitro*[23]. Accordingly, we suggest that the genetic alterations of Kit in our patient led to loss of protein function and to alterations in ICC architecture, and therefore, reduced bowel peristalsis. This

hypothesis provides a novel intriguing explanation for congenital interference with ICC development in cases of STC.

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CASE REPORT

Gastrojejunostomy followed by induction chemotherapy for incurable gastric cancer with outlet obstruction

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Abstract

A 72-year-old male gastric cancer patient with outlet obstruction underwent laparoscopic exploration. The examination disclosed intraperitoneal free cancer cells with no overt peritoneal, lymphatic, or hepatic metastasis. The patient underwent laparoscopy-assisted gastrojejunostomy (LAGJ) and started chemotherapy with S-1 plus cisplatin on postoperative day 13. Three course of the chemotherapy shrank the tumor markedly. Then, the patient underwent gastrectomy with a curative intent. Laparotomy revealed no intraperitoneal free cancer cells, and microscopically complete resection was achieved. The patient received S-1 chemotherapy as postoperative adjuvant treatment for 1 year, and is still alive with no evidence of peritoneal recurrence. LAGJ followed by S-1 plus cisplatin is one of the optional treatments that should be considered for patients with outlet obstruction as it may widen opportunities for potentially curative resection.

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Key words: Gastric cancer; Outlet obstruction; CY1; Laparoscopy-assisted gastrojejunostomy; S-1 plus cisplatin

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INTRODUCTION

In gastric cancer, intraperitoneal free cancer cells, described as CY1 in the Japanese Classification of Gastric Carcinoma (JCGC), 2nd English Edition, are one of the incurable factors[1]. The term "CY1" means histologically remnant tumors and gastric cancer with CY1 is diagnosed as stage Ⅳ and associated with poor prognosis. It has been reported that the survival time of gastric cancer patients with CY1 but not with overt peritoneal and other incurable metastases (P0CY1) is almost the same as that of patients with gross peritoneal metastases[2]. However, several reports have revealed that S-1, an oral agent consisting of tegafur, gimeracil, and oteracil potassium at a molar ratio 1:0.4:1[3], might improve prognosis in gastric cancer patients with P0CY1[4,5]. Furthermore, the recent phase Ⅲ clinical trial named SPIRITS trial demonstrated that S-1 plus cisplatin prolongs the survival time of patients with advanced or recurrent gastric cancer compared to S-1 alone[6].

Gastric cancer with outlet obstruction (GCOO) is a type of advanced cancer arising from the distal third of

the stomach. GCOO is associated with not only food intake inability but also metastatic disease[7]. In particular, Japanese patients with GCOO are faced with an oncology-specific issue, namely the patients cannot receive the most promising chemotherapy with S-1 plus cisplatin for incurable gastric cancer because of the inability to ingest S-1 capsules. In such patients, palliative gastrectomy is commonly selected and chemotherapy with S-1 plus cisplatin is subsequently prescribed, if possible. However, the SPIRITS trial also revealed that a considerable number of patients administering S-1 plus cisplatin suffered from severe toxic events and withdrew from the treatment[6]. Palliative gastrectomy seems to be unsuitable for inducing patients with incurable GCOO swiftly to the highly effective chemotherapy with S-1 plus cisplatin.

To solve such a practical problem, we have devised a pioneering therapeutic strategy to facilitate early induction and reliable continuation of S-1 plus cisplatin as an induction treatment for GCOO patients with P0CY1. The strategy consists of two steps. The first step is laparoscopy-assisted gastrojejunostomy (LAGJ) to allow the patient to ingest food and induce chemotherapy with S-1 plus cisplatin, the second step is gastrectomy for complete resection of the tumor and postoperative chemotherapy using S-1 alone.

Here, we present a successfully treated patient with incurable GCOO under our new therapeutic strategy.

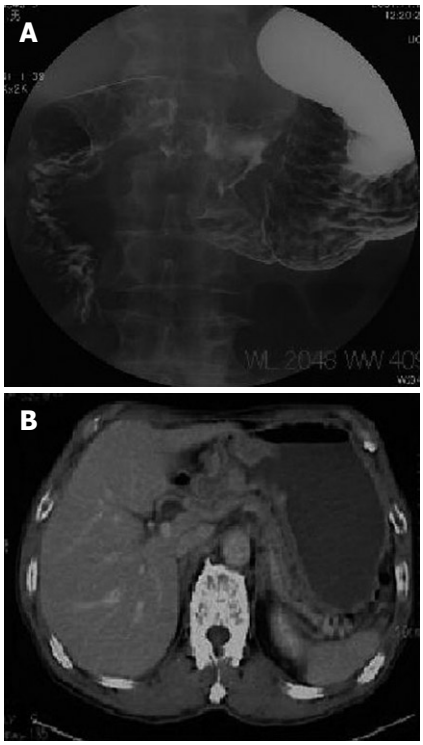

Figure 1 Gastrografin meal study revealing a tumor with ulceration at the gastric antrum, causing gastric outlet obstruction (A), and abdominal computed tomography scan showing remarkable thickness of gastric wall with no evidence of direct invasion to the pancreas head, and lymph node metastasis or distant metastasis (B).

CASE REPORT

A 72-year-old male suffering from GCOO was referred to our hospital. A gastrografin meal study revealed a type 3 tumor existing at the gastric antrum and causing gastric outlet obstruction (Figure 1A). Abdominal computed tomography scan showed neither lymph node metastasis nor distant metastasis (Figure 1B). Laboratory tests revealed that all the data were within normal limits.

Considering the high possibility of existing peritoneal metastases, we conducted laparoscopic exploration. Laparoscopic examination disclosed P0CY1, and we decided to conduct our new therapeutic strategy for GCOO with P0CY1. Laparoscopic examination was immediately converted into LAGJ with the intention of early induction of chemotherapy with S-1 plus cisplatin and the subsequent radical surgery was planned, that would enable potentially curable resection. The LAGJ was made in a Roux-en Y fashion in an antecolic manner (Figure 2A). We partitioned the stomach using a linear stapler, creating a small tunnel at the lesser curvature. Anastomosis was made between the distal stump of the proximal stomach and the jejunum. We located the partition at the upper part of the stomach, with the intent that cutting the tunnel would be a single procedure in reconstruction of the next surgery to be performed after chemotherapy (Figure 2B). The patient recovered swiftly and chemotherapy with S-1 plus cisplatin was started on postoperative day 13. The daily dose of S-1 was 120 mg/body (3 wk on and 2 wk off). Cisplatin (90 mg/body) was given intravenously on day 8 of S-1 administration. The patient received 3 cycles of the chemotherapy at the outpatient clinic without significant adverse events.

After 3 courses of the chemotherapy, examination revealed that the tumor shrank markedly although the gastric outlet obstruction still remained. The patient underwent laparotomy with a curative intent. Surgical exploration revealed that there was no metastasis to the peritoneum or the liver, and lavage cytology was negative. The tumor was excised with distal gastrectomy and D2 lymph node dissection (Figure 2C). Reconstruction was not necessary because we could retain the proximal gastrojejunostomy as a reconstruction route, which was previously made at the LAGJ.

Grossly, the resected specimen had a shallow depressed lesion at the antrum, which appeared to be only fibrosis (Figure 3). Histopathological examination revealed that the fibrotic change generated from the chemotherapy extended widely and live cancer cells were found throughout the whole gastric wall. Out of the 34 dissected lymph nodes, cancer metastasis was found in a single lymph node at station No. 7. The gastric cancer was finally diagnosed as T3, N2, H0, P0, CY0, M0, stage ⅢB, based on the JCGC, 2nd English Edition.

The patient was discharged on postoperative day 8 and underwent subsequent postoperative chemotherapy with S-1 alone for 12 mo. The patient is still alive and shows no evidence of peritoneal recurrence 20 mo after the initial surgery.

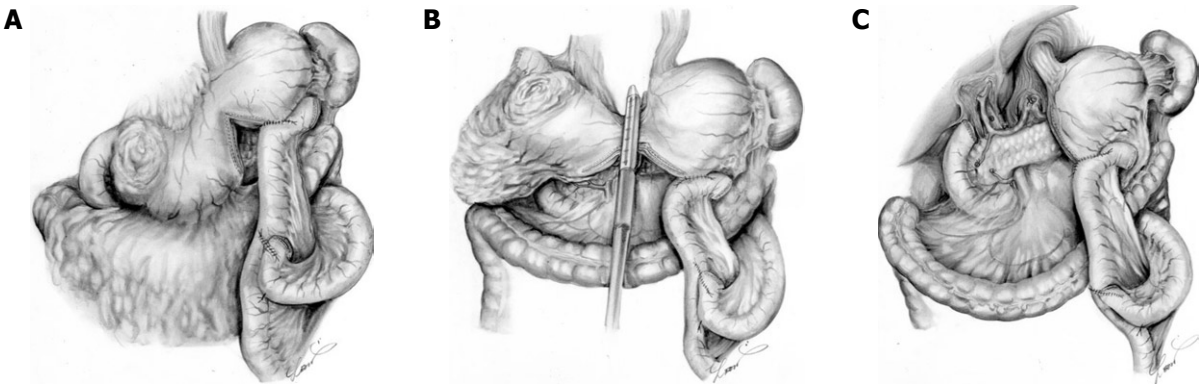

Figure 2 Laparoscopy-assisted partitioning gastrojejunostomy conducted in a Roux-en Y fashion in an antecolic manner (A), cutting the tunnel as a single procedure in reconstruction of the second surgery after chemotherapy (B), and the completely excised tumor with distal gastrectomy and D2 lymph node dissection (C).

Figure 3 Resected specimen showing a grossly shallow depressed lesion as only fibrosis at the antrum with live cancer cells found in the fibrotic tissue throughout the whole gastric wall at histopathological examination.

DISCUSSION

We presented a treated patient suffering from unresectable GCOO. The patient was treated with our new therapeutic strategy for GCOO with P0CY1. He underwent LAGJ and was induced swiftly to chemotherapy with S-1 plus cisplatin as an induction treatment, followed by potentially curative gastrectomy. We reported the present case because we believe in its importance in devising a novel therapeutic strategy for GCOO with P0CY1.

In Japan, it is considered important to facilitate immediate induction and reliable continuation of S-1-based chemotherapy for the treatment of unresectable and recurrent gastric cancer. In particular, S-1 plus cisplatin, demonstrating a response rate (RR) of 54% and a median survival time (MST) of 13 mo in the SPIRITS trial[6], is recommended as the first-line chemotherapy. Recent clinical trials in other countries, such as the V325 study and the REAL-2 trial, demonstrated that RRs of docetaxel, cisplatin, and 5-fluorouracil (DCF) and epirubicin, oxaliplatin, and capecitabine (EOX) are 37.5% and 47.9%, MSTs of DCF and EOX are 9.2 and 11.2 mo, respectively[8,9]. S-1 plus cisplatin is more favorable than the other regimens tested in Western countries, and is the first choice of treatment for unresectable and recurrent gastric cancer in Japan.

There are two possible options for induction to S-1 plus cisplatin for unresectable GCOO. First, palliative gastrectomy is initially done, followed by chemotherapy with S-1 plus cisplatin. Second, methods other than gastrectomy, such as bypass surgery or metallic stent insertion to enable patients to ingest food and S-1 capsules, are performed with S-1 plus cisplatin subsequently prescribed[10,11]. There is a major problem in the first option, i.e. the feasibility of S-1 plus cisplatin after recent gastrectomy has not been established yet. S-1 plus cisplatin is a toxic regimen and the SPIRITS trial demonstrated that more than 30% of the patients assigned S-1 plus cisplatin suffered from grade 3 to 4 anorexia and myelosuppression, and withdrew from the trial because of toxic events[6]. It is possible that patients who undergo palliative gastrectomy for GCOO fail in immediate induction and reliable continuation of chemotherapy with S-1 plus cisplatin. In contrast, the second option possibly enables patients to more immediately receive chemotherapy with S-1 plus cisplatin and more reliably continue it. In the present case, we conducted LAGJ to induce immediate ingestion of food and S-1 capsules, considering that LAGJ was reported to minimally suppress the patients' immune function and enable earlier recovery of bowel movement[12]. In fact, the present patient started S-1 plus cisplatin on day 13 after LAGJ and did not suffer from severe toxicities.

In the present case, we chose S-1 plus cisplatin as the induction treatment for gastric cancer with P0CY1, and CY1 were eventually eliminated. CY1 are one of the most chemosensitive lesions among the metastases. Nakagawa *et al*[13] revealed that 61% of patients who receive preoperative chemotherapy have no free cancer cells at the time of the second surgery. Satoh *et al*[14] reported that CY1 observed by staging laparoscopy can be eliminated by preoperative chemotherapy with S-1 plus cisplatin in 7 of 10 patients. Based on these favorable data, we intended to perform curative gastrectomy for the present patient and were actually able to do so. However, whether this result contributes to survival benefits is unclear. Satoh *et al*[14] also

described that 4 of 7 responders to preoperative chemotherapy for CY1 remain free from peritoneal metastasis. Our patient presented with no signs of peritoneal recurrence even though he initially had stage Ⅳ gastric cancer. However, whether the therapeutic strategy adopted in the present case truly provides survival benefits or not, needs a longer follow-up of the patient.

Chemotherapy with S-1 plus cisplatin is suitable for gastric cancer with P0CY1. Thus, LAGJ followed by induction chemotherapy with S-1 plus cisplatin is a possible strategy if the patient has GCOO and complies strictly with the regimen.

In conclusion, LAGJ, followed by induction chemotherapy with S-1 plus cisplatin and subsequent gastrectomy with curative intent, is one of the relevant strategies for GCOO with P0CY1.

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 35 September 21, 2010

EDITORIAL

4371 Natural orifice transluminal endoscopic surgery: Where are we going?
Whang SH, Thaler K

4374 Esophagogastric junction gastrointestinal stromal tumor: Resection *vs* enucleation
Coccolini F, Catena F, Ansaloni L, Lazzareschi D, Pinna AD

TOPIC HIGHLIGHT

4377 Liver transplantation for alcoholic liver disease
Varma V, Webb K, Mirza DF

REVIEW

4394 Diagnosis and management of interstitial pneumonitis associated with interferon therapy for chronic hepatitis C
Ji FP, Li ZX, Deng H, Xue HA, Liu Y, Li M

ORIGINAL ARTICLE

4400 Pegylated interferon α-2b plus ribavirin for older patients with chronic hepatitis C
Kainuma M, Furusyo N, Kajiwara E, Takahashi K, Nomura H, Tanabe Y, Satoh T, Maruyama T, Nakamuta M, Kotoh K, Azuma K, Shimono J, Shimoda S, Hayashi J, The Kyushu University Liver Disease Study Group

4410 Diagnostic value of glypican-3 in serum and liver for primary hepatocellular carcinoma
Liu H, Li P, Zhai Y, Qu CF, Zhang LJ, Tan YF, Li N, Ding HG

BRIEF ARTICLE

4416 Measuring episodic abdominal pain and disability in suspected sphincter of Oddi dysfunction
Durkalski V, Stewart W, MacDougall P, Mauldin P, Romagnuolo J, Brawman-Mintzer O, Cotton P

4422 Decreased serum essential and aromatic amino acids in patients with chronic pancreatitis
Adrych K, Smoczynski M, Stojek M, Sledzinski T, Slominska E, Goyke E, Smolenski RT, Swierczynski J

4428 Exercise-provoked esophageal motility disorder in patients with recurrent chest pain
Budzyński J

4436 High-dose-rate intraluminal brachytherapy during preoperative chemoradiation for locally advanced rectal cancers
Tunio MA, Rafi M, Hashmi A, Mohsin R, Qayyum A, Hasan M, Sattar A, Mubarak M

4443 Clinical significance of *Helicobacter pylori cagA* and *iceA* genotype status
Amjad N, Osman HA, Razak NA, Kassian J, Din J, bin Abdullah N

4448 *p73* G4C14 to A4T14 polymorphism is associated with colorectal cancer risk and survival
Lee KE, Hong YS, Kim BG, Kim NY, Lee KM, Kwak JY, Roh MS

4455 Antacid effects of Chinese herbal prescriptions assessed by a modified artificial stomach model
Wu TH, Chen IC, Chen LC

4460 Evaluation of cross-reactive antibody response to HVR1 in chronic hepatitis C
Xiu BS, Feng XY, He J, Wang GH, Zhang XY, Zhang HQ, Song XG, Chen K, Ling SG, Zhu CX, Wei L, Rao HY

4467 Diabetes mellitus and hepatocellular carcinoma: Comparison of Chinese patients with and without HBV-related cirrhosis
Gao C, Zhao HC, Li JT, Yao SK

4476 HOGG1 polymorphism in atrophic gastritis and gastric cancer after *Helicobacter pylori* eradication
Sun LM, Shang Y, Zeng YM, Deng YY, Cheng JF

4483 Paeonol inhibits tumor growth in gastric cancer *in vitro* and *in vivo*
Li N, Fan LL, Sun GP, Wan XA, Wang ZG, Wu Q, Wang H

CASE REPORT

4491 Febrile cholestatic disease as an initial presentation of nodular lymphocyte-predominant Hodgkin lymphoma
Mrzljak A, Gasparov S, Kardum-Skelin I, Colic-Cvrlje V, Ostojic-Kolonic S

4494 Delayed internal pancreatic fistula with pancreatic pleural effusion postsplenectomy
Jin SG, Chen ZY, Yan LN, Zeng Y

LETTERS TO THE EDITOR

4497 Walled-off pancreatic necrosis: Wishing our pancreatitis nomenclature was correct
Wysocki AP

World Journal of Gastroenterology
Volume 16 Number 35 September 21, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

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EDITORIAL

Natural orifice transluminal endoscopic surgery: Where are we going?

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Abstract

The foundation for natural orifice transluminal endoscopic surgery (NOTES) is to access the peritoneal and other body cavities through the wall of the alimentary tract *via* natural orifices, with the goal of performing procedures within the peritoneum and other cavities, without the need to make incisions in the abdominal wall. We have made great progress in the field of NOTES since the publication of the White Paper in 2006. There are still major fundamental goals as outlined by the Society of American Gastrointestinal and Endoscopic Surgeons/American Society for Gastrointestinal Endoscopy joint committee that need to be evaluated and answered before NOTES is ready for widespread clinical use. These include prevention of infection, instrument development, creation of a multitasking platform, and the ability to recognize and treat intraperitoneal complications such as hemorrhage and other physiological adverse events. In response to this need, recent abstracts and papers have focused on the management of intraoperative complications. The next phase is to focus on controlled prospective multicenter clinical trials that compare defined NOTES procedure to standard laparoscopy. The goal is to produce reliable and convincing data for the United States Food and Drug Administration, insurance companies, the physician community and the general public. At the present time, we still have many important milestones that still need to be met. Most investigators agree that a hybrid technique and not a pure NOTES practice should be advocated until devices can meet the current and new challenges in this field.

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Key words: Natural orifice transluminal endoscopic surgery; Endoscopic surgery; Gastrointestinal surgery; Laparoscopy

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INTRODUCTION

"Surgery, gaining much from the general advance of knowledge, will be rendered both knifeless and bloodless." (John Hunter, 1762). Dr. John Hunter, an accomplished anatomist and surgeon during the 18th century was many years ahead in his thinking. The foundation for natural orifice transluminal endoscopic surgery (NOTES) is to access the peritoneal and other body cavities through the wall of the alimentary tract *via* natural orifices. This allows one to perform procedures within the peritoneum and other cavities, without the need to make any incisions in the abdominal wall. The concept has generated much excitement and controversy over the past decade and has

revolutionized our thinking about what is possible, from simple peritoneoscopy to resection of solid organs.

WHERE HAVE WE BEEN

In 2005, members of the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) and the American Society for Gastrointestinal Endoscopy (ASGE) met to form the Natural Orifice Consortium for Assessment and Research (NOSCAR). This led to the production of the White Paper on NOTES in 2006, which laid down the fundamental objectives and goals that need to be addressed before NOTES will be ready for clinical application[1]. Since then, it has become clear that the field of NOTES research would create quite a stir in the gastroenterology and surgical worlds. This is reflected by the number of abstracts devoted to NOTES at the annual Digestive Disease Week (DDW), which has demonstrated a 20-fold increase from 2004 to 2007. There were 55 NOTES-related abstracts presented at DDW/Society for Surgery of the Alimentary Tract in 2009, which was the highest number reported over the last 6 years[2]. This indicates that numerous investigators have performed fundamental and noteworthy advances in a short period of time.

There have been some 60 human NOTES cases reported in the United States; the majority in the context of transgastric and transvaginal appendectomy and cholecystectomy[3], all approved by review boards to be performed under research conditions. In South America, this number has already reached the hundreds. The Brazilian NOTES Registry from 2008 highlights their experience with 116 institutional-review-board-approved procedures[4]. The majority of the procedures (94) were transgastric and transvaginal cholecystectomies. Other procedures reported in the database are transvaginal appendectomy, nephrectomy, and gynecological procedures. The bulk of reported human NOTES procedures have been limited to hybrid, laparoscopic-assisted approaches in the setting of case reports and phase I studies. Most of these studies have demonstrated safety and feasibility with variable completion rates. The major criticism to current NOTES research is the heavy emphasis on "proof of concept" without addressing management of potential complications and providing solid data on its benefits compared to laparoscopic surgery. A recent study by a German group completed a retrospective case-controlled study which compared transvaginal cholecystectomy (47 patients) to conventional laparoscopic cholecystectomy (46 patients)[5]. Hensel *et al*[5] reported similar complication rates but the NOTES group had a lower need for postoperative analgesia, faster recovery, and better cosmetic results compared to their laparoscopic counterparts. Although this was a retrospective study, the group aimed to evaluate the presumed benefits of NOTES compared to conventional laparoscopic procedures through a comparative study. These results are very exciting but will need to be confirmed and reproduced through prospective clinical trials. In addition, a recent study by Federlein *et al*[6] has combined the use of NOTES with traditional laparoscopy by performing video-assisted transvaginal cholecystectomy with rigid instruments[6]. They have determined that transvaginal cholecystectomy is safe and easy to learn but the potential complications are different from those of standard laparoscopy. The authors acknowledge that future studies including randomized trials need to be done to delineate the exact advantages of this procedure over standard laparoscopy.

FUTURE GOALS

We have made great progress in the field of NOTES since the inception of the White Paper. It is fair to state that major goals outlined by the SAGES/ASGE joint committee, such as safe access to the peritoneal cavity and closure techniques, have been sufficiently addressed. However, there are many fundamental challenges as outlined in the White Paper that need to be evaluated and answered before NOTES is ready for widespread clinical use. These include prevention of infection, instrument development, creation of a multitasking platform, and the ability to recognize and treat intraperitoneal complications such as hemorrhage and other physiological adverse events[1]. In response to this need, recent abstracts and papers have focused on the management of intraoperative complications. Investigators have offered novel strategies and techniques to manage complications such as the use of a flexible bipolar forceps to obtain hemostasis in a quick and reliable fashion[7]. Furthermore, additional studies need to be performed to address the role of NOTES in the setting of intraperitoneal contamination and infection, ensuring the maintenance of spatial orientation, and assessing the safest method of specimen retrieval. Advanced flexible platforms and endoscopic suturing devices still need to be developed, which is heavily dependent on industry collaboration. Training has been assessed recently in this novel area of minimally invasive approaches, with the first abstract presented at DDW on the development of a skills assessment model for NOTES by Vassiliou *et al*[8]. Clearly, this is an exciting time and must be balanced by healthy skepticism and caution to ensure patient safety and maximum benefit. These efforts should be undertaken by experienced multidisciplinary teams with the interest of patients and societal gain at heart.

Our group has recently completed our experience with transvaginal flexible peritoneoscopy with laparoscopic assistance in women with chronic pelvic pain[9]. A complete diagnostic peritoneoscopy was performed in five women with a mean intra-abdominal insufflation pressure of 7 mmHg to visualize pelvic and abdominal structures, with minimal postoperative pain and high cosmetic satisfaction. However, we discovered that the flexible biopsy forceps was not sharp enough alone to achieve a reliable peritoneal biopsy and required laparoscopic assistance in three of four patients. Other therapeutic interventions that were undertaken included appendectomy, simply ovarian cyst drainage, and lysis of adhesions. We concluded that transvaginal peritoneoscopy might provide advantages over standard laparoscopy specifically as it applies to visualization, pelvic accessibility, and postoperative outcome. How-

ever, we recognize the need for improved instrumentation to forgo laparoscopic assistance. Potential advantages such as minimal anesthesia requirements, shorter recovery time, and integration into an ambulatory procedure can only be implemented once we have the means to answer these looming questions with solid evidence.

CONCLUSION

Is NOTES ready for prime time? At present, the answer is no because we still have many important milestones that need to be met. What we have learned from past research is that the human NOTES experience is feasible but limited by current instruments and techniques. Many procedures have been tested in animal models but very few have confirmed their safety and feasibility in humans. We do know that in the current state, longer operative times and a steep learning curve are part of the package when it comes to NOTES procedures. Most investigators agree that a hybrid technique and not a pure NOTES practice should be advocated until devices can meet the current and new challenges in this progressive field. As we have learned from the introduction of past new innovative surgical techniques, such a feat is naturally met with heavy scrutiny. Such undertakings require a large amount of effort, time, and cost to be taken on by investigators, collaborating industry and society. We believe it will be paramount to find the complementary value of NOTES to existent minimally invasive and endoscopic procedures as we continue to make advances in this field.

The first NOSCAR research grants were awarded in 2006, and since then, four rounds of NOTES funding has been completed by NOSCAR for a total of 63 grants[10]. These have mainly been preclinical studies and some human phase I studies. The next phase is to focus on controlled prospective multicenter clinical trials to compare defined NOTES procedure to standard laparoscopy. The goal is to produce reliable and convincing data for the United States Food and Drug Administration, insurance companies, the physician community, and the general public. We are at a crucial crossroads to develop a systematic and strategic plan to address fundamental complexities if NOTES is to move forward. Only then will we be able to take the next important and final step and implement NOTES into clinical practice.

As we make strides towards Hunter J's prophecy and apply it to the current state of minimally invasive surgery, we must proceed with healthy optimism balanced with sound caution.

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EDITORIAL

Esophagogastric junction gastrointestinal stromal tumor: Resection *vs* enucleation

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Abstract

Esophageal gastrointestinal stromal tumors (GISTs) are extremely uncommon, representing approximately 5% of GISTs with the majority of esophageal GISTs occurring at the esophagogastric junction (EGJ). The treatment options available for these GISTs are fairly controversial. Many different options are nowadays at our disposal. From surgery to the target therapies we have the possibility to treat the majority of GISTs, including those which are defined as unresectable. The EGJ GISTs represent a stimulating challenge for the surgeon. The anatomical location increases the possibility of postoperative complications. As the role of negative margins in GIST surgery is still controversial and the efficacy of target therapy has been demonstrated, why not treat EGJ GISTs with enucleation and, where indicated, adjuvant target therapy?

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Key words: Esophagogastric junction; Gastrointestinal stromal tumor; Surgical approach; Resection; Enucleation

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What is an esophagogastric junction (EGJ) gastrointestinal stromal tumor (GIST) and, more importantly, how do you effectively treat it? These questions have no definitive answers. In the available literature on the subject, GISTs of the EGJ are often classified among gastric or esophageal lesions, without specifying the precise anatomical site. The aim of this article is not to establish the anatomical parameters for classifying EGJ GISTs, but rather to consider the available treatment options.

The treatment options available for EGJ GISTs are fairly controversial[1]. Esophageal GISTs are extremely uncommon, representing approximately 5% of all GISTs with the majority of esophageal GISTs occurring at the EGJ[2,3]. Relevant literature reports only a few cases of these kinds of tumors, some treated with esophageal resection and others treated with enucleation. We all know how difficult it is to accurately assess the aggressive behavior of a GIST using the official classification criteria of 2002[4]. This classification considers two parameters: tumor size and mitotic index. Tumors are classified using a ranking system, grouping tumors into very low-, low-, intermediate-, and high-risk categories based on size (< 2 cm, 2-5 cm, 5-10 cm, and > 10 cm) and on number of mitoses within 50 high-power fields (HPFs); such

measurements typically being reported as less than 5, 5 to 10, or greater than 10[5]. For patients who suffer from a localized and resectable condition, surgery should be the initial stage of treatment. The goal of surgical intervention should be complete resection, leaving a negative margin and an intact pseudocapsule. Anatomical positioning should be considered so as to avoid inadvertently increasing intra- and post-operative morbidity and mortality rates. GISTs typically have promising survival prognoses given the many therapeutic options at our disposal. The use of inhibitors of KIT, PDGFR-α, ARG, c-FMS, ABL and BCR-ABL such as imatinib mesylate[6,7] during surgery has dramatically improved the prognosis of both operable and inoperable GISTs[8]. For patients who develop a resistance to imatinib, it is also possible to begin therapy using a multi-target tyrosine kinase inhibitor (sunitinib)[9].

The main obstacle preventing a comprehensive understanding of EGJ GISTs and their various methods of treatment is the condition's rarity and the subsequent shortage of literature on the subject. The need for more in-depth clinical studies from experienced treatment centers is of utmost importance.

The original approach to surgically treating general GISTs, particularly EGJ GISTs, was initially influenced by distinctly "oncological-oriented" surgical techniques. Such methods included extended resections with complete lymphadenectomies. However, after taking note of the surprisingly low local and lymphatic diffusion rates of these tumors, the current approach has gradually become less aggressive. Nowadays one of the "hot topics" for EGJ GISTs is the ongoing debate between resection and enucleation, both treatments having been incorporated within target therapy. The significance of microscopically negative margins remains a very controversial topic[10]. In 2004, the GIST Consensus Conference defined such margin negativity as being the primary goal of surgical management of GISTs, agreeing that positive margins had not been conclusively proven to affect the patient's survival[11]. In the same year, National Comprehensive Cancer Network (NCCN) guidelines stated that the objective of surgical treatment should be the macroscopic resection of the tumor[12]. Later, in 2007, the NCCN further ratified these guidelines, introducing a set of criteria which ultimately established negative microscopic margins as being the key objective in surgical treatment[13]. The topic remains highly controversial with many notable authors having yet to come to a general consensus. Some authors, such as Langer *et al*[14], maintain that the negative microscopic margins serve as a reliable prognostic indicator of tumor recurrence while others, such as DeMatteo *et al*[15], suggest that recurrences are due more to the biological behavior of the tumor itself than to the microscopic margins. Finally, several authors agree that negative macroscopic margins often require too invasive a procedure, especially if extended surgery could potentially damage neighboring viscera thereby increasing the intra- and post-operative morbidity and mortality rates[16,17]. Another issue that should be considered is the fact that tumors located in the stomach often have more favorable recovery outcomes after undergoing such treatment[8].

Regardless of the technical expertise of the surgeon, esophageal surgery remains a very difficult and precarious procedure. Various studies have been conducted regarding the complication rates of esophageal surgery. For benign pathologies that do not require radical resections or lymphadenectomies, distinct complication rates have been reported. Morbidity and mortality rates for both laparoscopic and open surgery procedures range from 6.4% to 24% and from 0% to 1.3%, respectively[18-21].

In conclusion, no definitive treatment recommendation can be made due to the fact that the literature supporting each approach has been derived from small case studies from which reliable conclusions cannot be drawn. The inherent risks of aggressive gastroesophageal surgery, a relatively invasive procedure to address a pathology that has demonstrated an increasingly positive response to pharmacological treatments, have to be considered. Moreover, the lack of substantial evidence supporting the claim that extensive resections are correlated with better survival rates has to be kept in mind. Why not consider the possibility of treating EGJ GISTs with enucleation and, if indicated, adjuvant target therapy, thereby reducing esophageal and gastroesophogeal resections which undoubtedly result in higher morbidity and mortality rates? A prospective multicenter evaluation focusing on EGJ GIST outcome following different surgical and medical treatments might be the best way to better understand this peculiar pathology.

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TOPIC HIGHLIGHT

Abdul-Wahed Meshikhes, Dr., MD, FRCS, *Series Editor*

Liver transplantation for alcoholic liver disease

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Abstract

Alcoholic liver disease (ALD) is the second commonest indication for liver transplantation after viral hepatitis in the United States and Europe. Controversies surround the indications and allocation of scarce and expensive resource for this so called self inflicted disease. Controversies stem from the apprehension that alcoholic recipients are likely to relapse and cause damage to the graft. There is a need to select those candidates with lower risk for relapse with the available predictive factors and scores. Substance abuse specialist and psychiatrists are mandatory in the pre-transplant evaluation and in the post-transplant follow-up. There is conflicting evidence to support a fixed period of pretransplant abstinence, although most units do follow this. Alcoholic hepatitis (AH) continues to be a contraindication for transplantation, however there is a need for further research in this field as a subset of patients with AH who do not respond to medical treatment, have high early mortality and could benefit from transplantation. One year, 3-year, and 5-year survival post-transplant is similar for both ALD and non-ALD recipients. The incidence of post-transplant rejection and retransplantation is also similar to other recipients. ALD with viral hepatitis especially hepatitis C virus leads to a more aggressive liver disease with early presentation for transplantation. ALD patients are more prone to develop *de-novo* malignancy; this is attributed to the long term effect of alcohol, tobacco combined with immunosuppression. Post-transplant surveillance is important to detect early relapse to alcoholism, presence of *de-novo* malignancy and treat the same adequately.

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Key words: Alcoholic liver disease; Orthotopic liver transplantation; Pre-transplant abstinence; Acute alcoholic hepatitis; *De-novo* malignancy; Predictors of relapse; Alcoholic liver disease; Hepatitis C virus

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the leading causes of chronic liver disease and accounts for 50% of deaths from end stage liver disease (ESLD) in western countries[1]. It is the main indication for orthotopic liver transplantation (OLT) in males and after viral hepatitis, is the second commonest indication overall in the United States and Europe[2] (Figures 1-4). ALD accounts for approximately 17%-25% of all transplants performed in the United States and Europe[3,4]. Without transplant 5-year survival in patients with ALD is as low as 23% which improves to 88% with OLT[1,5].

OLT for ALD continues to be controversial because of the ever increasing demand for donor organs and the inadequate rate of organ donation, combined with the

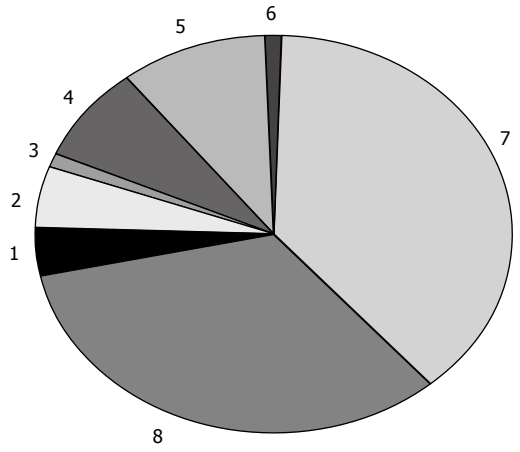
concern that alcoholic patients might relapse to drinking, thereby damaging the transplanted liver. There was an apprehension that the outcome of transplantation in these patients may not be as expected in other indications for OLT. In the initial reports, post-transplantation survival in ALD was poor (20% at 3 years), which was attributed to excessive alcohol consumption causing significant extra-hepatic organ damage, such as pancreatitis, cardiomyopathy, and cerebral dysfunction. Poor nutritional state along with the above co-morbidities was thought to impair the chances for post-transplantation survival[6]. However, there is increasing evidence that most ALD patients selected for transplantation have similar, if not better survival than those who undergo transplantation for other indications (1 year survival of 86% and 5 years survival of 74%)[7].

Patient selection for liver transplantation has always been a demanding responsibility for the transplantation professional. Less than 4% of patients with cirrhosis due to alcohol were listed in the United States in 2007. This pattern of referral may lead to as many as 12000 deaths per year[8,9]. Reasons for poor referral of these patients are multi-factorial and occur at all levels. Poor patient self identification, referring clinician misinformation, delayed intervention in alcohol cessation and counselling, premature and absolute attribution of liver disease to another aetiology (hepatitis C/B) are just some of the factors limiting effective management of alcohol related cirrhosis[4,10,11].

HISTORICAL PERSPECTIVE

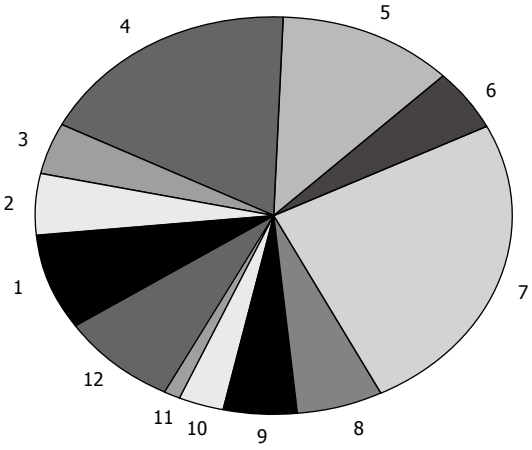
The National Institute of Health (NIH) Consensus Conference on Liver Transplantation in 1983 concluded that ALD is an appropriate indication for OLT, provided the patient is judged likely to abstain from alcohol after transplantation[12]. Following this, there was an increase in the number of transplants being performed for ALD. Starzl *et al*[13] reported that 73% of ALD patients who received a liver transplant were surviving 1 year following the procedure and that only 3% of those patients had relapsed to alcoholism. This was a convincing argument in favour of OLT for ALD patients. The Health Care Financing Administration in 1991 identified ALD as one of the seven conditions for which it approved payment for OLT, but it recommended a "significant" period of abstinence for alcoholics before undergoing the procedure as well as the availability of a reasonable social support system. Beresford *et al*[14] proposed a selection method to identify alcoholic patients suitable for OLT. Lucey *et al*[15] reported on a multidisciplinary collaboration of transplant hepatologists, surgeons and psychiatrists that identified psychosocial predictors of long term sobriety and compliance after OLT in alcoholics.

The NIH workshop in 1996 on OLT for patients of ALD concluded that liver transplantation provides a good outcome in alcoholic patients and that relapse rates after OLT were lower if the patient had successfully completed conventional alcohol rehabilitation program prior to OLT[2].



1 Viral + alcoholic 1759 (4%)
2 Autoimmune 1786 (5%)
3 Secondary biliary 495 (1%)
4 Unknown causes 3274 (8%)
5 Primary biliary 4277 (10%)
6 Others 531 (1%)
7 Virus related 15936 (38%)
8 Alcoholic 13638 (33%)

Figure 1 Indications for orthotopic liver transplantation according to the European Liver Transplant Registry (2008). Alcoholic liver disease (ALD) was an indication in 33%, 4% had combined aetiology of ALD and hepatitis C/B.



1 Cryptogenic 8140 (8%)
2 Cong cholestasis 5261 (5%)
3 Metabolic dis 3967 (4%)
4 Others 17384 (18%)
5 Alcoholic 11641 (12%)
6 Alcoholic + HCV 4946 (5%)
7 Viral 24762 (25%)
8 PSC 5918 (6%)
9 PBC 4932 (5%)
10 Autoimmune 3314 (3%)
11 Sec biliary 586 (1%)
12 Malignancy 8360 (8%)

Figure 2 Indications for orthotopic liver transplantation according to the United Network for Organ Sharing (2009) data. ALD was an indication in 17% of recipients, 5% had combined indication of ALD and viral cirrhosis. HCV: Hepatitis C virus; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

ABSTINENCE BEFORE TRANSPLANTATION

Since Starzl *et al*[13] first reported on transplantation for ALD, the evidence has continued to strengthen the merit in selecting appropriate ALD candidates for transplantation[16]. Nevertheless the issue of which candidates are considered "appropriate" remains a topic of debate[17]. Without known exception, what has been accepted as standard across trans-

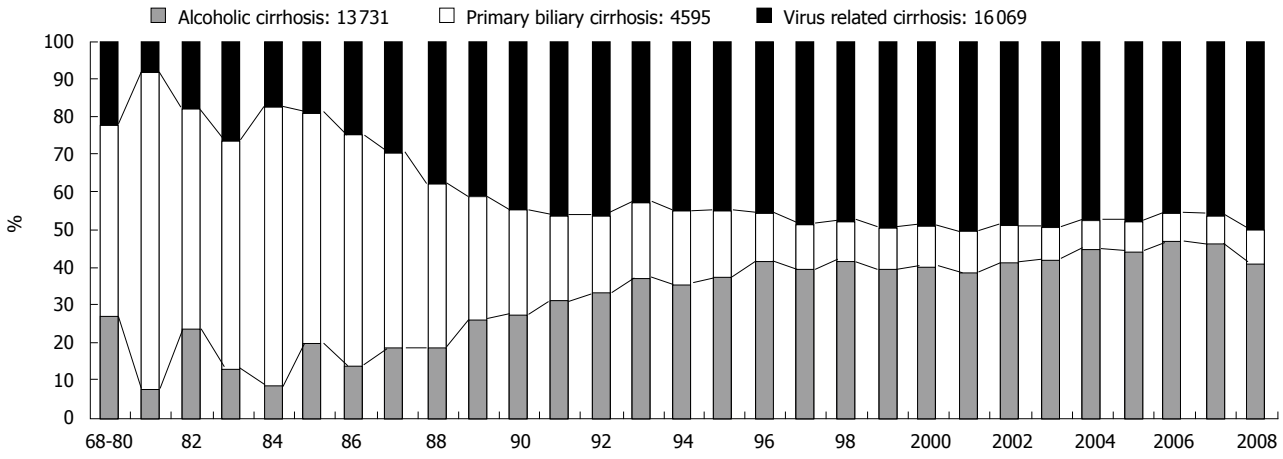


Figure 3 Evolution in the indication for orthotopic liver transplantation in European Liver Transplant Registry (2008), alcoholic liver disease is the second common indication after viral cirrhosis.

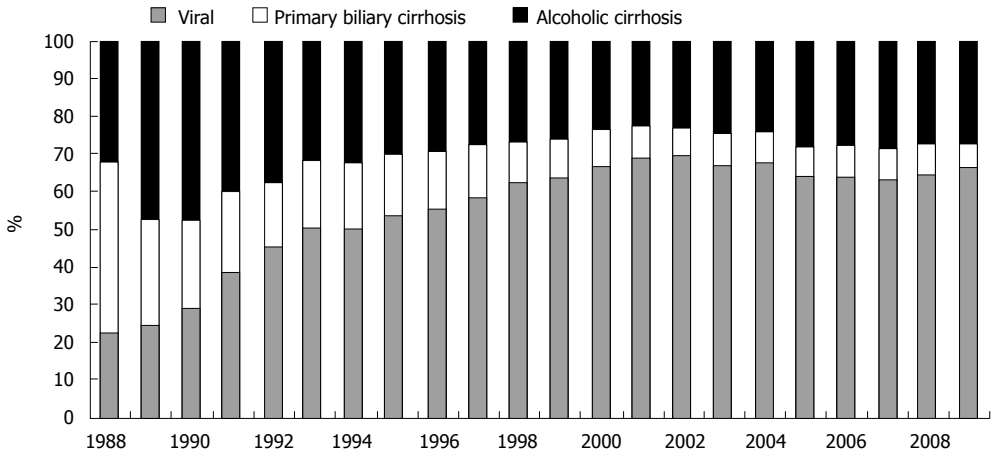


Figure 4 Evolution in the indications for orthotopic liver transplantation in the United Network for Organ Sharing (2009) data, viral cirrhosis and alcoholic liver disease are the main indications.

plant centres has been the insistence on abstinence from alcohol at the point of listing[18,19], however, the debate on the required length of pretransplant abstinence continues.

Pretransplant abstinence broadly achieves two goals; it allows a window of opportunity for the liver to stabilize, and it allows opportunity to examine the patient's commitment. This period of abstinence is important as it not only gives time for the addiction team to assess the patient and organise any support measures, it also improves the patient's condition in so much so that a few of them may no longer require transplantation. Many transplant programs (85%) in the United States require 6 mo of abstinence before transplantation[20]. About 75% of centres would expect the patients to sign a formal contract, in addition, for alcohol rehabilitation. This has however changed after 2005, following UNOS and French Consensus Conference on LT, in view of absence of enough evidence to support the 6 mo sobriety[21]. It is unclear whether this is an effective predictor for post transplant abstinence or simply a method of consistent selection - popular with insurance companies. Pfitzmann *et al*[5] in their study identified less than 6 mo period of abstinence prior to LT as a significant factor associated with relapse to harmful drinking, which was an important factor associated with reduced long term survival. Six months abstinence is mandatory in their centre before listing for LT. Gedaly *et al*[22] in a large retrospective study identified a significant association between post-transplant relapse and less than 12 mo of sobriety before transplantation. Many liver transplant programs in Europe also require pre-transplant abstinence of 6 mo to a year.

Even where there is evidence that shorter prelisting abstinence correlates to shorter time to first drink post transplant, an optimal period of pretransplant abstinence remains unclear[20,22-24]. According to the Liver Advisory Group in the United Kingdom, a fixed period of abstinence allows the addiction team to assess the patient and also provides an opportunity for improvement in these patients with ALD. UK guidelines propose that both length and context of abstinence are among factors to be considered in the wider psychosocial assessment and literature appears to support this approach[19,20,22-26] (Appendix-1[23]).

There is controversial evidence to suggest that patients with family history of alcoholism have an increased rate of relapse[8,25,27]. However, there is no strong evidence to suggest that patients of ALD with no family history of

alcoholism should be listed notwithstanding the period of abstinence.

INDICATIONS FOR OLT

Indications for OLT in patients with ALD are not different from any other cause of end stage liver disease. Minimal listing criteria include a Child-Turcotte-Pugh score greater than or equal to 7, an estimated 1 year survival without transplantation to be less than 90%, single episode of spontaneous bacterial peritonitis or the presence of stage Ⅱ hepatic encephalopathy in the presence liver failure. Allocation of organs is according to the Model for End-Stage Liver Disease regression equation which takes into account serum bilirubin, serum creatinine, and international normalized ratio and calculates a score which predicts 3 mo survival[28]. The UK Liver Transplant Units have developed a new scoring system to predict the waiting list mortality, the UKELD score (United Kingdom Model for end-stage liver disease) which is calculated from the patient's serum bilirubin, INR, creatinine and sodium. A UKELD score more than 49 is a predictor of greater than 9% 1-year mortality and is the minimum criteria for entry to the waiting list under this category. This scoring system is being followed for listing patients for OLT throughout UK liver Transplant Units[26,29,30].

The contraindications to listing were those factors which would result in poor outcome for the graft. (1) Alcoholic hepatitis (AH) which is a clinical syndrome of jaundice and coagulopathy in the presence of active alcohol intake and not a histological diagnosis is a contraindication for listing; (2) Repetitive episodes (more than 2) of noncompliance with medical care where there was no satisfactory explanation. This should not be confined to management of their liver disease; (3) Return to drinking following full professional assessment and advice (this includes permanent removal from the list if found drinking while listed); and (4) Concurrent or consecutive illicit drug use.

Once the multi-disciplinary team (MDT) opines that the patient is to be listed, then the patient is asked to sign an agreement that they will continue to abstain from alcohol in the post transplant period and will comply with follow-up. Signing of agreement is not being followed universally in all the transplant centres.

Immediate vs delayed listing

It is universally recognised that liver transplantation improves survival in patients with end stage liver disease due to alcoholic aetiology. However, for those patients whose liver function would spontaneously improve with alcohol withdrawal and conservative treatment there are no studies to compare the outcome of liver transplantation *vs* conservative treatment, especially so for patients with Child-Pugh stage B cirrhosis. It is important in an era of organ shortage to recognise which group of patients could be offered standard treatment and which group of patients should be immediately listed. The present system of organ allocation in the United States and Europe gives highest priority to the sickest patients. There have been proposals that outcome in these patients would be better if they were transplanted in the earlier stage of the disease and that this might reduce the mortality of patients on the waiting list. We have the results of a recently conducted multi centre randomized controlled trial which compared immediate listing for liver transplantation *vs* standard care for patients with Child-Pugh Stage B alcoholic cirrhosis. Patients on standard care were listed for transplantation once they progressed to Child-Pugh stage C cirrhosis[31].

This study provides four relevant results: (1) Immediate listing for liver transplantation was not associated with improved survival in patients with Child-Pugh stage B alcoholic cirrhosis. Available medical therapies are effective in preventing death not only in patients with Child-Pugh stage C disease but in those at earlier stages as well. Immediate listing for liver transplantation was in itself ineffective in preventing liver-related mortality; (2) Patients who received liver transplantation had an unexpectedly high rate of *de-novo* extrahepatic cancer, which included many upper aerodigestive tract neoplasias. These are known to be associated with alcohol intake and smoking. The occurrence of these tumors was associated with high risk of mortality. This was a deleterious effect of transplantation and immunosuppressive agents; (3) Patients with continued alcohol consumption had poor outcome regardless of the treatment received; and (4) Child-Pugh score greater than 7 was the cut-off value for predicting poor survival whereas recovery from Child-Pugh stage C was associated with a better survival. The study concludes that patients with Child-Pugh stage B alcoholic cirrhosis should not be listed for liver transplantation, especially when alcohol withdrawal is associated with recovery of liver function or when the Child-Pugh score is less than 8. The best strategy would be to consider liver transplantation on the basis of patient outcome and to actively screen these patients for extrahepatic cancer before and after liver transplantation. The results of this study support the current policy of giving priority for organ allocation to the sickest patient. There are other studies in the past which have stated that Child-Pugh stage C patients following transplantation had a higher 1- and 5-year survival than their matched controls, whereas among those with Child-Pugh stage A or B, there was no statistically significant survival difference between transplanted and their matched and simulated controls[31,32].

PRE-TRANSPLANT EVALUATION

Amidst all controversies, where there does appear to be agreement is in the timeliness of referrals to transplant centres[33,34]. Assessment from both a medical and psychosocial perspective takes time. Later referrals leave little scope to explore further medical management options or allow time to work with the substance misuse or psychiatric team. Family support may be more difficult to engage, monitoring of treatment concordance or substance misuse treatment engagement is less likely and medical conditions such as advanced hepatic encephalopathy rule out any reasonable psychotherapeutic treatment opportunities. It is a common practice - and indeed encouraged by guidelines - for units to employ the services of psychiatrists, psychologists, mental health nurses and social

Table 1 Michigan alcoholism prognosis scale

Criterion	Points
Acceptance of alcoholism	
Patient and family	4
Patient only	3
Family only	2
Neither	1
Prognostic indices	
Substitute activities	Yes 3, No 1
Behavioral consequences	Yes 3, No 1
Hope/self-esteem	Yes 3, No 1
Social relationship	Yes 3, No 1
Social stability	
Steady job	1
Stable residence	1
Does not live alone	1
Stable marriage	1
Rating	/20[1]

[1]Maximum score.

Table 2 High-risk alcoholism relapse scale

Item	Score
Duration of heavy drinking (yr)	
≤ 11	0
11-25	1
≥ 25	2
Daily drinks[1] (*n*)	
≤ 9	0
9-17	1
≥ 17	2
Prior alcoholism inpatient treatments (*n*)	
0	0
1	1
≥ 1	2

[1]One drink = 12 g of ethanol.

workers in the pretransplant assessment and evaluation of candidates with ALD[19,21,23,30].

UK Liver Transplant Group Recommendations for ALD, states that all these patients should be assessed by a specialist in substance misuse, who should have dedicated time for this purpose.

Formal pretransplant substance misuse evaluations require a broad psychosocial and substance misuse assessment which will commonly examine the nature and pattern of previous alcohol use, diagnose an alcohol use disorder, length of abstinence and factors which are likely to indicate risk of future alcohol consumption[22,35]. A number of predictive tools have been considered as part of the assessment. The University of Michigan Alcoholism Prognosis Scale examines a number of psychosocial domains with a higher score suggesting an increased stability linked to improved prognosis (Table 1), and Lucey *et al*[36] have recommended such a broad based tool as a useful alternative to a pre-transplant fixed abstinence period. Other tools include the alcohol abstinence self-Efficacy Scale which rates an individual's ability to self-determine in the context of relapse precipitants[37]. Though it shows good reliability and validity in alcohol treatment settings, it has yet to be proven in the liver transplant setting. A recent French study proposes the high-risk alcoholism relapse scale as a simple and useful predictor to be incorporated into assessment screening[38] (Table 2). The use of agreed clinical guidelines and candidate selection criteria offer the assessment team a framework upon which to base complex decisions and an opportunity to explain the assessment and decision making process. Transplant centres have a responsibility to audit their selections and outcomes against accepted listing criteria and the bodies approving the criteria have a subsequent duty to review guidance within an acceptable timeframe.

Psychiatric evaluation

Liver transplantation is a demanding procedure both in the acute stage and in the long-term. Early factors are considered to be the stress of waiting for a liver transplant - with its uncertainty in terms of both timing and outcome - as well as the physical and psychological demands of the procedure in the pre- and post-transplant period. Long term demands are linked to general quality of life (QOL) and treatment adherence. Much has been written of the need for psychological support in transplantation, though whether the ALD patient requires more input than a young person with acute liver failure or a fulminant patient secondary to a paracetamol overdose is open to debate. Dobbels *et al*[35] argue that pretransplant psychosocial screening in all transplant candidates highlights predictors and risks associated with post transplant adherence and clinical outcome, though do not single out the ALD cohort specifically. Psychiatric assessment of the ALD transplant candidate has been both undertaken and recommended for many years and the role of the psychiatrist and psychiatric team has developed and evolved, with a focus on assessment, objectivity and support - to both transplant team and patient - but at the same time caution against acting as the ethicist for the transplant team[39-42]. People judged suitable for OLT included patients with severe ESLD who showed a clear understanding of the risks and benefits of the procedure, had a favourable psychiatric assessment including acceptance of alcoholism, and had favourable prognostic factors for the future sobriety.

Results vary on the psychosocial outcomes of the transplant recipient. A single centre study of 30 UK transplant recipients reported improved QOL post transplant but not at levels consistent with the general population[43]. This is at odds with a contemporary study from another centre in the same city of a cohort of 20 subjects which found that ALD graft recipients do not have higher levels of psychiatric morbidity than other graft recipients and also found that psychiatric symptoms abated in their cohort over time[44]. A larger and more recent UK study prospectively assessed psychiatric "caseness" in 155 transplant assessment candidates. Higher rates of psychological distress were associated with greater severity of liver disease, unemployment and tobacco smoking. A DSM-IV diagnosis of alcohol abuse or dependence was not a significant predictor of psychiatric morbidity[45].

Table 3 Comorbidities associated with alcohol related liver disease

Cardiovascular	Alcoholic cardiomyopathy
	Cirrhotic cardiomyopathy
	Coronary artery disease
Musculoskeletal	Myopathy
	Osteopenia
Neurologic	Wernicke-Korsakoff psychosis
	Alcoholic dementia
	Alcoholic cerebellar degeneration
	Peripheral neuropathy
Malnutrition	
Chronic pancreatitis	
Hepatocellular carcinoma	
Hepatitis B or C infection	
Other malignancy	Upper aerodigestive tract malignancy
Psychiatric	Depression or mood disorders
	Personality disorders
	Anxiety disorders
	Psychosis

Comorbidities associated with ALD

It is seen that only a small percentage of patients with ALD, who are likely to benefit from OLT, actually undergo transplantation[46]. One of the potential reasons for the low rate of transplantation in these patients is the presence of comorbid medical conditions which might contraindicate transplantation. Comorbid medical conditions maybe either as a direct effect of alcoholism or they may be conditions commonly occurring in alcoholics (Table 3).

Cardiovascular

Patients with alcoholic cirrhosis may have alcohol related heart disease (alcoholic cardiomyopathy), heart disease associated with cirrhosis *per se* (cirrhotic cardiomyopathy), or coincidental heart disease (coronary artery disease, CAD). CAD is more common overt problem than either alcoholic or cirrhotic cardiomyopathy. Alcoholic cardiomyopathy is related to the total lifetime amount of alcohol intake[47]. Clinically resembles idiopathic dilated cardiomyopathy and is the major type of secondary dilated cardiomyopathy in Western world. Whereas idiopathic dilated cardiomyopathy is associated with progressive deterioration, alcoholic cardiomyopathy may reverse on stopping alcohol before severe heart failure develops.

Criteria for the diagnosis of alcoholic cardiomyopathy include the presence of alcohol dependence and the following cardiac findings: (1) Large left ventricular diameter on echocardiography; (2) Left ventricular ejection fraction less than 50% as measured on radionuclide angiography; (3) Normal coronary arteries on coronary arteriography; and (4) Characteristic histological changes in endomyocardial biopsy[48,49].

Alcoholic cardiomyopathy is generally associated with active alcohol intake, hence is uncommon in patients referred for OLT.

Cirrhotic cardiomyopathy is the syndrome of high output heart failure associated with impaired ventricular contractile function seen in patients with both alcoholic and non-alcoholic end stage liver disease[50]. Cirrhotic cardiomyopathy is usually mild or latent in these patients as the associated peripheral vasodilatation reduces the after load of the ventricle. OLT with shunting of large volumes of venous return back to the heart may precipitate overt heart failure and contribute to postoperative mortality[45]. The mechanism of cirrhotic cardiomyopathy involves impaired β adrenergic receptor function, alteration in plasma membrane fluidity and hyper dynamic circulatory state.

CAD is found more often (5.6%-27%) than expected in patients with ESLD being considered for OLT than in the general population. Factors proposed for such finding include: older age, preponderance of males, and frequent concomitant cigarette smoking. Associated diabetes mellitus if present is an important risk factor[50-52].

Management dilemma is posed when a patient with ESLD, who is otherwise a good candidate for OLT, is found to have moderate to severe CAD. Consensus is to treat the CAD before OLT, as OLT poses the risks of myocardial ischemia or infarction particularly in patients with triple vessel disease or left main CAD[51]. If CAD cannot be treated by percutaneous transluminal coronary angioplasty, then coronary artery bypass grafting (CABG) can be considered. Patients with ESLD might experience deterioration of hepatic functions after CABG, including portal hypertensive bleeding and worsening coagulopathy. Prophylactic placement of transjugular intrahepatic portosystemic shunt has been proposed, before CABG. Few patients have had CAD and ESLD treated with both CABG and OLT immediately following each other[51].

Currently in most of the European Transplant centres, echocardiography and electrocardiography are used routinely in pretransplant evaluation. About 50% utilise exercise or dobutamine stress tests. Radionuclide or invasive testing is not routinely undertaken. Although most centres consider cardiomyopathy as a relative contra-indication for OLT, the limits of left ventricular ejection fraction below which OLT is contraindicated is variable from 20%-50%[4]. Routine testing to exclude cardiomyopathy is not justified in asymptomatic patients[52,53].

Myopathy

Approximately half of active alcoholics have a myopathy that is related to alcohol intake, nutritional deficiency and neuropathic damage. Muscle strength is inversely related to the lifetime ingestion of alcohol[54]. Alcohol myopathy usually improves with abstinence and is not a factor for consideration in patient selection or outcome of OLT.

Neurologic

Neurologic disease with fixed deficits may be found in patients with ESLD and long standing alcoholism, and it may be difficult to differentiate it from hepatic encephalopathy, which is reversible following OLT[55]. Korsakoff's psychosis, which is characterised by profound deficits in retentive memory and learning, is a late manifestation of the Wernick-Korsakoff's syndrome, with features of opthalmoplegia, ataxia, and confusion occurring earlier

and is not always recognised. Complete recovery is uncommon. "Alcoholic dementia" is the term used for late stage Korsakoff's psychosis, which is characterised by cognitive impairment, memory dysfunction, and is associated with cerebral atrophy on imaging.

Alcoholic cerebellar degeneration is a form of cerebellar ataxia, which occurs in patients with prolonged and heavy alcohol intake. This is partly caused by nutritional deficiency, affects the stance and gait, and is usually not reversible[55]. Patients having significant cerebral or cerebellar dysfunction usually are not considered for OLT.

Peripheral neuropathy associated with alcoholism usually improves with nutritional therapy and is not a factor influencing the selection or outcome following OLT. Autonomic neuropathy is not uncommon in patients with ESLD of any aetiology, and improves with OLT. Imaging studies of the brain and psychometric testing may be required in patients with atypical hepatic encephalopathy or symptoms suggestive of organic brain dysfunction for differential diagnosis.

Chronic pancreatitis

Acute and chronic pancreatitis, an important clinical problem in alcoholic patients, seldom has an impact in the selection process for OLT. Patients with significant chronic pancreatitis are excluded from consideration for OLT. Chronic pancreatitis is less common in patients with ALD as compared to alcoholics without liver disease (1% *vs* 5%)[56]. Morbidity and mortality associated with pancreatitis following OLT is substantial and any evidence of active pancreatitis is a reason to abandon OLT[55].

Malnutrition

Malnutrition is common in patients with ESLD irrespective of the aetiology; it is one of the factors which leads to consideration for OLT. About half of the patients with alcoholic cirrhosis have protein calorie malnutrition[57]. Factors responsible for malnutrition in these patients include a poor diet, increased catabolism of carbohydrates, proteins and lipids, impaired absorption of nutrients, cholestasis with associated interruption of bile flow, pancreatic dysfunction, bacterial overgrowth and alcohol induced intestinal mucosal injury[58]. Various studies have indicated that the degree of malnutrition affects the outcome following hepatobiliary surgery and OLT, including the stay in intensive care unit, duration of ventilation, hospital stay and mortality after OLT[59-61]. Nutritional support before OLT is important for obvious reasons, and severe malnutrition may require postponement of OLT until a better state of nutrition is achieved[59].

Osteopenia

Long standing alcoholism and ALD is associated with osteopenia and reduced bone mineral density which remains unrecognised until fracture occurs. Spinal and peripheral fractures are common in patients with ESLD. Spinal and forearm osteoporosis is also more often seen in patients with ESLD. Reduced bone mineral density in various studies at various sites range between 10%-42%[62,63]. Factors attributed to osteoporosis in these patients include alcohol induced impairment of osteoblastic function hypogonadism, reduced body mass index and limited physical activity[64,65]. Pain and recurrent fractures particularly vertebral collapse are indications for transplantation in these patients[55]. It is imperative to check serum 25-hydroxyvitamin D levels in patients with ESLD and initiate vitamin D replacement therapy if the levels are low, use oestrogen therapy *via* patch in postmenopausal women, screen for testosterone deficiency in men and administer exogenous testosterone in those with low levels[55]. Treatment with calcium and vitamin D can improve bone mineral density in patients with ALD.

Other liver diseases

Approximately 20%-30% of patients with ALD have chronic hepatitis C virus (HCV) infection and the majority have detectable serum HCV RNA[66]. Diagnosis of concomitant HCV infection in patients with alcohol related cirrhosis has important implications on the outcome of OLT. HCV infection recurs in almost all patients with OLT, and 5%-10% of these would go on to develop ESLD within 3-5 years[67,68]. Patients with ALD can have coexistent chronic hepatitis B virus (HBV) and hemochromatosis. These have important implications in the treatment of these patients following OLT. Patients with HBV infection require antiviral therapy with either immunoglobulin or lamivudine and those with hemochromatosis have reduced survival following OLT, and they may benefit from phlebotomy prior to OLT[69-71].

Hepatocellular carcinoma and ALD

There is increased risk of hepatocellular carcinoma (HCC) in any patient with ESLD, including those with alcohol related cirrhosis. Patients with chronic HCV, chronic HBV and hemochromatosis have highest risk[72,73]. HCC in these patients may be detected during the transplant operation, discovered by the pathologist in the explant histology (incidental HCC) or may be detected in the pretransplant imaging (coincidental HCC). Patients with a lesion less than 3-5 cm in diameter have a good prognosis with OLT as compared to those with a larger and symptomatic lesion[74,75] (Table 4).

Other malignancy

It is seen that patients with ALD undergoing OLT have significantly increased incidence of upper aerodigestive tract cancers as compared to those with non-alcoholic ESLD. These are a major cause for morbidity and mortality following OLT (as described later)[82]. It is important that these patients undergo a thorough pretransplant evaluation to rule out these tumors before OLT and also to have regular evaluation post-OLT[4] (Tables 4 and 5).

LIVER TRANSPLANTATION IN ACUTE ALCOHOLIC HEPATITIS

Many patients with severe AH, whether in the setting of

Table 4 Outcome of post-orthotopic liver transplantation in patients with alcoholic liver disease, combined alcoholic liver disease with hepatitis C virus and hepatitis C virus alone *n* (%)

Study	ALD	ALD + HCV	HCV	Others
Burra *et al*[76]	*n* = 9880	*n* = 1119	*n* = 6672	
Patient survival 1-yr, 3-yr, 5-yr, 10-yr	84%, 78%, 73%, 58%	84%, 75%, 65%, 52%	81%, 72%, 67%,54%	
Aguilera *et al*[77]	*n* = 107	*n* = 60	*n* = 170	
HCC	19 (18)	21 (35)	75 (44)	
Graft loss	35 (33)	25 (42)	95 (56)	
Severe recurrent HCV disease		22/49 (45)	54/122 (45)	
Retransplant	4 (4)	8 (13)	7 (4)	
De-novo tumors	14/107 (13)	2/67 (3)	10/67 (6)	
Rejection	17/100 (17)	9/60 (15)	32/169 (19)	
Patient survival 1-yr, 5-yr, 7-yr	90%, 76%, 67%	86%, 73%, 63%	72%, 49%, 43%	
Graft survival 1-yr, 5-yr, 7-yr	89%, 76%, 67%	83%, 63%, 56%	71%, 48%, 43%	
Mortality	29 (27)	21 (35)	95 (56)	
Cause of death				
Recurrent disease	3 (3)	9 (15)	44 (26)	
Sepsis	7 (6.5)	7 (12)	26 (15)	
De-novo tumors	9 (8)	2 (3.5)	6 (3.5)	
Neuberger *et al*[4]				
HCC (%)	11	26	28	
Yamauchi *et al*[78] Yamanaka *et al*[79]				
Risk of HCC (at 10-yr)	15%-20%	50%-80%		
Khan *et al*[80]	*n* = 14	*n* = 24	*n* = 40	*n* = 42
HCV RNA (Meq/mL)		2.3 ± 1.7	2.7 ± 2.9	2.3 ± 2.6
Necroinflammation	1.8 ± 0.7	3.1 ± 1.1	3.4 ± 1.6	2.9 ± 1.3
Fibrosis	2.9 ± 1.0	3.6 ± 0.7	2.9 ± 0.9	3.4 ± 1.0
Cirrhosis	5 (8.4)	16 (27.2)	10 (16.9)	28 (47.5)
HCC	5 (9)	14 (25.5)	10 (18.2)	26 (47.3)
Size of HCC	1.9 ± 0.8	2.5 ± 0.8	2.5 ± 1.0	2.4 ± 0.9
Donato *et al*[81]				
Relative risk for HCC	4.6	64.7	23.2	

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ALD: Alcoholic liver disease.

Table 5 Post-orthotopic liver transplantation events-rejection, infection, malignancy, retransplantation and cardiac events

Study	Rejection (%)		Infection (%)		Malignancy (%)		Retransplantation (%)		Cardiac events (%)	
	ALD	Non-ALD	ALD	Non-ALD	ALD	Non-ALD	ALD	Non-ALD	ALD + viral	Non-ALD
Burra *et al*[76]	7.6	10.1	15.5	17.6	13.7/5.4[1]	5.6/2[1]			8	5.3
Pfitzmann *et al*[5]			4.7-6.3[2]		9.4-18.8/3.8[2]				3-11.8[2]	
Wiesner *et al*[83]	Significantly less in ALD *vs* non-ALD		Bacteremia, overall fungemia, and CMV infection, comparable		*De-novo* tumors significantly increased in ALD *vs* non-ALD		3	9		
Bhagat *et al*[84]	23/2[3]	41/4[3]	43[2]	53	29[2]	0	3.6	5.6	7[2]	26

[1]*De-novo* tumors/upper aerodigestive tract tumors; [2]Cause of death; [3]Acute/chronic rejection. ALD: Alcoholic liver disease.

previous normal liver or in those with established cirrhosis, fail to recover even after abstinence and maximal medical treatment. The severe form of AH is associated with 35%-50% mortality at 1 mo following diagnosis. Any treatment for these patients requires identification of that subgroup of patients who have significant risk of death at 1 or 2 mo. Severity of acute AH is best assessed using Maddrey discriminant function (DF), which is a reproducible, objective criterion to predict the risk of early death. This is based on prothrombin time and serum bilirubin concentration (mg/dL). It is calculated using the formula [4.6 × (prothrombin time - control prothrombin time) + serum bilirubin]. DF > 32 indicates high risk of early mortality in the absence of treatment. Spontaneous survival at 1 mo in patients with a DF < 32 is approximately 90%[85,86]. To reduce the probability of early death, patients with a DF > 32 need to be offered treatment. American College of Gastroenterology and various other studies have observed that 2 mo survival of patients of AH with DF > 32 treated with corticosteroids was approximately 80%[86-88]. One simple criterion to identify the population of patients with AH who would benefit from corticosteroid treatment is termed as an early change in bilirubin levels (ECBL), which is defined as an ECBL at 7 d, which is lower than level on the first day of treatment. At 6 mo, patients with ECBL had a significantly higher survival compared to those without ECBL (83% *vs* 23%)[89]. Another treatment which is found to have an effect in

improving survival in the index admission, compared to placebo, is that with pentoxifylline (75.5% *vs* 53.5%). Development of acute renal failure in these patients with acute AH is a bad prognostic criterion. The benefit of pentoxifylline appears to be related to a significant reduction in the risk of developing hepatorenal syndrome[90]. There is insufficient data on the benefits of transplantation in patients with AH. Offering liver transplantation to those patients who are non responders to corticosteroid treatment is still a matter of debate. These patients require alternative strategies of treatment as they have a poor survival. Most transplant centres in United States and Europe require a period of abstinence before considering transplantation which is not possible in these patients. There is limited and mixed experience of transplantation in these patients[91-95].

According to the current consensus in most European and North American transplant centres, patients with acute AH are not considered for liver transplantation[23,96]. A recent French multi-centre pilot study examined the outcomes of transplantation in patients with AH who were corticosteroid non-responders. The selection criteria included first time presenters and acceptance from all members of the transplant MDT. Twenty-two patients were listed with 18 undergoing transplantation following listing. At 6 mo, survival was 83% in transplanted patients in comparison with 44% in patients not transplanted in a case-control group. There was no reported relapse to drinking at 1 year post transplant. This data is as yet only available in abstract form and long term follow-up is required[97].

HEPATITIS C VIRAL INFECTION AND ALD

Prevalence of hepatitis C viral (HCV) infection is seven times higher in patients with ALD than in the general population. About 20%-30% of patients with ALD are infected with HCV, and the rate of progression of liver disease and the long term outcome are worse for these patients as compared to those who are not infected with HCV[55,98]. Although the outcome of patients undergoing liver transplantation for ALD is good with an overall survival of 60% at 10 years, outcome in patients with HCV cirrhosis is impaired by recurrence of disease and progression to cirrhosis[99-101]. The combination of alcohol and HCV infection leads to a rapid progression of disease, with cirrhosis developing earlier than in patients with HCV infection alone[102]. Factors proposed for this accelerated disease course are higher viral load, alterations in the immune response, alcohol induced aggravation of histological lesions, interference in hepatocyte regeneration and ineffectiveness of Interferon in these patients making treatment even more difficult[98,103-105].

A study by Aguilera *et al*[77] compared the post-transplantation outcome in patients with HCV related cirrhosis, alcoholic cirrhosis and cirrhosis of mixed aetiology (HCV and ALD) (Table 4). It is important to know the natural history post-transplantation in this group of patients, to address the expectation of these patients prior to transplantation and the potential complications. About a quarter of patients undergoing transplantation for HCV related cirrhosis had a history of significant alcohol consumption. This partially determined the course of disease prior to transplantation. On the contrary, 36% of patients undergoing OLT for ALD had associated chronic HCV infection. Age at transplantation was lower in the subgroup with mixed aetiology and the Child-Turcotte-Pugh score was higher in patients with alcoholic cirrhosis. The prevalence of hepatocellular carcinoma was more in the two groups of patients with HCV infection compared to the alcohol alone group, re-iterating the oncogenicity of the virus. This has been reported by other studies as well, a combination of hepatitis C and alcohol leading to an increased risk of HCC compared with either entity alone (50% to 80% *vs* 15% to 20% at 10 years)[78,79,81]. Post-transplantation patient survival at 1-, 5- and 7-year was significantly lower in the group of patients undergoing transplantation for HCV related cirrhosis (72%, 49% and 43%) as compared to patients with alcohol related cirrhosis (90%, 76% and 67%) or those with mixed aetiology (86%, 73% and 63%). Histological damage which was assessed by protocol biopsy at 1, 3 and 5 years post transplantation revealed no difference in the incidence of severe recurrent HCV disease or progression of disease, in patients with HCV related cirrhosis and the mixed group. Graft loss was more and graft survival was significantly lower in patients undergoing transplantation for HCV related cirrhosis compared to those with alcohol related cirrhosis or those with mixed aetiology. Despite greater survival, recurrent hepatitis C progressed similarly in patients undergoing transplantation for cirrhosis of mixed aetiology and in those undergoing transplantation for HCV alone. The authors propose that this might be attributed to greater use of antiviral agents in the mixed group compared to HCV alone group (32% *vs* 18%, $P = 0.03$) and younger age of patients in this group who were able to tolerate the treatment[106]. Patients with mixed aetiology for cirrhosis (HCV and alcohol), have more severe liver disease and this is determined by alcohol intake prior to the transplant, post transplantation course of these patients is determined by the interaction between the HCV and the new milieu, where alcohol no longer determines the progression of recurrent disease[77].

Risk of occurrence of HCC was significantly higher in the mixed aetiology group compared to HCV infection alone. HCV infected patients with moderate to heavy alcohol intake had a 1.5-2.5 fold increased risk of HCC compared to alcohol free HCV infected patients[80]. This study also demonstrated that excessive alcohol intake increased the severity of liver disease - it accelerated the degree of hepatic fibrosis, the risk of liver cirrhosis and worsened the clinical outcome of liver disease with higher risk of HCC. HCV replication however was independent of severity of liver disease[80].

Other problems encountered in HCV infected patients with excessive alcohol intake are that if there is early presentation for transplantation, alcohol abuse may not be investigated as in a typical case of alcohol related cirrhosis, thus the follow-up measures to detect relapse

Table 6 Comparison of outcomes of patients with alcoholic liver disease post-orthotopic liver transplantation

Study	Time period	Number of patients	Survival (%)				Relapse (%) any (abusive)	Death due to relapse (%)
			1-yr	3-yr	5-yr	10-yr		
Burra *et al*[76]	1988-2005	ALD-9880	84	78	73	58	33 (11)	4.3
		ALD + HCV-1119	84	75	65	52		18.3
		ALD + HBV-309	89	85	81	64		
		Cryptogenic-2410	78	73	69	61		
Bhagat *et al*[84]	1997-2007	ALD-83	92	86	86	76[1]		
		NASH-71	82	79	75	62[1]		
Gedaly *et al*[22]	1995-2007	ALD-147	96.2	89.6	84.4		19	
Pfitzmann *et al*[5]	1989-2002	ALD-300	96		88	76	19 (8)	4.8
		Non-ALD	97		80	72		
OPTN/SRTR 2006[113]	1996-2005	All causes of cirrhosis	86.9		82.4			
Lim *et al*[101]	1988-1997	ALD-3063	81.9	73.9	67.9		13	
		Viral hepatitis-4267	80.3	71.7	65.3			
Bellamy *et al*[82]		ALD-123	84		72	63[2]	10	
Mackie *et al*[110]	1996-1999	ALD-64	82	82[3]			45.6 (6.5)	
		Non-ALD-335	83	82[3]				
Gerhardt *et al*[114]	1985-1991	ALD-67	90	84[4]	82[4]	76[4]	26 (4.8)	4.5
Lucey *et al*[15]	1985-1989	ALD-45	78	73[3]			4.4	
		Non-ALD-111	70	65[3]				
Kumar *et al*[115]	1982-1988	ALD-73	74				11.5	2
		Non-ALD	67					

[1]9 yr survival; [2]7 yr survival; [3]2 yr survival; [4]2-yr, 3-yr and 4-yr survival. HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALD: Alcoholic liver disease.

and supportive measures to maintain abstinence may not be available[76].

According to European Liver Transplant Registry (ELTR) data, patient survival for ALD patients is superior to those transplanted for HCV infection. Increasing donor age is found to have an adverse influence on patient and graft survival for ALD and HCV patients; it is more significant in HCV patients when the donor age is more than 40 years[107].

OUTCOME OF TRANSPLANTATION IN ALD

Studies have reported similar 1- and 5-year survival rated for patients undergoing OLT for ALD and for other indications, and in most studies alcohol relapse did not influence 1- and 5-year survival rates after OLD for ALD[108-112] (Table 6). The definition of relapse is not clear and this lack of consistent definition explains the varied relapse rates reported in the literature ranging from 7%-95%[108,112].

Heavy drinking has been shown to impair the long term survival (over 5 years) of patients with ALD following OLT[116]. Pfitzmann *et al*[5] retrospectively analysed 300 patients of ALD who had OLT for long term survival and risk factors for alcohol relapse. Recurrent alcohol consumption was observed in 10% of patients, of whom 30% had slipped, abusive drinking was documented in 41% and in the remaining 29%, severity of alcohol consumption was unknown. On multivariate analysis, duration of sobriety of less than 6 mo, poor social support, presence of young children and poor psychosomatic prognosis were associated with significantly increased risk of recurrent alcohol consumption. The overall survival

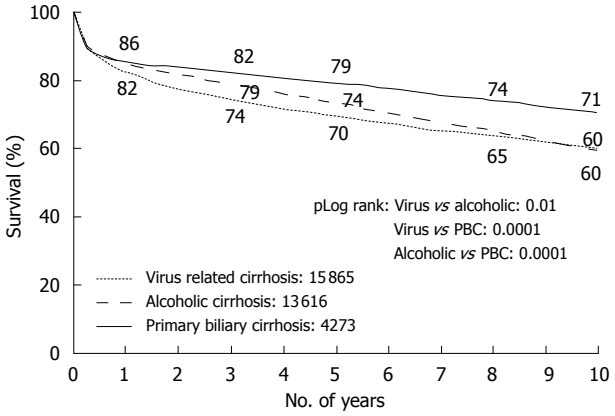


Figure 5 European Liver Transplant Registry (2008) data. One year, 3-year, 5-year, and 10-year survival following orthotopic liver transplantation for three common indications. PBC: Primary biliary cirrhosis.

of patients who underwent OLT for ALD was not statistically different from that of patients who had OLT for other indications. The 1-, 5-, and 10-year patient survival rates for ALD were 96%, 88%, and 76%, respectively as compared to 97%, 80%, and 72%, respectively for patients with other indications for OLT (Figure 5). Significantly better survival rates were observed for patients who remained abstinent when compared to those who resumed drinking after OLT. Further they observed that patients who resumed abusive drinking following OLT had the lowest survival. Recurrent alcoholic liver disease was responsible for the majority of deaths (87.5%) among patients who resumed abusive drinking[5] (Tables 4 and 6).

The impact of alcohol consumption on the outcome of OLT has been reported variedly. Several studies have

reported that recidivism has no significant impact on survival rate[114,117]. These studies did not account for the different patterns of drinking[5]. Recent studies have indicated that resumption of abusive drinking following OLT, leads to significantly reduced survival rates[116]. Patients who resumed heavy drinking have been reported to have 5- and 10-year survival rates of 69.5% and 20.1%, respectively compared to 90.3% and 81.5%, respectively in abstinent patients[5].

Numerous studies have reported that 5-year survival of patients undergoing OLT for ALD is comparable to the survival of patients transplanted for other indications[13,112,113]. According to the ELTR (2008), the overall 1- and 5-year survival of patients with ALD following OLT was 86% and 74%, and 1-year survival exceeded 90% in some centres (Figure 5).

Rejection in patients of ALD post OLT

ALD patients post OLT have reduced incidence of acute cellular rejection. Burra *et al*[76] report histologically proven acute cellular rejection in 14% of patients 23-180 d post OLT. Chronic ductopenic rejection is reportedly less common or the same in the patients receiving OLT for ALD from those for other indications[11,15]. Wiesner *et al*[83] in their study have reported significant decreases in the overall incidence of rejection in patients with ALD post-OLT as compared to those with non-alcoholic liver disease (Table 5).

Retransplantation in ALD

The incidence of retransplantation in patients with primary ALD is less as compared to other indications for transplantation. Wiesner *et al*[83] reported a significantly decreased incidence of retransplantation compared to non alcoholic liver disease recipients (3% *vs* 9%, $P = 0.04$). Retransplantation because of recurrence of disease is much less compared to those with HCV infection (where almost all recipients have recurrent disease at some point post-OLT), again justifying the allocation of scarce resources to patients with ALD (Table 5).

Medical complications

Infections are reportedly more common following OLT in patients with ALD. The incidence of bacterial infections is greater while the incidence of cytomegalovirus infection is similar to those patients transplanted for non-alcoholic liver disease. The incidence of hypertension and new onset insulin-dependent diabetes is again similar[22,115].

De-novo malignancies

Organ transplant recipients are at increased risk for developing *de-novo* malignancy, as they are exposed to prolonged and often lifelong immunosuppressive therapy. The incidence of *de-novo* malignancy reported in various series ranges from 6%-55% at 15 years following liver transplantation[118]. They are an important cause of delayed graft morbidity and mortality. Well documented risk factors for *de-novo* malignancy after liver transplantation include smoking and tobacco usage in any form, alcohol intake, and inflammatory bowel disease. Prolonged immunosuppression in these patients synergizes with known risk factors for malignancy.

Various studies have reported a higher incidence of *de-novo* malignancy in patients with ALD[119,120]. They are at high risk for developing upper aero-digestive tract malignancy. This has an important bearing in the pretransplant evaluation and post transplant follow-up. Oropharyngeal squamous cell carcinoma incidence of 17% has been reported in patients with ALD[121]. An incidence of 4.2% of oropharyngeal and oesophageal malignancies has been reported at 8 to 40 mo post transplantation in patients with ALD[122]. There is an increased incidence of basal and squamous cell carcinoma in these patients[119]. The incidence of oropharyngeal cancers is reportedly 25.5 times higher and the incidence of lung cancer 3.7 times higher for ALD patients[122]. There is no specific recommendation for post-OLT surveillance in these patients. In patients who have had OLT for ALD should have surveillance for upper aerodigestive tract malignancy at 1 year and thereafter annually[123,124] (Tables 4 and 5).

Cause of death

The vast majority (50%-87.5%) of deaths in patients who resume heavy drinking is due to recurrence of ALD and AH[5,82,112]. The cause of death in other patients who were abstinent was malignant tumors, infection, cardiovascular disease and cerebrovascular events. Malignant tumor of the upper aerodigestive tract was seen in patients who resumed heavy alcohol ingestion and in those who were abstinent after OLT[5,122]. Long term survival in the patients of ALD is affected by *de-novo* malignancy and this is the consequence of prolonged exposure to alcohol and tobacco[121-124]. These patients should be advised to discontinue smoking or intake of tobacco in any form.

Quality of life

QOL in all aspects, medical status, social status, employment status, or relationships shows improvement following transplantation for any indication[125]. There are conflicting results on the QOL of recipients of OLT for ALD as regards the return to work following recovery. There are studies proposing that the rate of return to work is as good as in those patients transplanted for any other cause, while there are others indicating that it is less for those transplanted for ALD[108,126]. Overall there is evidence that the QOL and return to work is similar or may be better, in patients transplanted for alcoholic and non-ALD. These patients seem to return to society to lead active and productive lives; however there is evidence that the societal re-integration may be less compared to those transplanted for other causes[127]. Post-transplant scores on QOL are poorer in patients who relapse to harmful drinking[128]. These patients have more sleep disturbances and are more prone to use benzodiazepines[129].

FOLLOW-UP AND RELAPSE

A proportion of patients grafted for end stage ALD

return to alcohol use post transplant. This statement in itself raises objection, concern, emotional responses and a sense of treatment failure[130]. The evidence is much less alarming, though worthy of further scrutiny. In the Dew *et al*[16] meta-analysis, 6 cases per 100 patients per year (PPY) return to any alcohol use post transplant, while less than 3 PPY return to heavy use. This describes cumulative rates of relapse and therefore the incidence rate at 5 years post transplant would stand at 28%. One Spanish centre has published data showing significant reduction in 10-year survival rates for ALD patients who relapse to alcohol use[116].

Transplant centres require assessment candidates to be abstinent at the point of listing and be committed to abstinence post transplant. If no ALD transplant recipients ever returned to alcohol use post-transplant the selection bar would clearly be set too high. If a significant number of grafts were lost due to a return to alcohol use - either directly or through poor treatment adherence - then this may suggest poor stewardship. Less than 5% of grafts are lost at 5-year post-transplant through direct or indirect consequences of alcohol misuse[32].

Criticism has been levelled at methods of monitoring alcohol use post-transplant. Literature has described retrospective case note review, biochemical markers, psychiatric interview, questionnaires and screening tools amongst others[131-133]. Inconsistent and unreliable methodology has been unhelpful in revealing a clear picture of alcohol use post-transplant, problematic use and untoward consequences.

Alcohol use in the non-ALD transplant candidate should not be overlooked. In a prospective study of 208 non-ALD transplant candidates in a UK centre, 80 (39%) met the DSM IV criteria for a lifetime diagnosis of alcohol abuse or dependence, highlighting the need for appropriate screening and assessment of this population[134].

Many transplant centres now have psychiatric/substance misuse specialists following patients up post-transplant in order to provide ongoing relapse prevention and support. This monitoring should be supplemented by appropriate alcohol screening by physicians, and Hepatology nurses as well as with effective biochemical markers. Where relapse has occurred a treatment plan should be effected and engagement with local substance misuse teams arranged. Such services should demonstrate willingness and a flexible response to this patient group.

CONTROVERSIES ABOUT "SLIPS" AND RELAPSES

Addiction specialists distinguish a relapse which is prolonged and harmful drinking behaviour from a minor lapse or slip which is a sporadic drinking event followed by re-establishment of abstinence[5]. It is also accepted that where a person has a diagnosis of dependence to a substance then it is likely that after a period of abstinence, further exposure to the substance provokes the risk of reinstatement, one of the features of dependence in which the person rapidly returns to the previously required level of drug use. This is a common feature of - for example - alcohol or tobacco dependence. Where a person has not been dependent then the risk of reinstatement is less profound and therefore it is argued in addiction treatment settings that with psychological interventions it is possible to modify the individual's behavioural responses to triggers such as cue response cravings, stress and other high risk situations using relapse prevention techniques largely based on cognitive behavioural therapies[135]. Evidence suggests that some of these techniques are of significant benefit[136].

ETHICAL ISSUES

There are medical and ethical concerns about the appropriate use of scarce resources, and the degree of priority given to patients with ALD has always been a controversial issue: (1) should an individual receive the same priority as others for a self inflicted disease; (2) whether the outcome of liver transplantation is as good in patients with ALD as in non-alcoholics; and (3) the possibility of recidivism and its influence on the graft.

The Oregon experiment highlighted the issue of permitting the voting public to select healthcare priorities, and a UK poll also identified the difference in candidate selection, based on seemingly emotional and moral grounds - in the case of the general public - rather than on clinical outcome and utilitarian use of the organ[11,137].

Doubts have been raised about the ethics of a "6-mo rule" of abstinence as a consequence of the lack of evidence of this approach and the "context" of the abstinence[20]. The argument has also been made that if alcohol dependence as an "addiction" carries a neurobiological component (i.e. a genetic influence) then it does not constitute a "self-induced" disorder but a medical one which should be managed accordingly. This argument is limited as it ignores the fact that clinical outcome is the most important factor and therefore if an "addicted" drinker is transplanted the argument would suggest that they may not be able to exert any degree of control over their drinking in the future[138]. In response to this, Berkalovich[139] argues the disease model of alcoholism and therefore advocates treatment of the addiction and treatment of the liver disease. If an ALD patient is as likely to have a favourable long-term prognosis as a non-ALD candidate with transplantation then there is no further issue[140].

FUTURE DIRECTIONS

Certain issues related to liver transplantation in ALD have remained unresolved despite the convincing reports of similar survival in these patients post transplant as compared to those who received transplant for other indications. Areas of future research are many, and these may help in resolving the controversies associated with OLT in these patients. (1) Not all patients who consume alcohol develop alcoholic liver disease. There have been studies indicating genetic predisposition in ALD patients to develop

chronic liver disease as the severity of liver damage is not uniformly related to the amount and number of years of consumption[141]. Studies are needed to further identify these genetic factors so that liver transplantation is taken as a curative procedure in these patients as they acquire a different set of genes in the new liver with different susceptibility even if there is relapse; (2) Though there are studies which have identified risk factors for relapse in patients with ALD, the controversy about the period of abstinence prior to transplant evaluation still continues. It is time to have a consensus about this so that there is uniformity in organ allocation for these patients and the listing criteria are better defined[142]. Comparison of data would be uniform if this period of abstinence is defined and followed; (3) AH is not yet considered as an indication for liver transplantation; however it is known that there is a subgroup of patients who do not respond to medical treatment and that they have a poor prognosis. This is the group where the benefit of liver transplantation is being argued. It is also known that histological recovery from features of AH is different from clinical recovery so whether these patients who have had acute AH are to be uniformly abandoned from listing or can be reconsidered for OLT when the liver disease worsens must be considered. Whether they have a similar outcome to other ALD patients who have not had AH is to be studied in a prospective manner. A blanket approach of contraindication ignores cases such as the young man with first episode liver damage, thus raising issues around age and opportunity; (4) Pretransplant psychological input need not be restricted to assessment and evaluation. Opportunities abound for therapeutic work during the assessment phase, particularly if transplantation is not imminent. Relapse prevention strategies can be woven into appointments and at least one transplant centre has attempted to engage with the transplant candidate and their families at the assessment phase through implementation of sessions of the psychosocial intervention social behavioural and network therapy[141,142]. Initial results appeared favourable and units should be creative in employing their specialist teams to develop and evaluate such approaches; (5) Follow-up of patients of ALD post-OLT as well as the pretransplant assessment necessitates psychiatric and psychosocial evaluation, which is important not only to identify a subgroup of patients with better outcome, but also is important to identify relapse and treat alcoholism in them. Not all transplant centres have a dedicated psychiatrist/addiction specialist to deal with these patients. Treatment of alcohol relapse in patients of ALD post-OLT is still not defined. Further research is required in this field for the treatment of alcoholism and alcohol dependency in these patients. This is important for graft survival; (6) Treatment of ALD before transplantation and considering OLT only once if it fails is important for better utilisation of organs. The concept of better outcome in patients who are not very sick at the time of transplantation was proven wrong in the recent prospective study on immediate listing *vs* delayed transplantation. Future research in the medical treatment of patients with ALD might help in reducing the long list of patients waiting for OLT; (7) *De-novo* malignancies post-transplant are more frequently identified in ALD patients, hence a better surveillance programme is required for these patients so that patient and graft survival is not affected in the long term. Longitudinal studies to determine the timing and frequency of surveillance in these patients are important to avoid delayed morbidity and mortality. Whether the immunosuppression protocol needs adjustment in these patients is an ingredient for further research; and (8) There is no definitive biochemical test to identify alcohol relapse and the tests available have poor sensitivity and specificity. More research is required in this field to identify sensitive tests for detection of harmful alcohol ingestion and the effect on the new liver.

CONCLUSION

ALD is an acceptable indication for liver transplantation as survival of these patients after transplantation is similar to that seen in patients who receive grafts for other causes. Patient selection is important for rationing scarce organs, hence the use of prognostic models for predicting risk of relapse to alcoholism. Rate of graft loss is no greater and rejection of the graft is even less so in patients transplanted for ALD. The disease recurs in a minority of patients but histologically proven disease recurrence is less frequent than with hepatitis C, primary biliary cirrhosis, auto-immune hepatitis, or primary sclerosing cholangitis.

Disease recurrence has little impact on graft survival rates within 7-10 years of transplantation, in contrast with hepatitis C. Abstinence before transplantation evaluation and listing is important to select patients who would benefit the most from transplantation, as some would get better in this period. There should be reservations in listing those patients with a lack of social support, active smoking, psychotic or personality disorders, or a pattern of nonadherence. Pretransplant evaluation and follow-up is a combined effort of clinicians, psychiatrists and substance abuse specialists.

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Figures 1-5 are downloaded from the ELTR and UNOS website. These are available for public view, and have been accessed in December 2009. These are incorporated in the manuscript for comparison of data. http://www.eltr.org/publi/results.php3; http://optn.transplant.hrsa.gov/latestData/viewDataReports.asp.

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REVIEW

Diagnosis and management of interstitial pneumonitis associated with interferon therapy for chronic hepatitis C

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Abstract

Interstitial pneumonitis (IP) is an uncommon pulmonary complication associated with interferon (IFN) therapy for chronic hepatitis C virus (HCV) infection. Pneumonitis can occur at any stage of HCV treatment, ranging from 2 to 48 wk, usually in the first 12 wk. Its most common symptoms are dyspnoea, dry cough, fever, fatigue, arthralgia or myalgia, and anorexia, which are reversible in most cases after cessation of IFN therapy with a mean subsequent recovery time of 7.5 wk. Bronchoalveolar lavage in combination with chest high resolution computed tomography has a high diagnostic value. Prompt discontinuation of medication is the cornerstone, and corticosteroid therapy may not be essential for patients with mild-moderate pulmonary functional impairment. The severity of pulmonary injury is associated with the rapid development of IP. We suggest that methylprednisolone pulse therapy followed by low dose prednisolone for a short term is necessary to minimize the risk of fatal pulmonary damage if signs of significant pulmonary toxicity occur in earlier stage. Clinicians should be aware of the potential pulmonary complication related to the drug, so that an early and opportune diagnosis can be made.

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Key words: Chronic hepatitis C; Interferon α; Interstitial pneumonitis; Management; Corticosteroid therapy

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INTRODUCTION

Combination of pegylated interferon (PEG-IFN) and ribavirin has been the standard program for hepatitis C virus

(HCV) infection. Pulmonary complications, although uncommon, are associated with the use of IFN for HCV infection[1-21]. The overall incidence of pulmonary toxicities is < 1%[10], and the unusual wide spectrum includes pulmonary sarcoidosis, interstitial pneumonitis (IP), bronchiolitis obliterans organizing pneumonia, pleural effusion, and exacerbation of bronchial asthma and acute respiratory distress syndrome[8,9,18-21]. The precise incidence rate of IFN-induced IP is still unclear. In a Japanese study by Okanoue *et al*[6], 3 of 667 IFN-treated patients with HCV infection developed IP, with an incidence of 0.45%, which is significantly higher than the reported annual incidence rate in the general populations of Japan, United States and other countries. Thus, the incidence rate of this complication is estimated to be 0.01%-0.3%[22].

With the increasing use of IFN, more and more IP cases will present. Based on this background, the present review was to investigate the clinical data, chest radiography, chest high resolution computed tomography (HRCT), pulmonary function test (PFT), histological findings, treatment and prognosis, with an attempt to propose an algorithm for the evaluation of suspected IP patients.

PATHOGENESIS

The mechanism underlying IFN-induced IP has not yet been clarified. It has been reported that IFN can provoke lung tissue fibrosis by inhibiting suppressor T cells, increasing cytotoxic T cells, inducing proinflammatory cytokines, and exaggerating release of fibrinogenic cytokines[23,24]. PEG-IFN provides a higher IFN level because of its prolonged half-life and increased area under the curve. However, increased risk of IP due to the use of PEG-IFN has not been reported since its first application for HCV in 2000[25,26]. The major side effects of ribavirin are dose-dependent hemolytic anemia, cough, dyspnoea, rash, depression, and dyspepsia. No case of IP caused by ribavirin has yet been described, and whether ribavirin plays a potential role in the development of IP remains theoretical.

CLINICAL FEATURES

English papers were searched from PubMed, Blackwell, Elsevier, Springer and OVID in 1986-2009 using various combinations of "IFN", "HCV", "IP" and "pulmonary toxicity". References in the papers were reviewed as additional sources.

Twenty-four cases (with detailed data available from 20) reported in 17 articles were included in this review. Their main characteristics are shown in Table 1. Of these cases, 11 (46%) were males and 13 (54%) were females. Their mean age was 54.2 years (range 39-72 years). IP occurred in a comparable proportion of females and males, and was more frequent in older patients. The mean IFN treatment time before the diagnosis of IP was 11.8 wk (range 2-48 wk) and less than 12 wk in 73% of them. Of

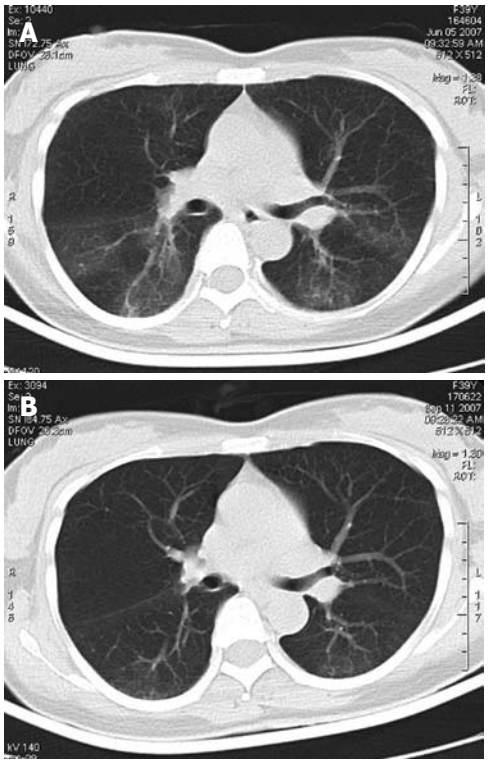

Figure 1 Chest computed tomography scanning showing symmetrical, bilateral interstitial abnormality with areas of ground glass opacity predominantly involving middle lung fields and lung bases (A), and visible resolution of inflammatory infiltration following symptom improvement with less dyspnoea and cough (B) in 14 out of 21 cases.

the 24 cases, 3 were given Sho-saiko-to (a Chinese herbal drug that increases the risk of IP), 12 were treated with ribavirin and IFN, and 9 were given IFN without any other medications. The most common symptoms were dyspnoea (80%, 16/20), dry cough (75%, 15/20) and fever (55%, 11/20), followed by fatigue (25%, 5/20), arthralgia or myalgia (20%, 4/20), and anorexia (15%, 3/20). Physical examination revealed unilateral or bilateral lung field fine crackles in 10 out of 11 patients.

Detailed PFT information was available from 14 cases with a restrictive pulmonary functional impairment pattern. Of the 14 cases, 8 were accompanied with an obstructive pathological change. Arterial blood gas (ABG) analysis showed different extents of hypoxemia in 88% (15/17) of the patients. Chest radiography showed no evidence of lung disease and HRCT showed diffuse infiltration in 4 cases after pulmonary symptoms occurred[10-12,16]. Chest CT scanning showed symmetrical, bilateral interstitial abnormality with areas of ground glass opacity predominantly involving middle lung fields and lung bases (Figure 1A), and visible resolution of inflammatory infiltration following symptom improvement with less dyspnoea and cough (Figure 1B), in 14 out of 21 cases.

Bronchoscopy with bronchoalveolar lavage (BAL) was performed for 19 cases. BAL fluid (BALF) analysis demonstrated that the number of lymphocytes, macrophages and other inflammatory cells was increased with an elevated proportion of CD8+/CD4+ cells[27]. Culture and

Table 1 Case reports of interstitial pneumonitis associated with interferon therapy for hepatitis C virus

Ref.	Yr	Age (yr)/sex	Administered	Duration of IFN therapy (wk)	Diagnostic method	Treatment	Outcome
[1]	1993	60/F	IFNα-2b	8	C, R, B	D	Resolved
[2]	1994	58/F	IFN-α	12	C, R, B	D	Resolved
		56/M	IFN-α	6	C, R, B	D + S	Resolved
		72/F	IFN-α	3	C, R	D + S	Improvement
[3]	1994	48/F	IFN-α	9	C, R, B	D + S	Resolved
[4]	1994	62/F	IFN-α	3	C, R, B	D + S	Resolved
[5]	1996	49/F	IFNα-2b, Sho-saiko-to	6	C, R, B	D + S	Resolved
		59/M	IFNα-2b, Sho-saiko-to	4	C, R, B	D + S	Resolved
		42/M	IFNα-2b	16	C, R, B	D + S	Resolved
[6]	1996	46/M	IFN-α, Sho-saiko-to	4	C, R	D	Resolved
		57/M	IFN-α	5	C, R	D	Resolved
		59/M	IFN-α	23	C, R	D	Resolved
[7]	2001	57/M	IFN-α, ribavirin	12	C, R, B	D	Resolved
[8]	2002	48/F	IFNα-2b, ribavirin	24	C, R	D	Resolved
			PEGIFNα-2a, ribavirin	6	C, R, B	D + S	Improvement
		50/M	IFNα-2b, ribavirin	4	C, R, B	D + S	Resolved
[9]	2003	49/M	PEGIFNα-2b, ribavirin	2	C, R, B	D + S	Death
[10]	2004	71/F	PEGIFNα-2a, ribavirin	20	C, R, B	D	Resolved
[11]	2004	51/M	PEGIFNα-2b, ribavirin	5	C, R, B	D + S	Death
[12]	2005	58/F	PEGIFNα-2b, ribavirin	12	C, R, B	D + S	Resolved
			PEGIFNα-2a, ribavirin	12	C, R	D	Resolved
[13]	2006	68/F	IFNα-2b, ribavirin	14	C, R, B	D + S	Resolved
[14]	2007	47/F	PEGIFNα-2b, ribavirin	10	C, R, B	D + S	Resolved
[15]	2008	43/F	PEGIFNα-2b, ribavirin	48	C, R	D + S	Death
[16]	2009	39/F	PEGIFNα-2a, ribavirin	36	C, R, B	D + S	Resolved
[17]	2010	51/M	PEGIFNα-2b, ribavirin	4	C, R, B	D + S	Resolved

B: Biopsy proven diagnosis; C: Clinical diagnosis; R: Radiological diagnosis; PEGIFN-α: Pegylated interferon α; D: Discontinuation IFN-α; S: Corticosteroids therapy.

staining of bronchial lavage were negative for bacteria, fungi, acid fast bacilli (AFB) and viruses in these cases. Of the 24 cases included in the review, 17 were diagnosed as IP, which was confirmed by lung biopsies including transbronchial, thoracoscopic lung and open lung biopsies. A biopsy was considered positive for IP when histology demonstrated interstitial infiltration, principally of lymphocytes with macrophages, interstitial fibrosis with thickening of alveolar walls and without evidence of granulomatous lesions in parenchyma[28], although bronchial biopsy showed normal epithelium in 3 cases[12,13,16].

DIAGNOSIS

The diagnosis of IFN-induced IP is not easy since no uniform international diagnostic criteria for IFN-induced IP are available at present. Based upon the classification of causal criteria for adverse effects of medications from the World Health Organization[29], we suggest that the diagnostic criteria for IFN-induced IP should include (1) dyspnoea, dry cough, fever, fatigue, arthralgia or myalgia, and anorexia during antiviral treatment; (2) patchy shadows on chest radiograph and ground glass shadows and/or reticular opacities on HRCT; (3) a restrictive pulmonary functional impairment pattern with/without hypoxemia; (4) no pulmonary infection with bacteria, fungi, AFB and viruses, and congestive heart failure; (5) symptom improvement of pneumonitis after cessation of IFN therapy; and (6) one of the following positive results, including bronchoscopy with BAL, lung biopsy and IP recurrence on re-challenge with IFN therapy. The diagnosis was certain, probable and possible, respectively, when the patient had the 6 major features, the first 5 features and the first 4 features.

EVALUATION OF DISEASE SEVERITY

The ATS/ERS guidelines[30] can accurately predict the clinical severity or prognosis of individual patients, and are generally used in patients with obstructive, restrictive and mixed pulmonary defects. Severe pulmonary functional impairment is defined as FEV1%pred < 50% in PFT or arterial oxygen pressure < 60 mmHg in ABG[30]. Mild-moderate pulmonary functional impairment is defined as FEV1%pred > 50% and arterial oxygen pressure > 60 mmHg. The 8 patients with severe pulmonary functional impairment[2,4,5,7,9,11] received IFN therapy for a shorter time (5.9 wk *vs* 14.4 wk, $P < 0.05$) and more of them were treated with corticosteroids than other cases (75% *vs* 56%). No significant difference was found in age and sex between the two subgroups (Table 2).

TREATMENT

The standard strategy for managing drug-induced IP is to immediately discontinue the drug and start corticosteroid pulse therapy, combined with supportive therapy for severe respiratory condition[6]. IFN therapy should be discontinued in all cases following the diagnosis of IP. Corticosteroids were prescribed for pneumonitis in

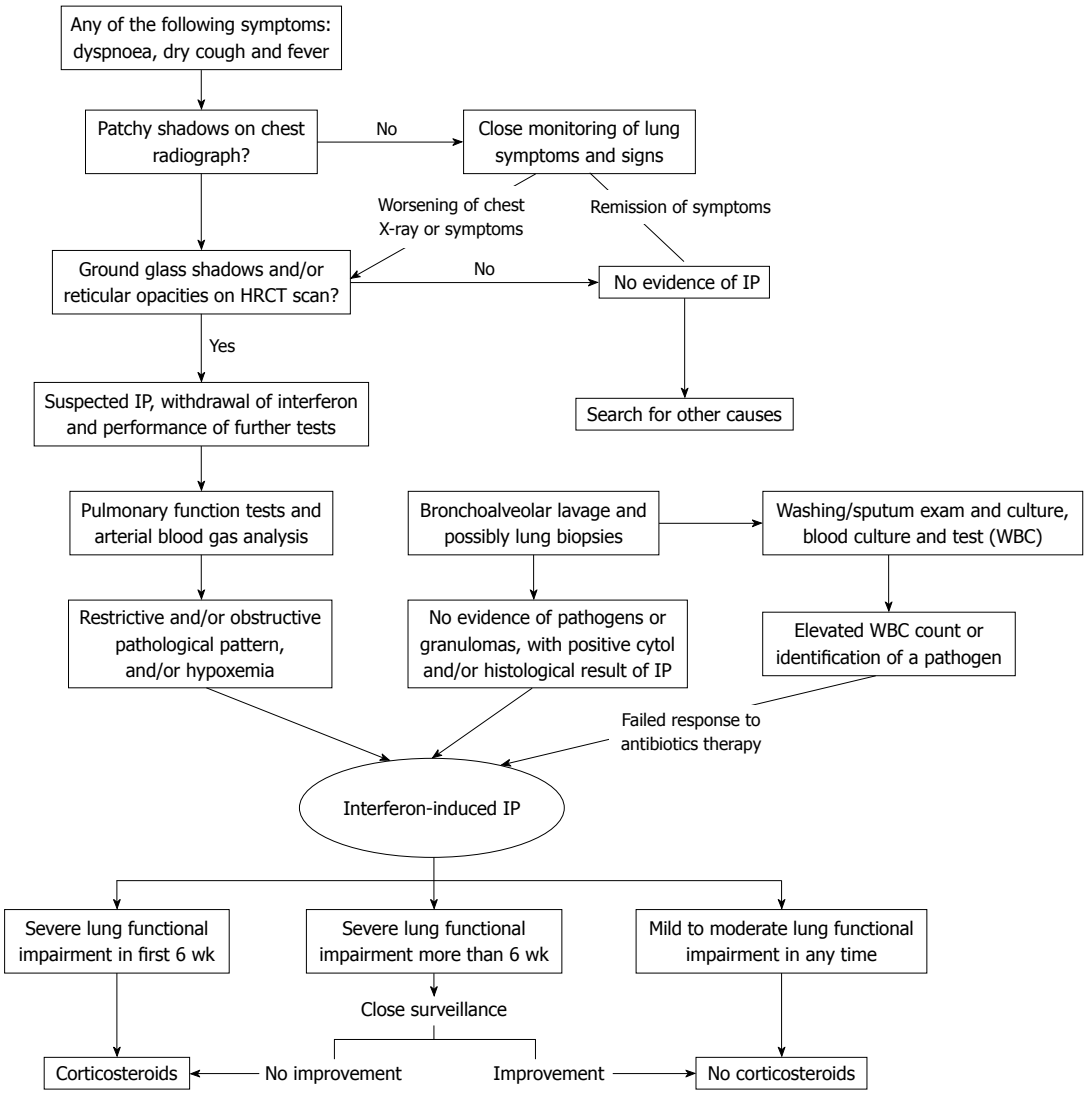

Figure 2 Algorithm for the diagnosis and management of interstitial pneumonitis associated with interferon therapy for hepatitis C virus infection. HRCT: High resolution computed tomography; IP: Interstitial pneumonitis.

Table 2 Clinical data about patients in two subgroups

Severity of IP	Cases, *n* (%)	Sex (M/F)	Age (yr, mean)	Duration of IFN therapy (wk, mean)	Steroid therapy
Severe group	8 (36)	4/4	57.1	5.9	6/2
M and M group	14 (64)	7/7	52.1	14.4	9/7
P value	--	1.00	0.201[1]	0.041[2]	0.43

[1]*U* = 31.5, *W* = 109.5; [2]*U* = 20.5, *W* = 53.5, *P* < 0.05 by Mann-Whitney test. M and M group: Mild or moderate pulmonary functional impairment group. IP: Interstitial pneumonitis; IFN: Interferon.

67% (16/24) cases included in our review. The dosage and length of corticosteroids are highly variable, usually started at a relatively high dosage. IP in a 39-year-old female patient was treated with methylprednisolone (160 mg/d for 3 d, 80 mg/d for 4 d), followed by prednisolone (30 mg/d), with the dosage gradually tapered over the next 12 wk[16]. A 56-year-old male patient was treated with methylprednisolone (2 g/d for 3 d) and prednisolone (40 mg/d for 2 d)[2]. Prednisolone (60 mg/d) and azathioprine (100 mg/d) were given after relapse of IP initially treated with prednisolone (30 mg/d)[4]. Some patients started on oral corticosteroid therapy (40 mg/d)[8,13,17], while other patients started on prednisolone (125 mg/d)[11,15] or on corticosteroid by inhalation[12]. Of the 8 out of the 24 patients (33%) included in our review, 6 suffering from mild-moderate pulmonary functional impairment had a resolution of IP after the drug was discontinued without administration of corticosteroids.

Corticosteroid treatment of IP associated with IFN therapy for HCV infection is controversial. Which patients need corticosteroid therapy, what dosage of corticosteroids should be chosen and how long the patients should be treated are unclear. Based on this review, corticosteroid therapy may not be essential for patients with mild-moderate pulmonary functional impairment. Methylprednisolone pulse therapy (160 mg/d for 3 d) followed by

prednisolone (30 mg/d) for a short term may be a good choice for patients with signs of significant pulmonary toxicity in earlier stage.

OUTCOMES

In our review, the prognosis of IFN-induced IP was different. Of the 24 patients, 19 had a complete resolution in a follow-up time of 7.5 wk (range 2-12 wk), 2 required permanent corticosteroid treatment to control their respiratory symptoms[2,8], 3 were treated with PEG-IFNα-2b and died due to acute respiratory failure, chronic hypoxia-induced cerebral edema and acute cholestatic hepatitis, respectively[9,11,15]. Of the 11 patients who developed IP within 6 wk, two cases died[9,11] and 2 cases required permanent corticosteroid therapy[2,8]. The other 13 patients who developed IP over 6 wk, one case died[15] and none required permanent corticosteroid therapy.

PROPOSED ALGORITHM

In general, IP occurs within the first 12 wk after IFN therapy for HCV infection. Clinical presentations such as dyspnoea, dry cough, fever, fatigue, arthralgias or myalgias, and anorexia during the treatment are attributed to the common side effects of IFN and ribavirin therapy. It is worth noting that these symptoms are usually present or exacerbated after elimination or remission of the general side effects of antiviral treatment. Laboratory assessment should include PFT and ABG analysis, blood test, culture of blood, sputum specimens, chest radiography and HRCT, BALF analysis and lung biopsy.

Chest X-ray plays an important role in the diagnosis of IP. Of the 24 patients included in this review, 4 showed no evidence of lung disease on chest radiograph after pulmonary symptoms occurred[10-12,16], indicating that chest CT scan should be performed for such patients. The occurrence of fatal evolution in the patients confirms this recommendation[11]. HRCT scanning may provide a high diagnostic value as it can show the lung interstitial infiltrations in earlier stage[31]. BAL has become a standard diagnostic procedure for the majority of patients with drug-induced interstitial lung diseases[27]. BAL with a pattern of lymphocytes, neutrophils, and eosinophils, or a mixed cellular pattern can be used as an adjunct to diagnosis[27]. Besides, negative culture and staining of bronchial washing help to exclude pneumonia resulting from infection with bacteria, fungi, AFB and viruses. BAL in combination with HRCT can increase the diagnostic power of both methods, and may even obviate more invasive procedures in some cases[27,31].

There is convincing evidence that corticosteroids increase HCV replication[32,33]. Whether corticosteroids should be included in the treatment modality for IP associated with IFN therapy for HCV infection remains controversial. We, thus, suggest an algorithm for the evaluation of suspected IP patients (Figure 2) and clinicians should be aware of the potential pulmonary complication related to the drug, so that an early and opportune diagnosis can be made.

CONCLUSION

There are some limitations in this review. All the information came from case reports, whereby cannot be controlled for confounding factors. Detailed data were not available from some cases, thus making it difficult to identify the risk factors for the occurrence of IP. Despite the limitations, several conclusions can be made based upon the available data.

IP can occur at any stage of IFN therapy for HCV infection. Close monitoring is necessary for the treatment of HCV with IFN in early stage, especially for older cases. Prompt discontinuation of medication is the cornerstone, and corticosteroid therapy may not be essential for patients with mild- moderate pulmonary functional impairment. The severity of pulmonary injury may be associated with the rapid development of IP, indicating that methylprednisolone pulse therapy, followed by low dose prednisolone therapy for a short term, is necessary to minimize the risk of fatal pulmonary damage if signs of significant pulmonary toxicity occur in earlier stage (within 6 wk) during antiviral therapy.

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ORIGINAL ARTICLE

Pegylated interferon α-2b plus ribavirin for older patients with chronic hepatitis C

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Abstract

AIM: To analyze the efficacy and safety of a combination therapy of pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) in older Japanese patients (65 years or older) infected with hepatitis C virus (HCV).

METHODS: This multicenter study included 938 patients with HCV genotype 1 who received 1.5 μg/kg per week PEG-IFN α-2b plus RBV 600-1000 mg/d for 48 wk and 313 HCV genotype 2 patients who received this treatment for 24 wk.

RESULTS: At 24 wk after the end of combination therapy, the overall sustained virological response (SVR) for genotypes 1 and 2 were 40.7% and 79.6%, respectively. The SVR rate decreased significantly with age in each genotype, and was markedly reduced in genotype 1 ($P < 0.001$). Moreover, the SVR was significantly higher in patients with genotype 1 who were less than 65 years (47.3% of 685) than in those 65 years or older (22.9% of 253) ($P < 0.001$) and was higher in patients with genotype 2 who were less than 65 years (82.9% of 252) than in those 65 years or older (65.6% of 61) ($P = 0.004$). When patients received a dosage at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target dosage of RBV, the SVR rate significantly increased to 66.5% in patients less than 65 years and to 45.2% in those 65 years or older ($P <$

0.001). Adverse effects resulted in treatment discontinuation more often in patients with genotype 1 (14.4%) than in patients with genotype 2 (7.3%), especially by patients 65 years or older (24.1%).

CONCLUSION: PEG-IFN α-2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum acceptable treatment dosage.

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Key words: Hepatitis C virus; Gerontology; Pegylated interferon; Ribavirin

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, affecting 170 million individuals worldwide[1]. It is well known that patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC)[2]. Previous studies have made clear that interferon (IFN) treatment is effective for eliminating HCV[3,4] and that it significantly reduces the progression of liver fibrosis and the risk of HCC[5,6]. Antiviral treatment for chronic hepatitis C has greatly improved, and the combination treatment of pegylated (PEG)-IFN α-2b plus ribavirin (RBV) has been approved and recommended in Japan since 2004, as the first choice for chronic hepatitis C. This combination treatment attained a sustained virological response (SVR) rate of 50%-60% for genotype 1 in the United States and Europe[7]. However, SVR was relatively low (42.4%) in Japan[8], where chronic hepatitis C patients are older, indicating that older patients did not respond well to IFN treatment[9]. Moreover, the combination treatment was associated with more adverse effects than IFN monotherapy[7,10]. Older patients who have decreased cardiovascular, pulmonary and renal function have a higher incidence of adverse effects than younger patients. The rate of discontinuation due to adverse effects was reported to be significantly higher in patients aged 65 years or more than in those less than 65 years[11]. Older patients with HCV infection are at risk for progressive liver disease. It was reported that clearance of HCV after IFN therapy significantly reduces the incidence of HCC and death in older chronic hepatitis C patients[6,12]. Ikeda *et al*[13] demonstrated that IFN treatment is needed for 65-70-year-old patients with chronic hepatitis C to prevent the occurrence of HCC. We also consider older patients to be acceptable candidates for antiviral treatment to prevent the development of HCC, and previously reported that monotherapy with natural IFN α was not effective in older patients[9]. Therefore, in an attempt to ameliorate these problems, we decided to treat older patients with a combination of PEG-IFN plus RBV therapy.

Little data concerning the response and safety of this combination treatment in a large number of older patients with chronic HCV infection has been published. A multicenter study of the efficacy and safety of antiviral treatments for Japanese patients with chronic liver disease, the Kyushu University Liver Disease Study (KULDS), was launched in 2003[8,14]. The present prospective study was carried out to analyze the efficacy and safety of the combination treatment of PEG-IFN α-2b plus RBV in older patients.

MATERIALS AND METHODS

Patients

Treatment of chronic hepatitis C with a combination of PEG-IFN α-2b plus RBV was accepted by the Japanese Ministry of Health in October, 2004. We used this combination treatment from December 2004 to July 2008, and enrolled chronic hepatitis C patients with exclusion criteria which included: (1) clinical or biochemical evidence of hepatic decompensation, advanced cirrhosis identified by bleeding, high-risk esophageal varices, history of gastrointestinal bleeding, ascites, encephalopathy, or HCC; (2) hemoglobin level < 11.5 g/L, white blood cell count < 3×10^9/L, and platelet count < 50×10^9/L; (3) concomitant liver disease other than hepatitis C (hepatitis B surface antigen positive or HIV positive); (4) excessive active alcohol consumption > 60 g/d or drug abuse; (5) severe psychiatric disease; or (6) antiviral or corticosteroid treatment within 12 mo prior to enrollment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 32 affiliated hospitals in the northern Kyushu area of Japan. We have treated 2270 Japanese patients aged 18 years or older with PEG-IFN α-2b plus RBV. All patients who were positive for both antibody to HCV and HCV RNA for over 6 mo were enrolled in KULDS. Three months before the start of treatment and every 3 mo during the treatment period, each patient was tested for α-fetoprotein (AFP) and had an abdominal ultrasonographic examination. If an abnormal AFP level of 40 ng/mL and/or focal lesions on ultrasonographic examination were found at any testing, further testing for HCC was carried out, which included dynamic computed tomography, and angiography. Patients confirmed to have HCC within 3 mo after starting treatment were excluded from this study (n = 14). Of 2270 patients, 1021 were currently under combination treatment or we were not yet able to judge the effect of the combination treatment. This left the data of 1251 patients (938 with genotype 1 and 313 with genotype 2) available for analysis.

Table 1 Characteristics of 938 chronic hepatitis C genotype 1 patients treated with a combination of pegylated interferon plus ribavirin according to age (mean ± SD)

	Group A (age < 65 yr) (*n* = 685)	Group B (age ≥ 65 yr) (*n* = 253)	*P*-value
Age (yr)	53.1 ± 8.9	68.6 ± 3.1	< 0.001
Male/female	374/311	122/131	0.090
Body mass index (kg/m^2)	23.7 ± 3.3	22.8 ± 2.7	< 0.001
Prior IFN monotherapy, *n* (%)	163 (23.8)	76 (30.0)	0.052
Prior combined IFN plus RBV treatment, *n* (%)	51 (7.4)	20 (7.9)	< 0.001
Alanine aminotransferase (IU/L)	80.2 ± 62.0	67.9 ± 46.6	0.004
γ-glutamyltranspeptidase (IU/L)	60.2 ± 56.6	57.1 ± 49.2	0.708
Albumin (g/dL)	4.1 ± 0.4	4.0 ± 0.4	< 0.001
White blood cell count (/mm^3)	5200.0 ± 1476.7	4756.3 ± 1458.9	< 0.001
Hemoglobin (g/dL)	14.1 ± 1.4	13. 5 ± 1.4	< 0.001
Platelet count (10^9/L)	16.6 ± 5.3	15.0 ± 5.2	< 0.001
Creatinine (mg/dL)	0.7 ± 0.6	0.8 ± 1.4	0.107
Creatinine clearance (mL/min)	105.5 ± 28.7	75.8 ± 17.5	< 0.001
Serum HCV-RNA level (kIU/mL)	1776.1 ± 1500.0	1986.9 ± 1604.5	0.125
Histological fibrosis			0.008
F0/F1/F2/F3/F4	36/155/121/61/30	9/46/49/31/17	

IFN: Interferon; RBV: Ribavirin; HCV: Hepatitis C virus.

Table 2 Characteristics of 313 chronic hepatitis C genotype 2 patients treated with a combination of pegylated interferon plus ribavirin according to age (mean ± SD)

	Group C (age < 65 yr) (*n* = 252)	Group D (age ≥ 65 yr) (*n* = 61)	*P*-value
Age (yr)	47.7 ± 10.4	69.2 ± 3.4	< 0.001
Male/female	124/128	28/33	0.671
Body mass index (kg/m^2)	23.1 ± 3.5	22.8 ± 2.9	0.577
Prior IFN monotherapy, *n* (%)	47 (18.7)	16 (26.2)	< 0.001
Prior combined IFN plus RBV treatment, *n* (%)	5 (2.0)	4 (6.6)	0.056
Alanine aminotransferase (IU/L)	79.9 ± 78.7	68.9 ± 52.9	0.821
γ-glutamyltranspeptidase (IU/L)	55.8 ± 64.7	44.3 ± 34.7	0.937
Albumin (g/dL)	4.2 ± 0.4	3.9 ± 0.5	< 0.001
White blood cell count (/mm^3)	5276.3 ± 1636.3	4958.0 ± 1495.6	0.005
Hemoglobin (g/dL)	14.1 ± 1.4	13.4 ± 1.3	< 0.001
Platelet count (10^9/L)	18.9 ± 6.3	15.6 ± 4.7	< 0.001
Creatinine (mg/dL)	0.8 ± 1.5	0.7 ± 0.2	0.581
Creatinine clearance (mL/min)	112.1 ± 31.4	74.6 ± 17.2	< 0.001
Serum HCV-RNA level (kIU/mL)	1588.3 ± 1628.7	1195.4 ± 1645.5	0.038
Histological fibrosis			< 0.001
F0/F1/F2/F3/F4	30/77/39/10/10	1/21/9/2/12	

IFN: Interferon; RBV: Ribavirin; HCV: Hepatitis C virus.

Informed consent was obtained from all patients before enrollment in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

Table 1 (genotype 1) and Table 2 (genotype 2) show the baseline characteristics of the enrolled patients, who were further classified into four groups according to age and genotype status: group A, genotype 1 aged less than 65 years (*n* = 685); group B, genotype 1 aged 65 years or older (*n* = 253); group C, genotype 2 aged less than 65 years (*n* = 252); and group D, genotype 2 aged 65 or older (*n* = 61). In group B, body mass index, prior combined IFN plus RBV treatment, alanine aminotransferase, albumin, white blood cell count, hemoglobin, platelet count, and creatinine clearance calculated using the Modification of Diet in Renal Disease equation[15] were significantly lower than in group A ($P < 0.010$). In group D, albumin, hemoglobin, platelet count, creatinine clearance and serum HCV RNA level were significantly lower than in group C ($P < 0.010$). The percentage of patients with platelet counts below 10 × 10^{10}/L was significantly higher in group B (36 of 253, 14.2%) than in group A (56 of 685, 8.2%) ($P = 0.006$), however, there was no significant difference between group C (16 of 252, 6.3%) and group D (7 of 61, 11.5%).

Liver histology

Liver biopsy was performed in 555 patients (59.2%) with genotype 1 and 209 patients (66.8%) with genotype 2. The other patients refused liver biopsy. Fibrosis was staged on a 0-4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. Liver fibrosis was more advanced in group B than in group A

and was more advanced in group D than in group C (P = 0.008, P < 0.001, respectively).

Treatment regimen

All patients were treated with a weight-based, 1.5 μg/kg weekly dose of subcutaneous PEG-IFN α-2b (PegIntron, Schering-Plough, Osaka, Japan), in combination with RBV (Rebetol, Schering-Plough), which was given orally at a daily dose of 600-1000 mg based on body weight (600 mg for patients weighing less than 60 kg, 800 mg for those weighing 60-80 kg, and 1000 mg for those weighing 80 kg or over). The length of treatment was 48 wk for patients with HCV genotype 1 and 24 wk for patients with genotype 2. The above duration and dosage are those approved by the Japanese Ministry of Health, Labor and Welfare. Patients were considered to have RBV-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of RBV was required. Patients aged 65 years or older had a significantly higher frequency of RBV dose reduction during the treatment period than those aged less than 65 years old (HCV genotype 1: group A *vs* group B, 41.2% *vs* 49.0%, P = 0.032, genotype 2: group C *vs* group D, 28.6% *vs* 54.1%, P < 0.001). Some patients also had PEG-IFN α-2b-induced psychological adverse effects or a decrease in white blood cell and platelet counts. In such cases, a reduction in the dosage of PEG-IFN α-2b was required. Both PEG-IFN α-2b and RBV were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1 × 10^9/L, and 25 × 10^9/L, respectively. The treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic disorders developed, continuation of treatment was judged not to be possible by the attending physician, or if the patient desired discontinuation of treatment.

Determination of baseline HCV RNA level and HCV genotype

The pretreatment, baseline, serum HCV RNA level was measured by a quantitative HCV RNA polymerase chain reaction (PCR) assay (COBAS Amplicor HCV Monitor Test v 2.0 using the 10-fold dilution method; Roche Diagnostics, Tokyo, Japan), which has a lower limit of quantitation of 5000 IU (13 500 copies)/mL (5 kIU/mL) and an outer limit of quantitation of 5 100 000 IU/mL (5100 kIU/mL). The HCV genotype was determined by type-specific primers of the core region of the HCV genome. The protocol for genotyping was carried out as previously described[3].

Efficacy of treatment

End of treatment (EOT) response and SVR were defined as serum HCV RNA undetectable at the end of treatment and at 24-wk follow-up after the end of treatment, respectively. EOT response and SVR were defined as non-detectable HCV-RNA as measured by qualitative COBAS Amplicor HCV Monitor Test v 2.0, with the results labeled as positive or negative. The lower limit of detection was 50 IU/mL (0.5 kIU/mL). The analysis of EOT and SVR was performed on an intention-to-treat basis.

Statistical analysis

Continuous data are expressed as mean ± SD. The statistics were carried out using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The χ^2 test, Fisher's exact test and Kruskal-Wallis test were used to determine the differences in baseline clinical characteristics, safety, efficacy of the combination therapy, adherence to the total dose, and the association between the adherence and SVR. Logistic regression analysis was used to identify the association between age and SVR. A P < 0.05 was considered significant.

RESULTS

EOT response rate by intention-to-treat analysis

Among patients with genotype 1, the EOT response rate was significantly higher in group A (497 of 685, 72.5%) than in group B (129 of 253, 45.0%) (P < 0.001). Among patients with genotype 2, there was no significant difference between groups C (239 of 252, 94.8%) and D (55 of 61, 90.1%).

SVR rate by intention-to-treat analysis

Of 1251 patients, 631 (50.4%) achieved SVR in the intention-to-treat analysis. The SVR rate was significantly higher for genotype 2 (249 of 313, 79.6%) than for genotype 1 patients (382 of 938, 40.7%) (P < 0.001). Among patients with genotype 1, the SVR rate was significantly higher in group A (324 of 685, 47.3%) than in group B (58 of 253, 22.9%) (P < 0.001). Among patients with genotype 2, SVR was also significantly higher in group C (209 of 252, 82.9%) than in group D (40 of 61, 65.6%) (P = 0.004). The rate of SVR was significantly higher for females (113 of 128, 88.3%) than for males (96 of 124, 77.4%) in group C only (Figure 1). Furthermore, we analyzed whether or not the SVR rate differed according to the age at which the combination treatment of PEG-IFN α-2b plus RBV was started. The results showed that the SVR rate decreased significantly with age for both genotype 1 and 2. SVR was achieved by 5.6%-26.3% of genotype 1 patients aged 70 years or older, and by 57.1%-100% of genotype 2 patients aged 70 years or older (Figure 2).

We previously reported a minimum acceptable dose of at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target dosage of RBV for the successful treatment of Japanese patients with genotype 1[8]. Therefore, we analyzed the SVR rates in patients with genotype 1 by the dosage they actually received during treatment (a total dose of at least 80% or more of PEG-IFN α-2b and 60% or more of RBV) (Table 3). The number who received at least this minimum acceptable dosage during treatment were 278 (40.6%) of 685 patients in group A and 62 (24.5%) of 253 in group B, significantly lower in group B than in group A (P < 0.001). Compared with patients who received less than the minimum acceptable dosage, in patients who received at least this minimum dosage, the SVR rates increased from 34.2% to 66.5% in group A patients and from 15.7% to 45.2%

Table 3 The comparison of the rate of sustained virological response of patients with genotype 1 receiving a dose of 80% or more of pegylated interferon α-2b plus 60% or more of ribavirin and the reduced dosage group *n* (%)

	Male		Female		Total	
	n	SVR	*n*	SVR	*n*	SVR
Group A						
Minimum acceptable	168	116 (69.0)	110	69 (62.7)	278	185 (66.5)
Reduced	206	73 (35.4)	201	66 (32.8)	407	139 (34.2)
Total	374	189 (50.5)	311	135 (43.4)	685	324 (47.3)
Group B						
Minimum acceptable	31	15 (48.4)	31	13 (41.9)	62	28 (45.2)
Reduced	91	18 (19.8)	100	12 (12.0)	191	30 (15.7)
Total	122	33 (27.0)	131	25 (19.1)	253	58 (22.9)

Minimum acceptable: patients who received 80% or more of the target dose of pegylated interferon (IFN) α-2b and 60% or more of ribavirin (RBV). Reduced: Patients who received less than 80% of pegylated IFN α-2b and less than 60% of RBV. SVR: Sustained virological response.

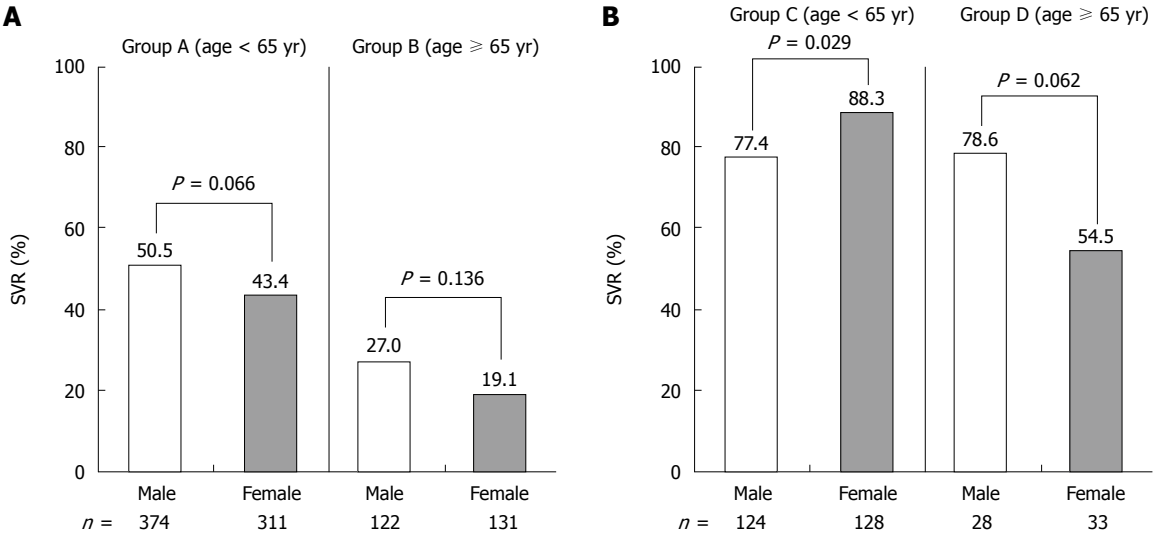

Figure 1 Virological response to the combination treatment by age and sex of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response.

($P < 0.001$) in group B patients. No significant difference between groups C and D was observed. On comparing patients whose platelet count was under 10×10^{10}/L, the SVR rate for genotype 1 was significantly lower in group B (2 of 36, 5.6%) than in group A (16 of 56, 28.6%) ($P < 0.001$). Among the patients with genotype 2, SVR was not significantly different between group C (9 of 16, 56.3%) and group D (2 of 7, 28.6%).

In a comparison of the SVR rate in patients with or without one or more previous courses of IFN plus RBV, there was no significant difference between the genotypes (genotype 1: 118 of 310, 38.1% *vs* 264 of 628, 42.0%, genotype 2: 44 of 72, 61.1% *vs* 141 of 241, 58.5%). Furthermore, we compared the EOT response rate and SVR rate of cirrhosis patients whose liver fibrosis was F4, and found no significant difference between groups A (EOT: 16 of 30, 53.3%, SVR: 7 of 30, 23.3%) and B (EOT: 6 of 17, 35.3%, SVR: 2 of 17, 11.8%). In addition, no significant difference was found between groups C (EOT: 8 of 10, 80.0%, SVR: 6 of 10, 60.0%) and D (EOT: 9 of 12, 75.0%, SVR: 5 of 12, 41.7%).

Discontinuation of PEG-IFN α-2b plus RBV treatment and adverse effects

Of 1251 patients, 314 (25.1%) did not complete PEG-IFN α-2b plus RBV treatment due to adverse effects or other reasons. The discontinuation rate was significantly higher in patients with genotype 1 (273 of 938, 29.1%) than in those with genotype 2 (41 of 313, 13.1%) ($P < 0.001$) (Tables 4 and 5). Furthermore, the rate of discontinuation due to adverse effects was significantly higher in patients with genotype 1 (135 of 938, 14.4%) than in those with genotype 2 (23 of 313, 7.3%) ($P < 0.010$). The rates of discontinuation due to lack of treatment efficacy and for economic reasons (loss of job, inability to pay the medical costs) were also significantly higher in patients with genotype 1 (55 of 938, 5.9%, 15 of 938, 1.6%) than in those with genotype 2 (1 of 313, 0.3%, 0 of 938, 0%) ($P < 0.001$ and $P = 0.025$, respectively).

For genotype 1 patients, the discontinuation rate was significantly higher in group B (106 of 253, 42.9%) than in group A (167 of 685, 24.4%) ($P < 0.001$), and the rate of discontinuation due to adverse effects was also significantly

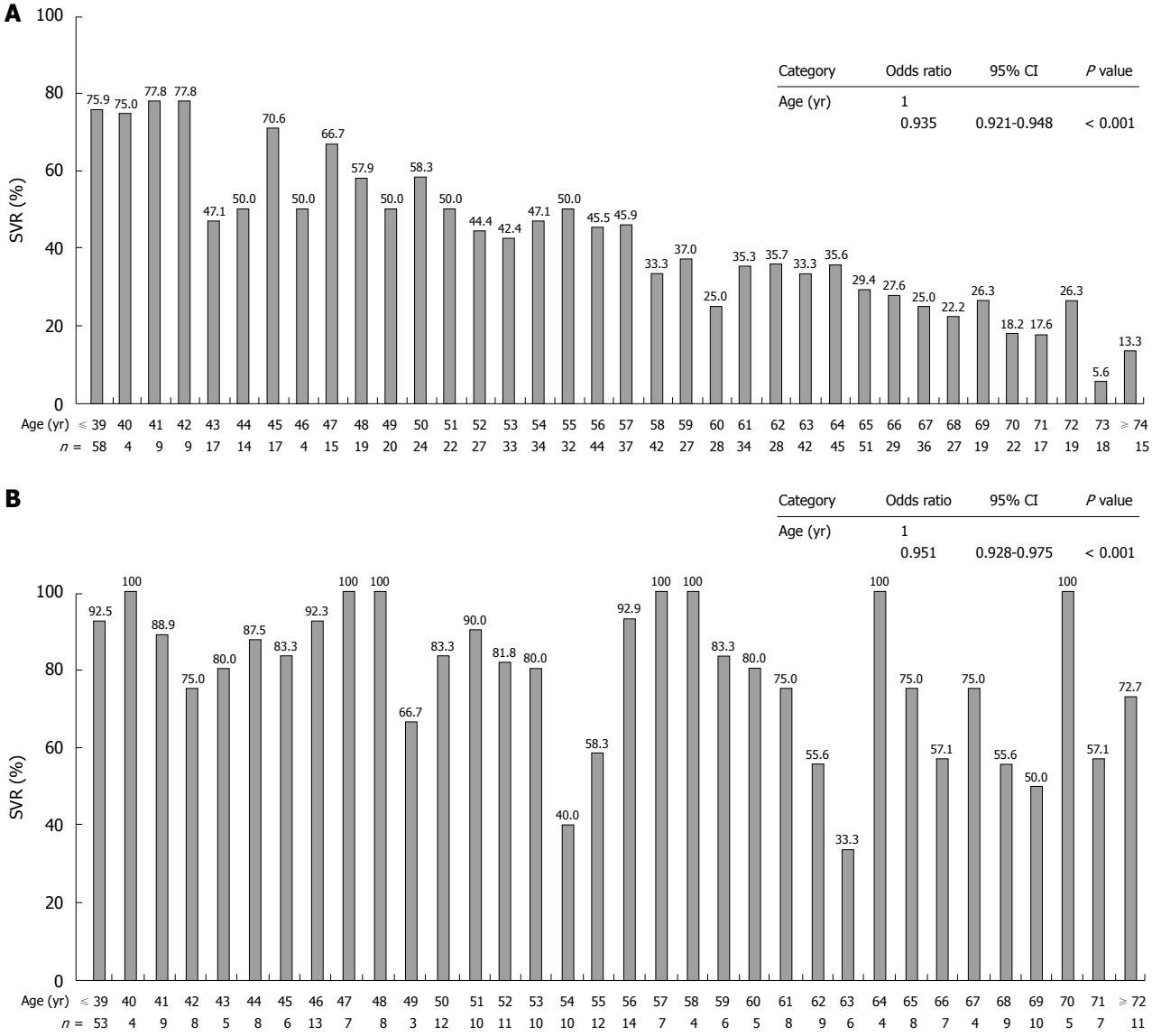

Figure 2 Virological response to the combination treatment by age of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response; CI: Confidence interval.

higher in group B (61 of 253, 24.1%) than in group A (74 of 685, 10.8%) ($P < 0.001$). General fatigue was the most frequent adverse effect, and was significantly more frequent in group B than in group A ($P < 0.001$). However, in these group 1 patients, RBV was reduced due to anemia in 12.5% (3 of 24) of group A and in 30.4% (7 of 23) of group B. Furthermore, rash and thrombocytopenia were significantly more frequent in group B than in group A ($P = 0.014$ and $P = 0.007$, respectively). In group A, depression was significantly more frequent in females than in males ($P = 0.012$). In genotype 2 patients, treatment discontinuation did not differ between group C (33 of 252, 13.1%) and group D (8 of 61, 13.1%), and the rate of discontinuation due to adverse effects did not differ between these groups (17 of 252, 6.7%, 6 of 61, 9.8%, respectively).

The mean time to discontinuation in group A (21.6 ± 11.9 wk) was not significantly different from group B (21.5 ± 12.6 wk), and the mean time in group C (11.0 ± 6.8 wk) was also not significantly different from group D (11.6 ± 6.0 wk). There was no significant difference between male and female patients in each group (male: 21.0 ± 12.4 *vs* female: 22.1 ± 11.8 in group 1, male: 11.3 ± 7.1 *vs* female: 10.9 ± 6.1 in group 2).

HCC was not seen in genotype 2 patients; only in patients with genotype 1 (29.5 ± 9.9 wk) and was more frequent in group B (5 of 253, 2.0%) than in group A (2 of 685, 0.3%) ($P = 0.008$).

DISCUSSION

In a large, national, multicenter Greek study involving 993 treated and 734 untreated patients with chronic hepatitis C, patients with cirrhosis, showed a protective effect of treatment even among those without SVR. For patients without cirrhosis, the beneficial effect of IFN α treatment was particularly evident in older patients; patients with the worst prognosis if left untreated. Therefore, IFN α-based treatment should be offered to older persons, as these are

Table 4 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 1 patients

	Group A (age < 65 yr)		Group B (age ≥ 65 yr)		Total
	Male (*n* = 374)	Female (*n* = 311)	Male (*n* = 122)	Female (*n* = 131)	
Discontinued number	101	66	52	54	273
Adverse effects	43	31	33	28	135
General fatigue	17	7	12	11	47
Depression	3	11	4	5	23
Appetite loss	1	0	1	0	2
Rash	3	2	3	4	12
Encephalopathy	1	0	0	0	1
Neutropenia	2	0	0	0	2
Anemia	3	2	4	1	10
Thrombocytopenia	1	0	3	1	5
Elevation of ALT	1	0	0	0	1
Hyperthyroidism	3	2	0	1	6
Hypothyroidism	0	1	0	0	1
Retinopathy	1	0	1	0	2
Interstitial pneumonia	2	0	1	1	4
Pulmonary disease (others)[1]	0	1	1	1	3
Psychoneurotic disorder[2]	2	0	2	0	4
Nervous disease[3]	1	1	0	1	3
Autoimmune disease[4]	0	2	0	1	3
Metabolic disease[5]	0	2	0	0	2
Digestive disorder[6]	2	0	1	1	4
Hepatocellular carcinoma	2	0	4	1	7
Malignancy (extra-liver)	0	1	1	0	2
No effect of treatment	22	18	7	8	55
Economic problem	9	3	0	3	15
Others[7]	25	13	7	14	59

[1]Includes pulmonary tuberculosis (*n* = 1), pneumonia (*n* = 1), tuberculous pleuritis (*n* = 1); [2]Includes psychiatric disorder (*n* = 2), disquiet (*n* = 1), insomnia (*n* = 1); [3]Includes nerve paralysis (*n* = 1), cerebral infarction (*n* = 1); [4]Includes rheumatoid arthritis (*n* = 2), myasthenia gravis (*n* = 1); [5]Includes diabetes mellitus (*n* = 1), hypertriglycemia (*n* = 1); [6]Includes cholecystitis (*n* = 3), pancreatitis (*n* = 1); [7]Includes 25, 13, 6 and 13 drop-outs from groups A, B, C and D, respectively: One for excessive alcohol consumption in group C and one was nursing in group D. ALT: Alanine aminotransferase.

the patients with the greatest potential benefit and may achieve SVR[16]. In Japan, the prevalence of chronic HCV infection increases with age, however, the optimal management of older patients has not yet been accurately defined. Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. In addition, the natural history of chronic hepatitis C in elderly patients is not accurately known, as the presence of comorbidity can affect illness progression and life expectancy. HCV became more prevalent in Japan decades before the United States[17]. Japanese patients with chronic hepatitis C treated with IFN are currently 10 to 15 years older than corresponding patients in the United States and European countries, where patients treated with antiviral treatment tend to average 45 years of age[18-20]. Therefore, our results can serve as a world-wide model for the treatment of older chronic hepatitis C patients.

It has been well documented that the combination therapy of PEG-IFN α-2b plus RBV is more effective than previous IFN monotherapy in chronic hepatitis C patients[7,8]. There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1, which revealed low rates of SVR (31.1%-51.9%)[21-24]. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. Because the present study was a large multicenter design, it is useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients. The present study confirmed the results of our previous study which showed that the SVR rate was significantly higher for genotype 2 than for genotype 1 patients[8]. Another important result was that the ability to take at least a minimum acceptable dosage during treatment increased the SVR rate by about three times in older patients with genotype 1. This result also confirmed previous studies which indicated the importance of giving at least the minimum acceptable treatment dosage in patients infected with HCV genotype 1, especially older patients[23,24].

Secondly, we compared discontinuation of treatment by genotype and sex. In genotype 1 patients, adverse effects were seen more often in older than in younger patients. This was the most important reason why the rate of treatment discontinuation was higher in older than in younger patients, and affected the outcome of PEG-IFN α-2b plus RBV combination therapy. General fatigue was the most common adverse effect in older patients. Because older patients often have impaired renal function, they have increased blood levels of RBV[25,26]. They are also inclined to be anemic and to have general fatigue. However, only a small number of older patients in the present study had reduced RBV due to anemia. Therefore, general fatigue is probably a direct adverse effect of PEG-IFN α-2b. We previously reported that herbal medicine

Table 5 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 2 patients

	Group C (age < 65 yr)		Group D (age ≥ 65 yr)		Total
	Male (*n* = 124)	Female (*n* = 128)	Male (*n* = 28)	Female (*n* = 33)	
Discontinued number	18	15	4	4	41
Adverse effects	6	11	3	3	23
General fatigue	1	3	1	0	5
Depression	0	2	0	0	2
Appetite loss	0	0	0	0	0
Rash	2	1	0	2	5
Encephalopathy	0	0	0	1	1
Neutropenia	0	2	0	0	2
Anemia	0	0	2	0	2
Thrombocytopenia	2	0	0	0	2
Elevation of ALT	0	0	0	0	0
Hyperthyroidism	0	1	0	0	1
Hypothyroidism	0	1	0	0	1
Retinopathy	0	0	0	0	0
Interstitial pneumonia	0	0	0	0	0
Pulmonary disease(others)	0	0	0	0	0
Psychoneurotic disorder	0	0	0	0	0
Nervous disease[1]	1	1	0	0	2
Autoimmune disease	0	0	0	0	0
Metabolic disease	0	0	0	0	0
Digestive disorder	0	0	0	0	0
Hepatocellular carcinoma	0	0	0	0	0
Malignancy (extra-liver)	1	0	0	0	1
No effect of treatment	1	0	0	0	1
Economic problem	0	0	0	0	0
Others[2]	10	4	1	1	16

[1]Includes nerve paralysis (*n* = 1), tetany (*n* = 1); [2]All patients were drop out. ALT: Alanine aminotransferase.

relieved the adverse effects of IFN, including general fatigue[27]. Herbal medicine may be useful for mitigating general fatigue during PEG-IFN α-2b plus RBV combination treatment, especially in older patients.

The rate of discontinuation was lower in patients with genotype 2 than in patients with genotype 1, and there was no difference between the older and the younger patients with genotype 2. These results are possibly a consequence of the shorter term of treatment in genotype 2 and the many genotype 1 patients who discontinued due to lack of efficacy.

Two of the characteristics of older patients in the present study were that both hemoglobin and platelet count were significantly lower than in younger patients. The SVR rate was significantly lower when the platelet count was less than 10×10^{10}/L. Furthermore, the older genotype 1 patients were often forced to discontinue treatment due to thrombocytopenia and the occurrence of HCC. These findings appear to result from advanced liver fibrosis in older chronic hepatitis C patients. Therefore, the possibility of HCC during long-term IFN treatment in older patients must be considered.

We previously reported that older female patients had a low response to IFN-α monotherapy[9], and other investigators have reported that older female patients have a poor response to PEG-IFN α-2b plus RBV[22,28]. Although our data showed that sex was not related to SVR, the reason for this finding was not fully elucidated. In any case, studies have conclusively shown that it is important to begin treatment with PEG-IFN α-2b plus RBV combination therapy as soon as possible. Our data suggest that age may be a more important factor than sex for increasing the rate of SVR. Resistance to treatment in older patients may be due to IFN-immunomodulation, advanced liver fibrosis, or reduced dosage.

To maximize adherence to the optimal treatment regimen, the treatment schedule can be modified or other therapeutic modalities added, such as hematopoietic growth factors[29] or the new thrombopoietin-receptor agonist, eltrombopag, for the antiviral treatment of older patients with chronic hepatitis C[30]. A further individualized treatment protocol based on viral kinetics might be more practical[31].

In conclusion, PEG-IFN α-2b plus RBV treatment was effective in the treatment of older chronic hepatitis C patients when they received at least the minimum acceptable treatment dosage. However, there were frequent adverse effects and treatment discontinuation. It is necessary to control for adverse effects that might interrupt treatment and to begin this combination therapy as soon as possible, especially in older patients.

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COMMENTS

Background

Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. However, there is little data concerning the response and safety of combination treatment for a large number of older patients with chronic hepatitis C virus infection. Therefore, in an attempt to ameliorate these problems, the authors decided to treat older patients with pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination therapy.

Research frontiers

The combination treatment of PEG-IFN α-2b plus RBV improved the sustained virological response rate in chronic hepatitis C patients. However, the current issue is whether or not to treat older patients because of low response and high dropout rate.

Innovations and breakthroughs

There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. This study is very useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients, because of its large scale, multicenter design.

Applications

The study demonstrated that PEG-IFN α-2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum required treatment dosage. Furthermore, this study suggested that the combination treatment and beginning this therapy as soon as possible are important, especially in older patients.

Peer review

The study has been well conducted and includes a large number of patients. Results have been described in a lucid and informative manner and are of clinical relevance.

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ORIGINAL ARTICLE

Diagnostic value of glypican-3 in serum and liver for primary hepatocellular carcinoma

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Abstract

AIM: To evaluate the diagnostic value of glypican-3 (GPC3) in serum and liver for primary hepatocellular carcinoma (HCC).

METHODS: Serum levels of GPC3 and α-fetoprotein (AFP) were measured in 75 patients with primary HCC and 32 patients with liver cirrhosis. Expression of GPC3 and AFP in 58 HCC and 12 cirrhotic specimens was detected with immunohistochemical staining.

RESULTS: When the cut-off value of serum GPC3 was set at 300 ng/L, its sensitivity and specificity for HCC were 47.0% and 93.5%, respectively. Among the 14 patients with HCC at stage according to the Barcelona Clinic Liver Cancer staging system, the serum GPC3 level was higher than 300 ng/L in 50% (7/14) patients, the serum AFP level was not ⩾ 400 μg/L in any patient. Combined serum AFP and GPC3 significantly increased the sensitivity to the diagnosis of HCC. The GPC3 expression was detected in cytoplasm of HCC cells but not in hepatocytes and bile ducts of benign tumors. Among the 58 HCC patients, the GPC3 was expressed in 100% (28/28) patients with their serum AFP level ⩾ 400 μg/L, and in 90% (27/30) patients with their AFP level < 400 μg/L, respectively. The GPC3 was weakly or negatively expressed in all paracarcinomatous and cirrhotic tissue samples. AFP positive HCC cells were only found in 1 out of the 58 HCC patients.

CONCLUSION: GPC3 protein is a sensitive and specific serum marker for diagnosis of early HCC. Its expression in liver tissues can be used to discriminate tumor cells from benign hepatic cells.

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Key words: Hepatocellular carcinoma; α-fetoprotein; Glypican-3; Diagnosis

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INTRODUCTION

The prognosis of patients with primary hepatocellular

carcinoma (HCC) is generally very poor with a 5-year survival rate of less than 10%-15% since most of them are diagnosed at their late stage using current strategy[1,2]. Surgical resection is still the most used treatment option for the diagnosed HCC patients[3]. Therefore, early detection and diagnosis of HCC would be of great clinical benefit. Screening strategy is to identify small tumors that may be successfully treated. Currently in China, detection of serum α-fetoprotein (AFP) and ultrasound examination every 6 mo in patients with liver cirrhosis have been recommended[4,5]. Since elevated serum AFP level is correlated with the occurrence of HCC, screening and surveillance for HCC in high-risk patients through periodic measurement of AFP have been proposed since the 1980s[5-7]. The cut-off value for HCC diagnosis in China is set at 400 μg/L according to the guidelines of clinical diagnosis and staging criteria for primary HCC established by Chinese Society of Liver Cancer in 2001[8]. However, 25%-30% of the diagnosed HCC patients have a normal AFP level (< 20 μg/L) or 40%-50% of HCC patients have a lower serum AFP level (> 20 μg/L - < 400 μg/L)[1,2]. On the other hand, the AFP level can reach 2500 μg/L in around 20%-25% of patients with chronic hepatitis, liver cirrhosis and other liver disease[9-11]. Although ultrasonography has been widely used in clinical screening of HCC, it is highly dependent on the experience of its operator[3,5]. Therefore, as AFP and ultrasonography only play a limited role in screening of HCC, some candidate biomarkers can be used in diagnosis of HCC including glypican-3 (GPC3). GPC3 is a heparan sulfate proteoglycan that is bund to the cell surface by glycosylphosphatidylinositol anchors (GPI) and highly expressed in fetal but not in adult liver. It has been shown that GPC3 is closely related to HCC[9,12]. It was reported that the frequency of GPC3 expression in AFP-negative HCC patients is as high as 90%, suggesting that it can be used in diagnostic of HCC[13,14]. However, serum GPC3 and its expression in liver tissue of patients with HCC at different stages have not been fully investigated. Therefore, the value of serum GPC3 expression in tumor/paracarcinomatous tissue for the diagnosis of HCC was explored in this study.

MATERIALS AND METHODS

Patients

One hundred and seven patients admitted to Beijing Youan Hospital, Capital Medical University from January 2006 to December 2008 were recruited in this study and divided into HCC group (n = 75) and liver cirrhosis group (n = 32) with a male to female ratio of 55:20 and 24:8, respectively. HCC was diagnosed based on at least one of the following criteria in the guidelines of clinical diagnosis and staging for primary hepatocellular carcinoma established by Chinese Society of Liver Cancer, 2001[8]: (1) hepatic space-occupying lesion with a serum AFP level ⩾ 400 μg/L; and (2) serum AFP level < 400 μg/L but with new hepatic space occupying lesions, arterial phase enhancement on computed tomography or magnetic resonance imaging. HCC was staged according to the Barcelona Clinic Liver Cancer (BCLC) staging system[15]. Liver cirrhosis was diagnosed based on physical examination, laboratory test, and B-ultrasonography or computed tomography (CT) scan. Patients in liver cirrhosis group were followed up at least for 2 years. The study was approved by the Ethical Committee of Beijing Youan Hospital, Capital Medical University. Investigators explained in detail to all the patients and/or their relatives. Written consent was obtained from all patients when they were recruited.

Serum and liver tissue samples

Serum samples were collected from the patients. HBV and HCV infectious markers were detected in clinical laboratory. HBV infection was determined by the presence of HBsAg using reagents from Roche Diagnostics-China (Shanghai, China) and HBV-DNA from Kehua (Shanghai, China). HCV infection was determined by the presence of anti-HCV using reagents from Diasorin Ltd. (Shanghai, China) and HCV-RNA from Da An Gene Co. Ltd., Sun Yat-Sen University (Guangzhou, China). The serum samples were stored at -20℃ for the measurement of serum AFP and GPC3 levels. Liver tissue specimens and needle biopsies from 58 HCC and 12 cirrhotic patients were fixed in 4% polyoxymethylene and embedded in paraffin.

Measurement of serum AFP and GPC3 level

Serum AFP and GPC3 levels were measured by electrochemiluminescence (Abbott, USA) and enzyme-linked immunosorbent assay (ELISA; BioMosaics Company, USA), respectively, following their manufacturer's instructions. The value of optical density (OD) was detected using a spectrophotometer (Multiskan Ascent V1.24, Switzerland).

Detection of AFP and GPC3 expression with immunohistochemistry staining

Expression of GPC3 in liver tissue and needle liver biopsy samples was detected with immunohistochemistry (IHC) staining. The slides were reviewed to confirm the diagnosis of HCC according to the guidelines of WHO criteria[16]. A mouse monoclonal anti-GPC3 antibody (clone number: sc-65443 1G12, Santa Cruz Inc., USA) and a mouse polyclonal anti-AFP antibody (Neomarker Inc., USA) were used in IHC staining. Normal mouse serum served as a negative control. Briefly, tissue sections were deparaffinized, rehydrated, and antigen was retrieved using heat-induced epitope in a 10 mmol/L citrate buffer, pH 6.0. After blocked with peroxidase, the sections were incubated with primary antibodies diluted at 1:50. The secondary antibody used was a horse-radish peroxidase (HRP)-labeled antibody (Zhongshan Golden Bridge, Beijing, China). Results were evaluated by pathologists at Beijing YouAn Hospital. Five high visual fields were observed with no less than 1000 cells in each visual field. Cells in cytoplasm

Table 1 Clinical data about the patients included in the study

Diagnosis	Numbers[a]	Age (yr)[a]	HBV infection[a]	HCV infection[a]	BCLC-stage A	BCLC-stage B	BCLC-stage C + D
HCC	75 (M: 55, F: 20)	55.4 ± 9.91	84.0% (63/75)	16.0% (12/75)	18.7% (14/75)	30.7% (23/75)	50.7% (38/75)
Cirrhosis	32 (M: 24, F: 8)	53.3 ± 5.81	78.1% (25/32)	21.9% (7/32)			

[a]$P > 0.05$. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; BCLC: Barcelona Clinic Liver Cancer.

taking on yellow or brown particles were considered positive.

Statistical analysis

All analyses were performed using the SPSS for Windows (version 11.5). The data were expressed as mean ± SD. χ^2 test and Student t test were used to compare the distribution of categorical and continuous variables, respectively, in the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical data about the patients

All the 107 patients recruited in this study were Chinese. Their clinical data are shown in Table 1. The patients were diagnosed as HBV-related HCC (84%) and HCV-related HCC (16%) or liver cirrhosis (78.1% and 29.1%), respectively. No significant difference was observed in HBV infection between the two groups. No double HBV and HCV-infected patient was found in the current study. Of the 75 clinically diagnosed HCC patients, 14 were at BCLC-stage A, 23 at BCLC-stage B, 16 at BCLC-stage C, and 22 at BCLC-stage D.

Limitation of serum AFP level in diagnosis of early HCC

AFP was widely used as a biomarker for the diagnosis of primary HCC. The cut-off value was set at 400 μg/L according to the guidelines of clinical diagnosis and staging criteria for primary hepatocellular carcinoma established by Chinese Society of Liver Cancer in 2001. The serum AFP level was below 400 μg/L in all patients with liver cirrhosis and ⩾ 400 μg/L in only 28 patients (37.3%) with HCC (Figure 1A). The serum AFP level was ⩾ 400 μg/L in none of the 14 HCC patients at BCLC-stage A and only in 7 patients (30.4%) at BCLC-stage B. The positive rate was only 18.9% in patients at BCLC-stage A and B with their AFP level ⩾ 400 μg/L, although it increased to 55.3% in patients at BCLC-stage C and D (Figure 1A). The AFP levels were compared between liver cirrhosis and HCC patients at BCLC-stage A and B (Figure 1B). Among the 32 patients with liver cirrhosis, the AFP level was higher in 2 patients with their liver cirrhosis progressed to HCC than in those with their liver cirrhosis not progressed to HCC. The sensitivity of AFP for the diagnosis of HCC did not significantly increase in HCC patients at different BCLC stages.

Serum GPC3 level for the diagnosis of early HCC

The cut-off value of GPC3 was set at 300 ng/L by receiver operating characteristic (ROC) curve analysis. At this cut-off value, the sensitivity and specificity of serum GPC3 for the diagnosis of HCC was 46.7% and 93.5%, respectively. Among the 32 clinically diagnosed liver cirrhosis patients, the serum GPC3 level was > 300 ng/mL in 9.4% (3/32) patients. Among the early HCC patients, 7 (50%) had BCLC-stage A HCC, and 9/23 (39.1%) had BCLC-stage B HCC with their serum GPC3 level > 300 ng/mL (Figure 2A and B). Among the 16 patients with their GPC3 > 300 ng/mL, the serum AFP level was ⩾ 400 μg/L in only 3 patients.

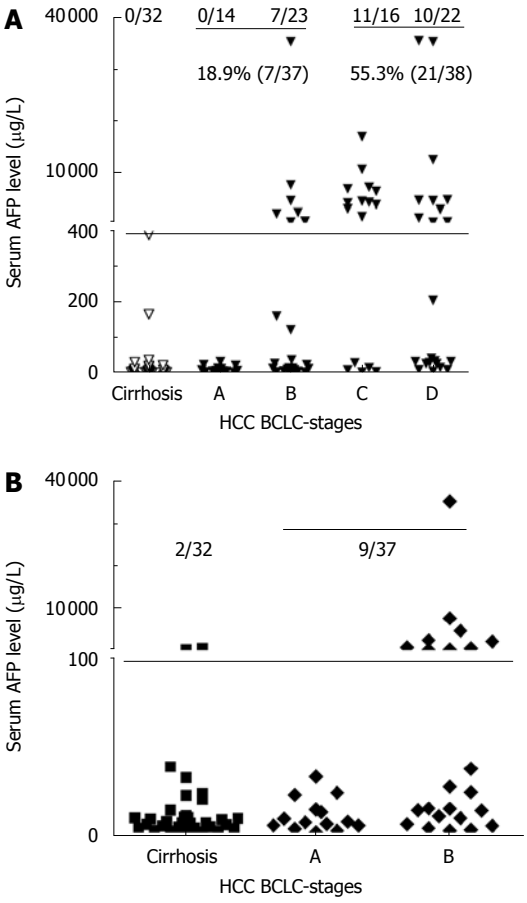
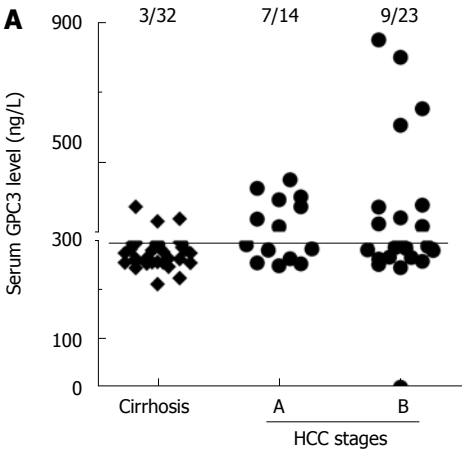


Figure 1 Serum α-fetoprotein levels in 107 patients with liver cirrhosis or hepatocellular carcinoma at late Barcelona Clinic Liver Cancer stages (A) and early Barcelona Clinic Liver Cancer stages (B). Each dot represents one patient. AFP: α-fetoprotein; HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer.

GPC3 expression in liver tissue

Liver cirrhosis was observed in all the examined specimens. With IHC staining, AFP positive cells were hardly



B

	AFP (+)	CPC3 (+)	*P* value
Stage A	0/14	7/14	< 0.01
Stage B	7/23	9/23	> 0.05

Figure 2 Serum glypican-3 levels in 32 liver cirrhosis patients and in 37 hepatocellular carcinoma patients at Barcelona Clinic Liver Cancer-stage A and B. A: Each dot represents one patient; B: Frequencies of α-fetoprotein (AFP) ⩾ 400 μg/L and glypican-3 (GPC3) > 300 ng/L in hepatocellular carcinoma (HCC) patients at Barcelona Clinic Liver Cancer-stage A and B.

detectable in HCC patients even with their serum AFP level ⩾ 400 μg/L (Figure 3) except in 1 tumor tissue sample. However, GPC3 was specifically expressed in cytoplasm of cancer cells but not or weakly expressed in paracarcinomatous tissue samples (Figure 3A-C). GPC3 was expressed in 94.8% (55/58) of HCC tissue samples and over-expressed in all the 28 HCC patients with their serum AFP level ⩾ 400 μg/L and in 30 HCC patients with their serum AFP level < 400 μg/L. GPC3-positive cancer cells were found in 90% of the 30 clinically diagnosed HCC patients with their serum AFP level < 400 μg/L. GPC3 was positively expressed in HCC tissue specimens and negatively expressed in all liver cirrhotic specimens.

The expression of GPC3 was also detected in needle biopsy samples. After the specimens were stained, some cells were positive for GPC3 but negative for AFP (Figure 4A-C).

Combined serum GPC3 and AFP

Combined serum GPC3 and AFP significantly increased the sensitivity for HCC diagnosis. The sensitivity of serum AFP increased from 18.9% to 54% for the diagnosis of HCC at BCLC-stage A + B, and from 55.3% to 81.6% for the diagnosis of HCC at BCLC-stage C + D (Figure 5).

DISCUSSION

The main etiological factor for primary HCC in China is HBV infection[17,18]. However, it has been shown that HCV infection plays a role in pathogenesis of primary HCC[19,20]. Of the 75 clinically diagnosed HCC patients enrolled in this study, 63 had HBV-related HCC. As HCC can develop in patients with HBV infection, chronic HBV carriers are also included in screening of HCC in China

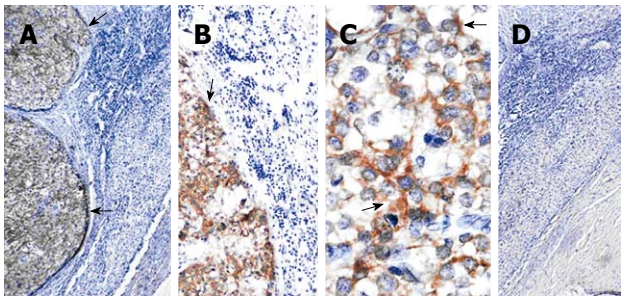


Figure 3 Immunohistochemistry staining of glypican-3 or α-fetoprotein in liver samples. Representative of the excised hepatocellular carcinoma samples stained with mouse anti-glypican-3 (GPC3) (A-C) or AFP (D, 4 ×); B (10 ×) and c (40 ×) are the amplified image of A (4 ×). The cells stained with yellow or brown particles were considered positive (arrows).

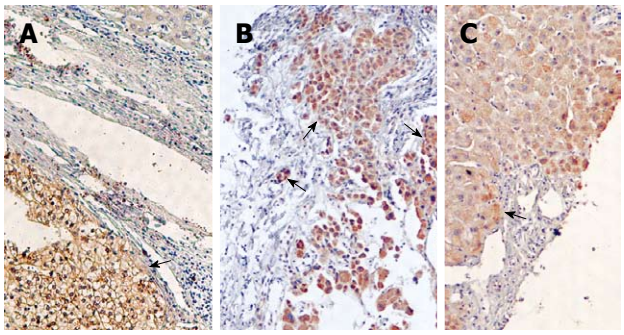


Figure 4 Immunohistochemistry staining of glypican-3 in needle biopsy. Three tissue sections (A-C, 10 ×) from patients with hepatic space occupying lesions with their serum α-fetoprotein < 400 μg/L. Glypican-3 was positively expressed in hepatocellular carcinoma (arrows).

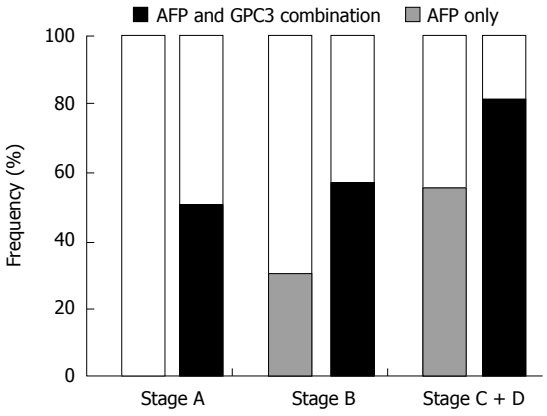


Figure 5 Frequencies of serum α-fetoprotein ⩾ 400 μg/L only (stripped bars), α-fetoprotein ⩾ 400 μg/L and glypican-3 > 300 ng/L (filled bars) in 75 hepatocellular carcinoma patients at different Barcelona Clinic Liver Cancer stages. AFP: α-fetoprotein; GPC3: Glypican-3.

with the measurement of AFP level and ultrasonography every 6 mo[5].

Screening of HCC is cost-effective in patients with cirrhosis since its annual incidence exceeds 1.5% per year[3]. However, most liver mass/nodular lesions are benign, such as cirrhotic regenerative nodules, adenoma, and focal nodular hyperplasia, but they may mimic malignant liver lesions[3]. Therefore, the differential diagnosis between

HCC and benign mimickers is difficult. AFP is the most commonly used indicator in screening and diagnosis of HCC as well as in follow-up of high-risk population. However, its sensitivity for the diagnosis of HCC is only 30%-40%[2,21]. In the current study, the diagnosis rate of HCC was 37.3% for serum AFP (≥ 400 μg/L), which is consistent with the reported findings[22,23], but lower than that reported by Dr. Yang (44.6%)[24]. The serum AFP level is significantly different in HCC patients with different tumor size, number, stage and etiology[25]. The results of this study confirm that serum AFP (≥ 400 μg/L) alone cannot be used as a diagnostic marker for HCC, especially early HCC[26]. Therefore, all these results indicate that the serum AFP level plays a limited role in diagnosis of HCC, especially early HCC.

It was recently reported that GPC3 is only detected in HCC cells but not in benign liver tissues, and can thus be used as a potential biomarker for the diagnosis of early HCC[9,14,26,27]. The serum GPC3 level was higher than 300 ng/L in 50% of early HCC patients, although their serum AFP level was below 100 μg/L in this study, suggesting that serum GPC3 level plays a potential role in diagnosis of early HCC and can be used in screening of HCC. Therefore, measurement of serum GPC3 level in combination with B-ultrasonography is useful in screening and diagnosis of HCC as well as in follow-up of high-risk population.

In this study, GPC3 was over-expressed in cytoplasm of HCC cells but not expressed in 58 paracarcinomatous and 12 liver cirrhotic tissue samples. GPC3 positive cells were found in 90% of patients with their serum AFP level < 400 μg/L. GPC3 was negatively expressed in only 10% of the examined samples. As all the HCC patients included in this study were accompanied with cirrhosis, the positive GPC3 expression rate was high, which is consistent with the reported data[14], suggesting that expression of GPC3 protein is a sensitive and specific histological marker for the diagnosis of HCC and can thus be used in biopsy in the presence of nodule lesions.

Serum GPC3 and AFP levels were increased in HCC patients included in this study. GPC3 protein was exclusively expressed in cancer cells and serum GPC3 level was increased in early HCC patients with their serum AFP level < 400 g/L. GPC3 protein expression was only detectable in liver cancer cells but not in benign hepatic cells. The sensitivity of combined serum GPC3 and AFP was increased for the diagnosis of HCC at all stages.

In conclusion, GPC3 is a sensitive, specific serum and tissue marker for the diagnosis of early HCC. Over-expression of GPC3 in liver tissues can be used in discriminating tumor cells from benign hepatic cells. Combined AFP and GPC3 should be considered in diagnosis of HCC.

COMMENTS

Background

Serum α-fetoprotein (AFP) plays a limited role in and detection and diagnosis of hepatocellular carcinoma (HCC). Some new candidate biomarkers for the diagnosis of HCC have been investigated.

Research frontiers

Since 2000, the frequency of hepatic glypican-3 (GPC3) expression in AFP-negative HCC patients has been increased to 90%. However, serum GPC3 and its expression in HCC tissue have not been fully studied.

Innovations and breakthroughs

GPC3 is a sensitive and specific tissue marker for diagnosis of early HCC. Over-expression of GPC3 in liver tissues can be used in discriminating tumor cells from benign hepatic cells. Combined AFP with GPC3 should be considered in detection and diagnosis of HCC.

Applications

The results of this study suggest that combined serum GPC3 and AFP is useful in screening and diagnosis of HCC as well as in follow-up of high-risk population. GPC3 protein is a sensitive and specific histological marker for the discrimination between HCC and benign nodule lesion.

Terminology

GPC3 is a heparan sulfate proteoglycan which is bund to the cell surface by glycosylphosphatidylinositol anchors and highly expressed in fetal but not in normal adult liver.

Peer review

This is a straightforward well conducted study. The results suggest that combined serum GPC3 and AFP is useful in screening and diagnosis of HCC as well as in follow-up of high-risk population, and the authors showed that GPC3 protein was a sensitive and specific histological marker for the discrimination between HCC and benign nodule lesion, thus making a contribution to diagnosis of early HCC.

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BRIEF ARTICLE

Measuring episodic abdominal pain and disability in suspected sphincter of Oddi dysfunction

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Abstract

AIM: To evaluate the reliability of an instrument that measures disability arising from episodic abdominal pain in patients with suspected sphincter of Oddi dysfunction (SOD).

METHODS: Although several treatments have been utilized to reduce pain and associated disability, measurement tools have not been developed to reliably track outcomes. Two pilot studies were conducted to assess test-retest reliability of a newly developed instrument, the recurrent abdominal pain intensity and disability (RAPID) instrument. The RAPID score is a 90-d summation of days where productivity for various daily activities is reduced as a result of abdominal pain episodes, and is modeled after the migraine disability assessment instrument used to measure headache-related disability. RAPID was administered by telephone on 2 consecutive occasions in 2 consenting populations with suspected SOD: a pre-sphincterotomy population (Pilot Ⅰ, n = 55) and a post-sphincterotomy population (Pilot Ⅱ, n = 70).

RESULTS: The average RAPID scores for Pilots Ⅰ and Ⅱ were: 82 d (median: 81.5 d, SD: 64 d) and 48 d (median: 0 d, SD: 91 d), respectively. The concordance between the 2 assessments for both populations was very good: 0.81 for the pre-sphincterotomy population and 0.95 for the post-sphincterotomy population.

CONCLUSION: The described pilot studies suggest that RAPID is a reliable instrument for measuring disability resulting from abdominal pain in suspected SOD patients.

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Key words: Sphincter of Oddi; Abdominal pain; Disability measurement; Reproducibility of results; Pain measurement; Episodic pain

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Durkalski V, Stewart W, MacDougall P, Mauldin P, Romagnuolo J, Brawman-Minzter O, Cotton P. Measuring episodic abdominal pain and disability in suspected sphincter of Oddi dysfunction.

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INTRODUCTION

The sphincter of Oddi controls the flow of bile and pancreatic secretions into the duodenum through the ampulla of Vater. Dysfunction of the sphincter can result in pain due to back pressure in the pancreas or biliary tree (especially after the gallbladder reservoir has been surgically removed)[1,2]. Classically these pains are felt in intermittent episodes, with symptom-free intervals, as emphasized by the Rome Ⅲ consensus[3]. Some of these patients have objective findings on laboratory studies or imaging (e.g. abnormal liver enzymes, or a dilated bile duct), and are categorized by the Milwaukee classification as sphincter of Oddi dysfunction (SOD) Types Ⅰ and Ⅱ[4]. Many of these are found at endoscopic retrograde cholangiopancreatography (ERCP) to have bile duct stones or fibrotic sphincter stenosis, and are effectively treated by standard endoscopic biliary sphincterotomy.

Patients who have similar symptoms, but who have no demonstrable abnormalities on standard imaging and laboratory tests, are categorized as SOD type Ⅲ. Presumably these patients suffer from intermittent SOD and related episodes of pain. These patients are very difficult to effectively evaluate and manage[5], given the absence of objective markers of the condition, and because ERCP treatments are not without risk[6].

The intermittent episodes of abdominal pain associated with SOD Ⅲ can be severe, but the condition is not life-threatening. Pain episodes often interfere with the ability to function in primary roles (e.g. work, homemaker, *etc.*) and have a significant impact on quality of life. Impairment of function in primary roles has been previously shown to be a dominant motivation for seeking care and an outcome of primary concern to both patients and providers[7]. Several treatments are used to reduce pain and associated disability. However, measurement tools have not been developed to reliably and validly measure pain severity, frequency of pain episodes, and the impact of pain on function in primary roles.

To advance research in this area, we aimed to define an appropriate clinical measurement of efficacy for SOD treatment, where efficacy is defined as reduced pain-associated disability, also referred to as pain burden. The following describes the development and testing of an instrument to measure the pain burden in this patient population.

An extensive search of the literature did not yield an appropriate and validated instrument to measure pain and related burden for patients with SOD. Published clinical studies of treatment for SOD have used various instruments. Two sham-controlled trials used a global assessment scale[8,9], as have several open-label studies of sphincterotomy[10-13], and a trial of Botox injection[14]. Graded outcome scales (e.g. 5 grades of outcome, from very much improved, to very much worse or simply improved, same or worse) have been reported, but details on the questions and method of administration (e.g. clinic, by telephone, self-administered) were not published. One crossover trial of nifedipine treatment used daily diaries[15], but the study lasted only 16 wk, so the feasibility of daily diary use to assess episodes of pain over a longer follow-up period was not assessed. An extensive report of surgical sphincteroplasty (for a variety of indications) stated outcomes as good or excellent, without specifying criteria for outcomes[16]. Finally, a large follow-up study of 313 patients treated endoscopically used the need for re-intervention (at that center) as the primary measure of failure[17]. Such an outcome depends on other factors (e.g. access, patient preferences) that are not directly related to the patient's pain experience and that would make it unsuitable for a multicenter trial, particularly if there is not a clearly defined and standardized method of measuring a threshold for re-intervention. This outcome could be made more robust if combined with an outcome measure of a patient's pain experience and related burden.

A review of previous work and literature in other areas outside of gastroenterology revealed several well validated instruments for the assessment of pain and related disability[18-22]. Comprehensive reviews, such as the recommendations of the IMMPACT group (Initiatives on Methods, Measurement and Pain Assessment in Clinical Trials)[23], correspondence with numerous published authorities, a thorough search for validated scales that had been developed specifically for the assessment of other intermittent pains and disabilities, such as back pain and arthritis[24,25] and review of measures of quality of life (e.g. SF-36) were also conducted. The use of the SF-36 instrument to measure pain burden was deemed to be too general and not sufficiently disease-specific. Hebert *et al*[26] validated and published a Digestive Disease Quality of Life measure (DDQ15) but this instrument covers patients with many digestive diseases, and has not yet been used in practice. A "pain-volume" scale i.e. days of pain episodes in a month multiplied by the average severity of the reported episodes, was also considered, but again the concept does not include assessment of disability due to pain. Daily/weekly pain and disability diaries utilized in other studies were considered, but concerns about compliance over a long follow-up period in the context of pain episodes that are intermittent in nature, and the potential for inconsistent reporting of their number, frequency and severity precluded the use of patient diaries.

An important criterion for the development of a pain burden assessment instrument (unlike a daily diary) is its ability to measure retrospectively baseline pain-related disability over a period of weeks or months prior to any intervention. This is paramount as patients are often referred to tertiary centers from considerable distances, expecting an immediate intervention.

The closest analogy to pain and disability experienced by SOD Ⅲ patients is in patients with migraine headache. Like SOD pain episodes, onset of migraine headaches is unpredictable, intermittent, and temporarily disabling. Frequency of migraine attacks vary substantially within individuals over time and among individuals. Moreover, migraine can be progressive and evolve to a chronic persistent pain state. The validated migraine disability assessment (MIDAS) questionnaire measures headache-related disability as lost time from paid work or school, household work or non-work activities as a result of headache over the previous 3-mo period. The justification for a 3-mo recall period came from prior studies that illustrated a good correlation between responses from a 90-d recall period and daily patient diaries in this patient population[27,28]. MIDAS, widely used in specialty care and available in 5 other languages, defines 4 levels (Grades) of disability ranging from "little or no disability" to "severely limiting disability"[7,29]. Based on the MIDAS terms and concepts, the Recurrent Abdominal Pain Intensity and Disability (RAPID) instrument was developed in collaboration with Dr. Stewart. This instrument is comprised of 5 questions, completed by the patient, which records time lost from paid work or school, household work or non-work activities as a result of abdominal pain episodes over the previous 3 mo (Table 1). Two additional questions ask about the average frequency and severity of abdominal pain episodes using a 3-mo recall period. The RAPID score is a 90-d summation of missed days, and days where productivity for paid work or school, household activities and non-work activities are reduced by half as a result of abdominal pain episodes. By analogy with the MIDAS instrument, RAPID grade 1 is a score of 0-5 and indicates little or no disability. Grade 2 is a score of 6-10 and indicates mildly limiting disability. Grades 3 and 4 are 11-20 (moderately limiting disability) and 21 or greater (severely limiting disability), respectively.

Table 1 Recurrent abdominal pain intensity and disability Instrument

1 On how many days in the last 3 mo did you miss work or school because of your episodes of abdominal pain? ____ days
2 On how many days in the last 3 mo did you miss work or school because of your episodes of abdominal pain (Do not include days you counted in question 1 where you missed work or school.)? ____ days
3 On how many days in the last 3 mo did you not do household work because of your episodes of abdominal pain? ____ days
4 On how many days in the last 3 mo was your productivity in household work reduced by half or more because of your episodes of abdominal pain (Do not include days you counted in question 1 where you did not do household work.)? ____ days
5 On how many days in the last 3 mo did you miss family, social or leisure activities because of your episodes of abdominal pain? ____ days
6 On how many days in the last 3 mo did you have episodes of abdominal pain (If the abdominal pain lasted more than 1 d, count each day.)? ____ days
7 On a scale of 0-10, on average, how painful were these episodes of abdominal pain? ____

MATERIALS AND METHODS

Feasibility and reliability studies

Prior to using the newly developed RAPID instrument in a large, multicenter, randomized trial, we assessed its feasibility of administration and used Lin's concordance correlation coefficient to measure test-retest reliability[30,31]. Two IRB-approved pilot studies were initiated at the Medical University of South Carolina (MUSC). One study (denoted Pilot Ⅰ) enrolled a total of 55 SOD Ⅲ patients from 6 centers in the United States. Potential participants were recruited through existing hospital referral networks. After providing consent, participants were asked to complete the RAPID questionnaire by telephone prior to receiving any treatment by the respective institution. RAPID was administered by telephone on 2 separate occasions at 2-3 wk intervals (Visits 1 and 2) to assess test-retest reliability and to examine the range of RAPID scores in the sample population. The 2-3 wk interval was chosen based on previous work on migraine headaches in which this period was deemed long enough so that respondents did not recall their answers to the previous interview and short enough to ensure that the recall time period was acceptable[32].

The second pilot study (Pilot Ⅱ) collected the telephone-administered RAPID from 70 consenting adult patients who had undergone a sphincterotomy with a final diagnosis of "papillary stenosis/spasm" at MUSC between January and December 2003. To measure test-retest reliability, RAPID was administered twice by telephone at 2-3 wk intervals, between 6 and 18 mo post-sphincterotomy.

RESULTS

Of the 55 enrollees in Pilot Ⅰ, 55% were female, 85% were Caucasian and the average age was 44 years (SD: 16 years). One enrolled patient did not complete the baseline questionnaire and was excluded from the analysis. At Visit 1, the average RAPID score for the 54 participants was 82 (median: 81.5, SD: 64, range: 0-255); average number of pain-days per 3-mo interval (Question 6 on RAPID) was 70 pain-days (SD: 29, range: 3-90); and 65% reported a pain severity level greater than 5 on a 10-point scale (Question 7 on RAPID; median rating: 7). Table 2 illustrates the number of participants in each RAPID grade at Visit 1 and the descriptive statistics of their RAPID score by grade. Thirty-one patients (57%) were contacted by telephone for the second interview (Visit 2). Table 3 illustrates the RAPID scores for the participants who completed both Visits 1 and 2. The test-retest reliability for the RAPID instrument, as measured by Lin's concordance, was 0.81 ($n = 31$).

Of the 70 enrolled participants in the Pilot Ⅱ study, the average RAPID score at the first visit was 48 (median: 0, SD: 90.76, range: 0-450). The pain episodes (questions 6-7 on RAPID) recorded at the first visit occurred at an

Table 2 Pilot Ⅰ recurrent abdominal pain intensity and disability score by migraine disability assessment grade at Visit 1 (*n* = 54)

RAPID	*n*	mean	SD	Median	Min	Max
MIDAS grade						
1	6	1.17	1.83	0.00	0.00	4.00
2	2	9.00	1.41	9.00	8.00	10.00
3	3	15.00	2.65	14.00	13.00	18.00
4	43	101.70	57.35	103.00	21.00	255.00

RAPID: Recurrent abdominal pain intensity and disability; MIDAS: Migraine disability assessment.

Table 3 Pilot Ⅰ recurrent abdominal pain intensity and disability score by Visit (for participants completing both visits)

RAPID	*n*	mean	SD	Median	Min	Max
Visit						
1	31	80.65	71.82	53.00	0.00	255.00
2	31	75.71	79.25	53.00	0.00	300.00

RAPID: Recurrent abdominal pain intensity and disability.

Table 4 Pilot Ⅱ recurrent abdominal pain intensity and disability score by migraine disability assessment grade at Visit 1 (*n* = 70)

RAPID	*n*	mean	SD	Median	Min	Max
MIDAS grade						
1	40	0.33	1.02	0.00	0.00	4.00
2	4	7.00	1.15	7.00	6.00	8.00
3	2	15.00	0.00	15.00	15.00	15.00
4	24	137.33	109.74	115.50	26.00	450.00

RAPID: Recurrent abdominal pain intensity and disability; MIDAS: Migraine disability assessment.

Table 5 Pilot Ⅱ recurrent abdominal pain intensity and disability score by Visit (for participants completing both visits)

RAPID	*n*	mean	SD	Median	Min	Max
Visit						
1	56	54.46	98.89	0.00	0.00	450.00
2	56	53.63	89.68	3.50	0.00	450.00

RAPID: Recurrent abdominal pain intensity and disability.

average frequency of 31 pain days per 3-mo interval (SD: 39, range: 0-90), with 43% of participants reporting a pain severity level greater than 5 on a 10-point scale. Table 4 illustrates the number of participants in each grade post-treatment and the descriptive statistics of their RAPID score by grade. The RAPID was collected at both interview visits on 56 (80%) participants. RAPID scores at each visit are shown in Table 5. The test-retest reliability as measured by Lin's concordance was 0.95 (n = 56).

DISCUSSION

The RAPID instrument was developed to provide a meaningful measurement of pain severity and related burden experienced by patients with suspected SOD Ⅲ. This patient population is very similar to patients that suffer from severe headaches with respect to the unpredictable, intermittent, and temporarily disabling episodes of pain. For that reason, the development of RAPID relied on the published experience with the MIDAS instrument which illustrated the reliability, validity, and clinical utility of measuring pain-related disability in the previous 3 mo in patients experiencing severe headaches. The present 2 pilot studies demonstrated the reliability of the RAPID disability measure as measured by Lin's concordance. The high levels of concordance between the RAPID scores indicate that the instrument is consistent within individuals when capturing disability due to abdominal pain in the past 3 mo. The higher test-retest concordance value in Pilot Ⅱ may result from participants being assessed post-sphincterotomy with several RAPID scores of 0 (no disability due to abdominal pain in the last 3 mo). The discrepancy in the completion rates between the 2 studies (55% Pilot Ⅰ, 80% Pilot Ⅱ) most likely arises from limited resources at the various participating sites in Pilot Ⅰ to repeatedly contact patients after the initial telephone attempt. Despite this limitation, the instrument is easy to administer and the concordance measurements support its reliability for consistently measuring pain disability in a suspected SOD Ⅲ patient population. Patients with SOD Ⅲ experience severe episodic pain that is highly disabling. In our studies, 80% of the Pilot Ⅰ population and 34% of the Pilot Ⅱ population were classified as having severely limiting disability (Grade 4). The difference in median baseline RAPID scores between the 2 populations (82 and 0 d) provides insight into the validity of the instrument (i.e. ability to measure treatment response) but formal instrument validation studies need to be conducted. Further studies should be conducted to assess how RAPID correlates with other relevant measurements of the impact of episodic abdominal pain in this patient population including quality of life (e.g. SF-36), and depression and anxiety.

SOD is not the only condition associated with intermittent abdominal pain. Further studies are needed to show whether the RAPID instrument is an appropriate measurement tool for pain/burden and the response to treatment in other abdominal conditions, such as irritable bowel syndrome.

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COMMENTS

Background

Patients diagnosed with suspected sphincter of Oddi dysfunction (SOD) suffer from intermittent sphincter dysfunction and related episodes of pain. Measurement tools have not been developed to reliably measure pain severity, frequency of pain episodes, and the impact of pain on function in primary roles for these patients.

Research frontiers

Although several treatments have been utilized to reduce pain and associated disability, measurement tools have not been developed to reliably track patient-related outcomes. In this study, the authors evaluated the feasibility of administration and estimate the test-retest reliability of a newly developed instrument, the recurrent abdominal pain intensity and disability, for the measurement of disability due to episodic abdominal pain in patients with suspected SOD.

Innovations and breakthroughs

A measurement tool that reliably measures disability due to pain has the potential to improve understanding of the impact of treatments. This manuscript reports the development and reliability testing of an instrument that measures pain severity and related burden experienced by patients with suspected SOD.

Applications

If shown to be reliable, then future studies can assess the clinical usefulness of this instrument in suspected SOD patients and potentially other similar abdominal conditions such as irritable bowel syndrome.

Peer review

Professor Durkalski *et al* have performed an interesting pilot study in order to evaluate abdominal pain and disability in patients with suspected sphincter of Oddi dysfunction.

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BRIEF ARTICLE

Decreased serum essential and aromatic amino acids in patients with chronic pancreatitis

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Abstract

AIM: To investigate the influence of chronic pancreatitis (CP) on serum concentrations of amino acids.

METHODS: Thirty-five male patients with alcoholic CP and 21 healthy male subjects were examined. Serum concentrations of amino acids were assayed by ion-pair high-performance liquid chromatography with mass detection.

RESULTS: Serum glutamate concentration was increased in CP patients as compared to controls. In contrast, serum concentrations of glutamine, histidine, tyrosine, proline, tryptophan and threonine were significantly decreased in CP patients. A trend towards decreasing concentrations of serum lysine, alanine, methionine and valine as well as for total serum amino acids was observed. The sum of aromatic and the sum of essential amino acid concentrations were significantly lower in CP patients than in controls.

CONCLUSION: CP leads to decreased serum concentrations of several amino acids, such as essential and aromatic serum amino acids, most likely due to decreased exocrine function.

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Key words: Amino acids; Body mass index; Chronic pancreatitis; Maldigestion; Malnutrition

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INTRODUCTION

Chronic pancreatitis (CP) is characterized by progressive depletion of acinar structures and subsequent replacement of endo- and exocrine pancreatic cells with fibrous tissue[1]. In the course of CP, pancreatic exocrine insufficiency usually develops earlier than endocrine insufficiency[2]. The secretion and, consequently, the activity of digestive enzymes decrease gradually, resulting in maldigestion. CP often causes weight loss due to reduced food intake (caused by pain and/or persistent alcohol abuse) and maldigestion of proteins as well as complex lipids and carbohydrates[3].

Malnutrition is thus common in patients with CP and its severity is one of the major factors predicting complications and outcome of the disease[1,3]. In a recent study, we demonstrated that several nutritional parameters are significantly decreased in patients with advanced CP[4]. It has been reported that protein calorie malnutrition is strongly associated with decreased serum concentrations of amino acids[5].

Amino acids are important substrates for: (1) protein synthesis; (2) glucose and urea synthesis; (3) energy production; and (4) synthesis of biologically active substances (e.g. nitric oxide, catecholamines, thyroid hormones), creatinine and carnitine[6]. They also play an important role in specialized metabolic and regulatory functions such as modulation of the carcinogenic pathway (proline)[7], regulation of insulin and glucagon secretion (leucine, lysine)[8,9], or synaptic maintenance and plasticity (glutamate)[10]. Moreover, some amino acids are cell signaling molecules and regulators of gene expression[6]. Thus, an adequate serum concentration of amino acids is necessary for the maintenance of human health and prevention of disease. The serum amino acid concentration depends on their influx to the blood (from food and/or muscle) and the capacity of the body to dispose of them (*via* uptake from the blood by various organs). It is influenced by both hormonal activity (mainly insulin secretion) and food intake. Insulin stimulates uptake and inhibits release of amino acids from muscle[11]. Recently, we reported that serum insulin concentration decreases in patients with CP[12,13]. Collectively, the above data suggest that maldigestion of protein and changes in serum insulin concentration during the course of CP may, in theory, have a significant impact on serum amino acid concentration. These processes, in turn, may influence the course of the disease. A recent study demonstrated significant abnormalities in serum amino acid profile in patients with acute pancreatitis[14,15]. In particular, the serum concentrations of arginine, citrulline, ornithine and glutamine were significantly decreased compared to the level after recovery. These changes may influence the inflammatory events and organ function in the course of acute pancreatitis[15]. Moreover, it has been suggested that supplementation of selected amino acids can be of value in severe acute pancreatitis[15]. Several studies assessing the serum amino acid concentration in patients with CP have been reported[16-23]. However, there is no clear consensus regarding the effect of CP on serum amino acid concentration. These reports concentrated mainly on the amino acid consumption test as a method of detecting exocrine pancreatic insufficiency. Most of these papers showed that total serum amino acid concentrations in patients with CP did not significantly differ from controls[16,18-23]. However, Dzieniszewski *et al*[17] showed that patients with advanced CP have lower total serum amino acid concentrations than control subjects. These contradictory results and the fact that most authors except Dzieniszewski *et al*[17] used the ninhydrin method, which is less accurate, for determination of total serum amino acid concentration, led us to revaluate serum amino acid concentration in patients with advanced CP using high-performance liquid chromatography with mass detection, a much more reliable method for measurement of amino acid concentration in biological samples.

MATERIALS AND METHODS

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Medical University of Gdansk Ethics Committee. All patients signed an informed consent form for this investigation. Of the patients treated for CP in the Department of Gastroenterology and Hepatology, Medical University of Gdansk during 2006-2008, we selected 35 males aged 33-72 years (mean age, 50 ± 10 years), with a history of alcoholic CP. All patients included in the study met diagnostic criteria for CP[24]. The diagnosis was based on clinical symptoms and typical results on imaging studies. Most patients included in the study were found to have the following abnormalities: pancreatic parenchymal calcifications, pancreatic duct stones, irregular dilation and/or stenosis of the pancreatic duct, fibrosis and parenchymal inhomogeneity. As determined by the results of endoscopic retrograde pancreatography (ERP), 28 patients displayed marked (grade 5 according to the Cambridge classification), 5 moderate (grade 4) and 2 mild (grade 3) stage of disease[25]. Patients with CP who had an exacerbation of the disease (based on clinical symptoms accompanied by significantly increased serum amylase and lipase, and urine amylase) and patients with liver cirrhosis were excluded from the study. Patients with CP were moderate or heavy drinkers. Twenty-one healthy male volunteers aged 23-61 years (mean age, 34 ± 13 years) formed the control group. Selected laboratory values in both groups are presented in Table 1. The body mass index (BMI) was calculated for all study participants. Fasting blood samples, from patients and healthy controls, were collected at 8 a.m.

Serum amino acid concentrations were determined using liquid chromatography/mass spectrometry (LC/MS) as described recently[26]. Briefly, an aliquot of plasma (0.4 mL) was deproteinized with 0.4 mL of 10% trichloroacetic acid (TCA). The tubes were centrifuged at 4℃, 12000 × *g* for 5 min. The supernatant was collected and TCA removed by diethyl ether extraction followed by freeze-drying. The material obtained was dissolved in 0.1 mL of 10 mmol/L nonafluoropentanoic acid in H_2O and analyzed by ion-pair high-performance liquid chromatography with mass detection. Chromatographic separation was performed using a 3 μm Hypersil BDS 150 mm × 2.0 mm column. The mobile phase was delivered at 0.2 mL/min in a gradient from 0% to 60% acetonitrile in 12 min. The mass detector (Thermo-Finnigan LCQ Advantage, Waltham, MA, USA) with electrospray (ESI) ion source was operated in positive MS2 mode for detection of amino acids with the collision energy setting at 25%. Electrospray cone voltage was set at 4.5 kV and the heated capillary temperature was 275℃. Sheath gas flow was set for 35 arbitrary units. Post-column sheet flow of methanol with 0.05% formic acid at 0.2 mL/min was used

Table 1 Selected laboratory values in patients with chronic pancreatitis and control subjects (mean ± SD)

	Control	Chronic pancreatitis	Statistical significance
No. of patients	21	35	
Age (yr)	34 ± 13	50 ± 10	< 0.01
Body weight (kg)	76 ± 4	64 ± 10	< 0.01
Body mass index (kg/m^2)	25 ± 2.7	22 ± 3.4	< 0.01
Serum total protein (g/L)	76 ± 4	72 ± 8	< 0.05
Serum albumin (g/L)	46 ± 3.6	42 ± 8	< 0.05
Serum triacylglycerol (mg/dL)	107 ± 30	119 ± 43	NS
Serum cholesterol (mg/dL)	195 ± 26	175 ± 44	< 0.05
Glucose (mmol/L)	5 ± 0.64	7.7 ± 2.8	< 0.01
Insulin (μU/mL)	12 ± 5.2	9.1 ± 4.4	< 0.05
Homeostasis model assessment	2.7 ± 1.3	2.6 ± 1.6	NS
Leptin (ng/mL)	6.6 ± 2.9	3.9 ± 2.5	< 0.01
Hemoglobin (mg/dL)	15 ± 2.9	13 ± 1.8	< 0.01
Blood urea nitrogen (mg/dL)	11 ± 2.8	14 ± 6	NS
Creatinine (mg/dL)	0.9 ± 0.13	0.89 ± 0.22	NS
Serum amylase (U/L)	54 ± 31	65 ± 35	NS
Urine amylase (U/L)	174 ± 66	344 ± 336	< 0.05
Serum lipase (U/L)	28 ± 16	69 ± 75	< 0.05
Serum alanine aminotransferase (U/L)	21 ± 9	38 ± 38	< 0.05
Serum aspartate aminotransferase (U/L)	19 ± 5	41 ± 45	< 0.05
Serum alkaline phosphatase (U/L)	80 ± 28	104 ± 65	NS
Serum-glutamyl transpeptidase (U/L)	26 ± 14	112 ± 152	< 0.05

NS: Not significant.

to improve ionization efficiency. The identity of individual amino acids was confirmed by the similarity of molecular weights, fragmentation pattern and chromatographic retention time.

Serum leptin and insulin concentrations were analyzed as previously described[12,13]. Serum alanine aminotransferase, aspartate aminotransferase, amylase, lipase, alkaline phosphatase, γ-glutamyl transpeptidase, glucose, triacylglycerols, total cholesterol, hemoglobin, total protein and albumin, blood urea nitrogen (BUN), creatinine as well as urine amylase were analyzed using standard procedures. The homeostasis model assessment score (HOMA score) was used to assess insulin resistance as described by Matthews *et al*[27].

Statistical analysis

Statistical analysis was performed using Microsoft Excel. The statistical significance of differences observed between patients with CP and controls was assessed using the two-tailed *t*-test.

RESULTS

Table 1 shows selected nutritional and laboratory parameters in patients with CP and controls. Body weight, BMI and hemoglobin concentration were significantly lower in patients with advanced CP as compared to control subjects. Moreover, CP patients had lower serum concentrations of total protein and albumin. Serum total cholesterol concentration was lower (P <0.05) in patients with CP compared to controls. Serum glucose concentration was higher in patients with CP. BUN and creatinine concentrations were essentially similar in CP patients and control subjects. Table 1 also shows that serum insulin concentrations in patients with CP were significantly lower than in control subjects. The changes in serum insulin concentration observed in patients with CP essentially paralleled changes in serum leptin concentration (Table 1). Table 1 also shows that no significant differences were observed in the HOMA score between patients with CP and control subjects. We also studied serum amylase, lipase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and γ-glutamyl-transpeptidase activity in patients with CP and control subjects. Serum lipase, alanine aminotransferase, aspartate aminotransferase and γ-glutamyl-transpeptidase activity were slightly elevated in patients with CP. Urine amylase activity in patients with CP was also higher than in control subjects (Table 1).

Table 2 Serum concentrations of amino acids in patients with chronic pancreatitis and control subjects (mean ± SD, range)

Amino acid (μmol/L)	Control	Chronic pancreatitis	Statistical significance
Threonine	125 ± 26	99 ± 33	0.0037
Tryptophan	67 ± 21	52 ± 16	0.0043
Tyrosine	59 ± 21	45 ± 17	0.0071
Histidine	48 ± 16	37 ± 14	0.0097
Proline	201 ± 82	157 ± 61	0.026
Glutamate	46 ± 24	66 ± 39	0.034
Glutamine	307 ± 97	256 ± 86	0.044
Lysine	114 ± 36	95 ± 36	0.059
Alanine	605 ± 128	535 ± 158	0.093
Methionine	15 ± 5.8	12 ± 5.7	0.13
Valine	93 ± 28	84 ± 23	0.17
Isoleucine	34 ± 11	31 ± 8	0.3
Glycine	154 ± 52	142 ± 46	0.37
Arginine	67 ± 37	61 ± 31	0.55
Cysteine	119 ± 42	112 ± 43	0.56
Serine	43 ± 13	41 ± 14	0.57
Asparagine	51 ± 22	47 ± 24	0.58
Leucine	67 ± 20	70 ± 16	0.62
Aspartate	22 ± 8	23 ± 11	0.67
Phenylalanine	57 ± 20	58 ± 24	0.87

Table 2 shows the serum concentrations of amino acids in control subjects and patients with advanced CP. Surprisingly, the serum concentration of glutamate, as opposed to other amino acids, was significantly increased in patients with CP. In contrast, there were significant decreases in serum glutamine, histidine, tryptophan, tyrosine, proline and threonine in patients with CP. A trend towards decreased concentrations of serum alanine, methionine, valine and lysine was also observed, however, the differences did not reach statistical significance. We did not find significant decreases in the serum concentrations of other amino acids such as: glycine, isoleucine, cysteine, serine, leucine, phenylalanine, arginine, aspartate and asparagine (Table 2). The total serum amino acid concentration was slightly lower in CP patients than in healthy subjects, how-

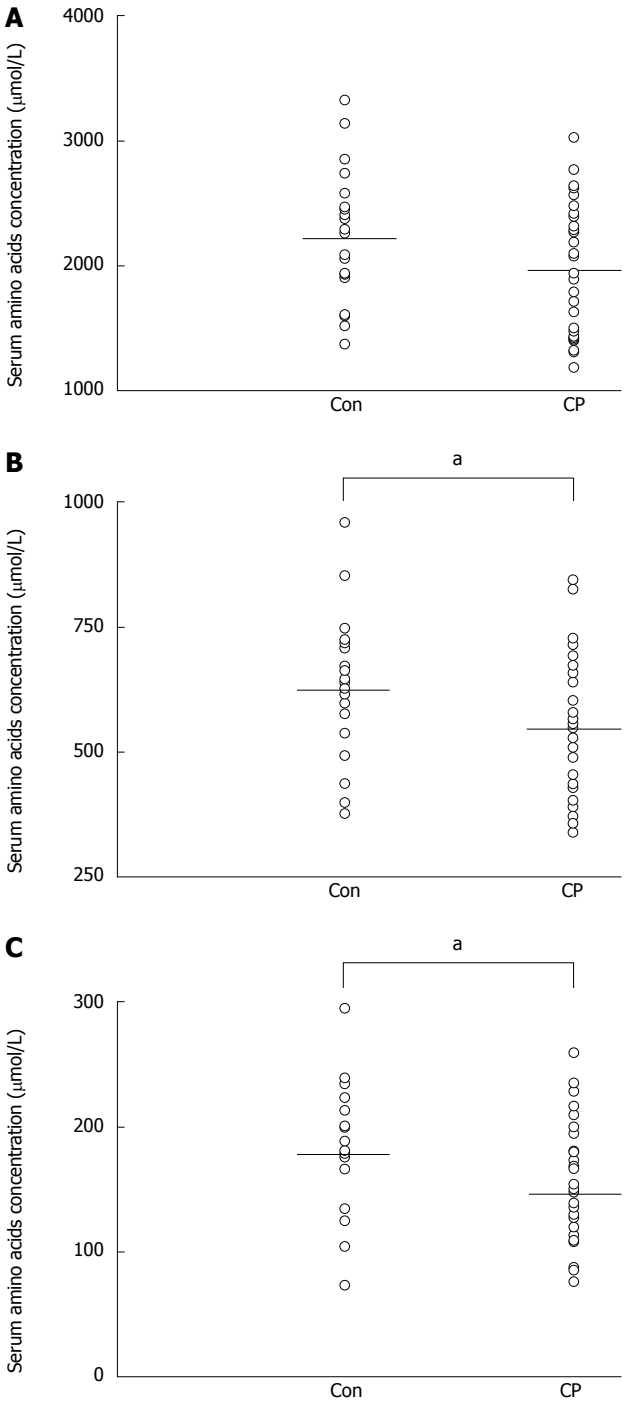

Figure 1 Concentrations of total serum amino acids (A), sum of serum essential amino acids (B), and sum of serum aromatic amino acids (C) in control subjects and patients with chronic alcoholic pancreatitis. Results are shown as mean (illustrated by the line). A: $P = 0.06$; B and C: $^{a}P < 0.05$, serum amino acid concentration *vs* patients and controls. Con: Control subjects; CP: Patients with chronic alcoholic pancreatitis.

ever, the differences did not reach statistical significance (Figure 1A). A significant decrease in the sum of selected amino acids such as essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine) (Figure 1B) and aromatic (phenylalanine, tryptophan, tyrosine) (Figure 1C) amino acid concentration in the serum of patients with advanced CP was found.

DISCUSSION

We have demonstrated, for the first time, higher serum glutamate concentration, and lower serum glutamine, histidine, tryptophan, tyrosine, proline and threonine concentrations in patients with CP as compared to healthy subjects. The serum concentrations of alanine, methionine, valine and lysine, were lower in CP patients, as was the total serum amino acid concentration, but the differences did not reach statistical significance (Figure 1A). Moreover, the results presented here suggest that the measurement of selected amino acids such as essential and aromatic amino acids could help to assess exocrine pancreatic insufficiency. Our measurements of total serum amino acid concentrations in patients with CP are generally consistent with the results of previous studies[16,18,21].

Since the secretion of digestive enzymes gradually decreases in the course of CP, resulting in protein maldigestion, a decrease in serum concentrations of some amino acids would be expected. It is not clear why serum glutamate concentration is higher in patients with CP than in control subjects. The serum glutamate concentration was also increased, whereas serum glutamine concentration decreased during the first 5 d after the onset of symptoms in patients with acute pancreatitis[15]. This phenomenon is probably due to the increase in intracellular conversion of glutamine to glutamate[15]. In patients with CP, the ratio of serum glutamine to glutamate was 7:1 and was 4:1 in controls (Table 2). It is therefore likely that increased intracellular metabolism of glutamine to glutamate is also present during the course of CP. Another possible explanation for the increase in serum glutamate concentration could be an increase in intracellular conversion of 2-oxoglutarate to glutamate or inhibition of glutamate oxidation.

The significant decrease in serum concentration of some amino acids, especially aromatic and essential amino acids suggests that protein maldigestion due to exocrine insufficiency could be one possible mechanism leading to this deficit. Among pancreatic proteases, chymotrypsin catalyzes the hydrolysis of only those peptide bonds in which the carboxyl (carbonyl) group is contributed by aromatic amino acids. Thus, one explanation of decreased concentration of aromatic amino acids would be that chymotrypsin is the most deficient of all pancreatic proteases during the course of CP. The unchanged concentration of phenylalanine and significant decrease in tyrosine (aromatic amino acids) suggest that the activity of phenylalanine hydroxylase is diminished, resulting in an increased serum phenylalanine to tyrosine ratio [1.0 ± 0.4 in control subjects *vs* 1.4 ± 0.7 ($P < 0.05$) in patients with CP]. Since tetrahydrobiopterin (BH_4), as a cofactor of phenylalanine hydroxylase, is necessary for the conversion of phenylalanine to tyrosine, one can suppose that the intracellular BH_4 concentration is diminished in patients with CP. Diminished phenylalanine hydroxylase activity, and consequently a lower rate of conversion of phenylalanine to tyrosine, should lead to an increase in serum phenylalanine. This was not the case. It is possible

that the impact of diminished phenylalanine hydroxylase activity on the serum concentration of phenylalanine is counterbalanced by the effects of protein malnutrition in the course of CP. However, this is just one possible explanation for this phenomenon and other mechanisms are not excluded.

Another mechanism affecting the serum concentration of amino acids could be an increased catabolism of skeletal muscle protein. CP, being an inflammatory disease, can shift muscle protein from anabolism to catabolism. Consequently, the decrease in the serum concentration of some amino acids due to protein maldigestion could be counterbalanced by release of amino acids from muscle. This is probably the reason why the serum concentrations of several amino acids remain unchanged (or only slightly decreased) despite protein maldigestion during the course of CP. The diminished serum concentration of some amino acids (glutamine, histidine, tryptophan, proline and tyrosine) may have profound effects on the rate of protein synthesis. This may explain the lower concentration of total serum protein (Table 1). Moreover, lower serum concentration of some amino acids may influence the course of CP. Therefore, it is reasonable to postulate that supplementation of selected amino acids could be of value in advanced CP.

The reason for the decrease in serum histidine concentration in patients with CP is unknown. However, one can suppose that the decrease of this amino acid could be disadvantageous because histidine is a precursor of histamine (besides being a substrate for protein synthesis). Consequently, decreased histidine concentration can lead to a decrease in histamine concentration. It is well known that binding of histamine to its receptor located on the surface of parietal cells (H2 receptor) stimulates gastric acid secretion. Diminished histamine production can lead to diminished protein digestion. Moreover, histidine inhibits production of proinflammatory cytokines by human monocytes[28]. It is therefore likely that diminished histidine concentration can promote inflammation. Collectively, the decrease in serum histidine concentration may worsen the course of CP.

The results reported by Pitkänen *et al*[29] indicate that the decrease in serum amino acid concentrations occurs in groups of subjects who are older than 60 years, but not in individuals under 60. Thus, it is unlikely that the different age of controls (34 ± 13 years old men) and CP patients (50 ± 10 years old men) could be a reason for the results presented in this paper.

Finally, it should be emphasized that the application of tandem mass spectrometry for analysis of non-derivatized amino acids used in this study, represents a unique approach with numerous advantages as compared to the techniques used so far[16,18-23]. Our technique has better sensitivity and specificity. It requires only a small volume of serum and limited sample preparation. This procedure can be highly recommended not only for research applications but also for clinical use.

In conclusion, the results reported here indicate that advanced CP interferes with serum amino acid concentrations. Surprisingly, serum glutamate concentration increases during the course of CP, whereas serum concentrations of glutamine, histidine, tryptophan, tyrosine, proline and threonine were significantly lower in patients with CP. A tendency towards a decreased concentration of total serum amino acids was observed without reaching statistical significance. A significant decrease in selected amino acids such as essential and aromatic serum amino acid concentrations in patients with advanced CP was found. Decreased serum concentration of some amino acids is probably due to protein maldigestion. However, the effect of malnutrition and systemic chronic inflammation on serum amino acids cannot be excluded. It is tempting to speculate that, first, accurate measurement of selected amino acids such as aromatic and/or essential amino acids could help assess pancreatic exocrine insufficiency in the course of advanced CP, and, second, that supplementation of selected amino acids could be of therapeutic value in advanced CP.

COMMENTS

Background

In the course of chronic pancreatitis (CP), the secretion of digestive enzymes gradually decreases, resulting in maldigestion. In the advanced stage of disease, in turn, maldigestion of proteins could lead to a decrease in serum amino acid concentrations.

Research frontiers

Malnutrition is common in patients with CP and its severity is a factor predicting complications and outcome of the disease. However, there is no clear consensus regarding the effect of CP on serum amino acid concentration. In this study, the authors demonstrate that CP is associated with a decrease in the serum concentration of essential and aromatic amino acids, most likely due to decreased pancreatic exocrine function.

Innovations and breakthroughs

This is the first study to report that serum glutamate concentration increases and serum essential and aromatic amino acid concentrations decrease in the course of CP. The decrease in amino acids concentration is probably the result of protein maldigestion typical in advanced CP. A new method: ion-pair high-performance liquid chromatography with mass detection was used to assay non-derivatized amino acids.

Applications

Measurement of aromatic and essential amino acids could help to assess pancreatic exocrine insufficiency in the course of advanced CP. Moreover, supplementation of selected amino acids could be of therapeutic value in advanced CP.

Terminology

Essential amino acids are those that have to be supplied in the diet as they cannot be synthesized in the human body. Aromatic amino acids contain aromatic structures in the side chain (i.e. phenylalanine, tyrosine, tryptophan). Ion-pair high-performance liquid chromatography with mass detection - an analytical technique based on the separation of molecules and selective detection of its ions.

Peer review

The authors describe the decreased essential and aromatic amino acid content in the sera of patients with chronic pancreatitis and give several possible explanations. The manuscript is well written and conveys the message appropriately.

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BRIEF ARTICLE

Exercise-provoked esophageal motility disorder in patients with recurrent chest pain

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Abstract

AIM: To investigate the relationship between exercise-provoked esophageal motility disorders and the prognosis for patients with chest pain.

METHODS: The study involved 63 subjects with recurrent angina-like chest pain non-responsive to empirical therapy with proton pump inhibitor (PPI). In all, a coronary artery angiography, panendoscopy, 24-h esophageal pH-metry and manometry, as well as a treadmill stress test with simultaneous esophageal pH-metry and manometry monitoring, were performed. Thirty-five subjects had no significant coronary artery lesions, and 28 had more than 50% coronary artery narrowing. In patients with hypertensive esophageal motility disorders, a calcium antagonist was recommended. The average follow-up period was 977 ± 249 d.

RESULTS: The prevalence of esophageal disorders, such as gastroesophageal reflux or diffuse esophageal spasm, was similar in patients both with and without significant coronary artery narrowing. Exercise prompted esophageal motility disorders, such as a decrease in the percentage of peristaltic and effective contractions and their amplitude, as well as an increase in the percentage of simultaneous and non-effective contractions. In 14 (22%) patients the percentage of simultaneous contractions during the treadmill stress test exceeded the value of 55%. Using Kaplan-Meier analysis and the proportional hazard Cox regression model, it was shown that the administration of a calcium channel antagonist in patients with such an esophageal motility disorder significantly decreased the risk of hospitalization as a result of a suspicion of acute coronary syndrome after the 2.7-year follow-up period.

CONCLUSION: In patients with chest pain non-responsive to PPIs, a diagnosis of exercise-provoked esophageal spasm may have the effect of lowering the risk of the next hospitalization.

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Key words: Noncardiac chest pain; Esophageal motility; Calcium antagonist; Exercise; Provocative test; Follow-up

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INTRODUCTION

Chest pain, including its angina-like form, is a common problem in health care because of its frequency, recurrence, the utilization of resources consistent with costs of medical procedures, and diagnostic difficulties[1-7]. There are many causes of chest pain and in respective patients they may coexist, and even overlap[8,9]. For this reason, chest pain in patients with coronary artery disease (CAD), including that which is exercise-provoked, may originate not only from the myocardium, but also from noncardiac sources. The most frequent causes of noncardiac chest pain (NCCP) are diseases of the upper part of the digestive tract, such as gastroesophageal reflux disease (GERD), esophageal motility disorders, and gastric and duodenal ulcer disease[2]. Their prevalence in patients both with and without cardiovascular diseases seems to be similar[9,10], although some authors have reported a lower percentage of esophageal disorder diagnoses in patients with CAD than in individuals without CAD[11,12].

The importance of esophageal motility disorders in exercise-provoked, angina-like chest pain pathogenesis is still uncertain[5]. Pharmacological provocative tests have not been diagnostically useful[1,5], whereas the use of exercise in order to provoke symptoms in patients with NCCP suspected of being related to esophageal motility disorders has not been sufficiently investigated[13,14]. However, it is known that exercise can induce myocardial ischemia as well as alterations in esophageal motility and gastroesophageal reflux (GER), and in such a way reproduce chest pain[14]. Therefore, the simultaneous monitoring of esophageal function and electrocardiography (ECG) during a treadmill stress test, besides potentially shortening the diagnostic procedure time, seems to have additional benefits in providing an opportunity to carry out cardiac and esophageal investigations at the same time. It is also of great importance to exclude the possibility of life-threatening conditions (by revealing potential ischemic ECG changes), and to simulate the circumstances of angina-like (effort-provoked) chest pain appearance.

The aim of this study was to compare the effect of a treadmill stress test on esophageal motility in patients with and without angiographic signs of CAD. The objective was also to evaluate the influence of recommended treatment on the risk of hospitalization due to a suspicion of acute coronary syndrome over a 2.7-year follow-up period. To the author's knowledge, this is the first report concerning this aspect of recurrent angina-like chest pain.

MATERIALS AND METHODS

The analysis was carried out in 63 consecutive patients hospitalized in order to perform scheduled diagnostic procedures because of recurrent angina-like chest pain, defined as precordial symptoms induced by exercise and receding after rest or the taking of nitroglycerine. The symptoms were diagnosed as being noncardiac in origin by the cardiologist, who was not related to the researcher, and who had referred his patients to a gastroenterologist because of recurrent, angina-like symptoms. These symptoms had been resistant to treatment orientated towards coronary reserve improvement (in patients with CAD) and empirical therapy with proton pump inhibitors (PPIs). In all individuals, a coronary angiography was performed prior to all gastroenterological procedures. The studied group consisted of 28 (44%) patients who had significant angiographic changes, with > 50% of the coronary vessels being narrowed but not suitable for revascularization, and 35 (66%) subjects having normal coronary angiograms or no obstructive lesions. The purpose of referring patients with CAD to a gastroenterologist was to diagnose possibly overlapping gastroenterological and cardiological chest pain causes. This was of particular concern in subjects with CAD who did not present significant ST interval ECG changes accompanying chest pain occurrence during an ambulatory stress test, or who did not suffer from esophageal symptoms other than chest pain. Patients with CAD and those without significant coronary artery narrowing did not differ according to the majority of demographic and clinical data (Table 1).

The inclusion criteria were as follows: (1) aged between 40 and 70 years; (2) prior coronarography performance; (3) angina-like chest pain to the degree of class Ⅱ or Ⅲ according to the Canadian Cardiovascular Society (CCS); and (4) persistent symptoms despite adequate anti-angina treatment and therapy for at least 1 mo with a double dose of omeprazole. The exclusion criteria were as follows: (1) changes in the ECG which made it impossible to estimate ischemic signs (e.g. left bundle branch block or pre-excitation syndrome); and (2) the taking of medicines which would affect gastric acid secretion or digestive tract motility up to 2 wk prior to the examination, with the exception of the *ad hoc* use of nitroglycerine tablets.

The well-being of all 63 individuals, diagnosed during 2004-2007, was followed in outpatient cardiology clinics. The mean observation period between the day of gastroenterological diagnostic performance and 31 September 2008, when the follow-up period finished, amounted to 977 ± 249 d. The data concerning date, duration and cause of eventual hospitalization were obtained from the National Health Foundation (NHF) on the basis of social security numbers. Fortunately, the standard primary endpoints, such as death or myocardial infarction, did not occur within the observation period, so hospitalization due to suspected acute coronary syndrome was established as the end-point of the analysis. The author had no influence on the decision of patients' hospitalization. The mean time before the first hospitalization due to suspected acute coronary syndrome was 437 ± 356 d (in hospitalized individuals). None of the patients died during the course of their hospitalization and in none were observed signs of myocardial infarction (i.e. an increase in troponin level). None of the subjects needed an emergency coronarography or percutaneous coronary intervention (PCI).

Gastroenterological work-up at the beginning of the study

In all subjects the medical history, physical examination,

Table 1 Comparison of demographic and clinical data for 63 analyzed patients divided in relation to the presence of significant (> 50%) coronary artery narrowing and features of exercise-provoked esophageal spasm *n* (%)

Parameter	CAD- (*n* = 35)		CAD+ (*n* = 28)	
	EPES (+) (*n* = 8, 23%)	EPES (-) (*n* = 27, 77%)	EPES(+) (*n* = 6, 21%)	EPES (-) (*n* = 22, 79%)
Male gender	2 (25)	8 (30)	3 (50)	10 (45)
Age (yr)	56.3 ± 10.0	52.9 ± 7.3	54.5 ± 11.1	54.8 ± 8.8
Body mass index (kg/m^2)	31 ± 3.5	27.6 ± 4.8	30.2 ± 6.2	27.7 ± 4.0
Waist to hip ratio	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
> 50% narrowing of coronary vessels	0	0	6 (100)	22 (100)
Smoking	0	3 (11)	1 (16)	1 (5)
History of myocardial infarction	0	0	1 (16)	6 (27)
History of hypertension	3 (38)	10 (38)	3 (50)	8 (36)
History of diabetes (*n*)	1 (13)	1 (4)	2 (33)	4 (18)
Blood glucose (mg/dL)	106 ± 18.7	95.5 ± 13.7	91 ± 6.3	116.7 ± 22.2
LDL cholesterol (mg/dL)	127 ± 15.8	125.3 ± 44.2	116.6 ± 38	128 ± 34.3
Triglycerides (mg/dL)	120 ± 22	134.4 ± 58.3	154.8 ± 69.2	181.4 ± 104
Angina-like chest pain during the stress test	4 (50)	2 (15)[c]	4 (67)	4 (18)
Horizontal ST interval depression ⩾ 1 mm	6 (75)	15 (56)	4 (75)	8 (36)
Duration of stress test (s)	477 ± 145	408 ± 158[a]	501 ± 186	607 ± 175
Echocardiographically determined EF (%)	60 ± 7	63 ± 3	65 ± 3	69 ± 8
Helicobacter pylori infection	4 (50)	12 (44)	2 (33)	11 (50)
Esophagitis	0	4 (15)	2 (33)	2 (9)
Pathological acid GER	0	6 (22)	2 (33)	5 (23)
Acid epGER	0	2 (7)	6 (100)	0
Calcium antagonist recommendation	8 (100)	20 (74)	6 (100)	16 (73)
Event-free period (d)	904 ± 352[a]	866 ± 356	887 ± 374[a]	541 ± 400
Hospitalization	0	10 (37)	3 (50)	11 (50)
Time to first hospitalization (d)[1]	494 ± 372	226 ± 124[b]	604 ± 335	309 ± 210

[1]Calculated only for hospitalized patients. [a]$P < 0.05$, significance level for differences in relation to CAD+EPES- group; [b]Significance level for differences in relation to CAD+EPES+ group; [c]In relation to CAD-EPES+ group. CAD: Coronary artery disease; CAD-: Patients without significant coronary artery narrowing; CAD+: Patients with significant (> 50%) coronary artery narrowing; EPES: Exercise-provoked esophageal spasm; GER: Gastroesophageal reflux; epGER: Exercise-provoked GER; IEM: Ineffective motility disorders; EF: Ejection fraction; LDL: Low-density lipoprotein.

24-h esophageal pH-metry and manometry, and a pan-endoscopy with gastric and esophageal biopsy (after the removal of pH-metric and manometric probes) were performed. An investigation of ambulatory esophageal function was conducted by means of a multi-use antimony probe (Synetics Medical AB, Sweden), and a manometry catheter (Synectics, Medtronic) with three pressure sensors (separated by 5 cm) and a Synectic Digitrapper. An esophageal pH-metric sensor, after calibration to pH 7 and 1, and following nasal and esophageal intubation, was positioned 5 cm above the monometrically-determined lower esophageal sphincter (LES). A pressure probe was placed through the other nostril at 3, 8 and 13 cm above the LES. During esophageal pH and pressure monitoring, all patients eventually recorded the occurrence of symptoms. None of the patients reported disturbances in nasal breathing. The following day, during continuous esophageal pH-metry and manometry monitoring, a treadmill stress test on a running track was carried out at approximately 7 am, after patients had become accustomed to the pH-metric and manometric probes. The exercise test was performed using a device manufactured by Schiller, Switzerland, according to the Bruce protocol (the speed and gradient of the running track were increased every 3 min, respectively: 2.7, 4, 5.5, 6.8 km/h; and by 10°, 12°, 14° and 16°).

After the stress test, the data obtained during esophageal function monitoring were downloaded to a PC and analyzed using GASTROSOFT software. Standard parameters of esophageal pH-metry and manometry were calculated according to the software settings. As the normal values of exercise-induced esophageal disorders were unknown, the researcher's own definitions were proposed as follows. GER in 24-h pH-metry was defined as the time that intraesophageal pH < 4 exceeded 4.5% of the total duration of the examination. Gastroesophageal acid reflux provoked by exercise (epGER) was defined as a decrease in esophageal pH < 4 for more than 10 s during an exercise stress test. Simultaneous contractions were determined as being contractions when the delay between adjacent transducers separated by 5 cm was less than 0.25 s (with a propagation speed higher than 20 cm/s). Peristaltic contractions were defined as the increase of esophageal pressure in which the delay between contractions beginning on the adjacent transducers was in the range 0.25-7 s. Effective contractions were defined as complete (detected by all three sensors) peristaltic contractions with adequate amplitude. This last definition, according to the GASTROSOFT settings, is represented by the following: 20 mmHg at 13 cm above the LES, 25 mmHg at 8 cm above the LES, and 30 mmHg at 3 cm above the LES[15]. Ineffective esophageal motility (IEM) was defined as more than 30% of water swallows (during a meal) provoking contractions with an amplitude of less than

Table 2 Comparison of manometric parameter values during the treadmill stress test and during the 24-h monitoring period in patients with and without significant (> 50%) coronary artery narrowing

Parameter	CAD- (n = 35)			CAD+ (n = 28)		
	24 h	ext	P	24 h	ext	P
Contractions per minute	1.5 ± 0.8	2.7 ± 1.7	0.001	1.9 ± 1.5	2.7 ± 2.2	0.04
Peristaltic contractions (%)	64.3 ± 9.6	47.2 ± 8.4	0.001	67.7 ± 28.6	56.9 ± 18.7	0.03
Complete peristalsis (%)	43.9 ± 15.3	35.6 ± 18.5	0.140	51.9 ± 10.9	49.3 ± 26.5	0.70
Reduced peristalsis (%)	18.3 ± 13.2	16.2 ± 14.8	0.630	13.3 ± 6.3	10.1 ± 7.2	0.30
Interrupted peristalsis (%)	37.8 ± 10.8	40.1 ± 30.0	0.690	34.8 ± 8.5	36.8 ± 24.1	0.70
Simultaneous/mixed contractions (%)	35.7 ± 9.6	49.3 ± 23.8	0.010	32.3 ± 8.6	43.0 ± 18.7	0.02
Complete contractions (%)	16.6 ± 5.2	35.6 ± 28.5	0.010	14.9 ± 4.4	30.6 ± 30.1	0.03
Simultaneous contractions (%)	83.4 ± 5.3	71.1 ± 28.7	0.040	85.1 ± 4.9	69.4 ± 30.1	0.03

CAD: Coronary artery disease; CAD-: Patients without significant coronary artery narrowing; CAD+: Patients with significant (> 50%) coronary artery narrowing; 24 h: Examination within one whole day; ext: Examination during the exercise test.

30 mmHg and/or with a non-transmission rate to the distal esophagus[16]. In patients with a diagnosis of hypertensive esophageal motility disorders (nutcracker esophagus or diffuse esophageal spasm), a calcium channel antagonist - amlodipine 1 × 10 mg or diltiazem retard 2 × 120-180 mg, depending on the subject's resting heart rate - was recommended.

Ethics

The study protocol was approved by the local Bioethics Committee of Nicolaus Copernicus University in Toruń and Ludwik Rydygier Collegium Medicum in Bydgoszcz, Poland. All subjects gave their informed consent prior to the start of the investigation. All procedures have been conducted in compliance with the Declaration of Helsinki.

Statistical analysis

Statistical analysis was conducted using a licensed version of statistical software STATISTICA PL 8.0 for Windows. The results were mainly presented as the mean ± SD or n, %. The statistical significance of differences between patients with and without CAD, as well as with a diagnosis of exercise-provoked esophageal spasm (EPES) and those without EPES, was checked using an unpaired t-Student test, χ^2 or Fisher's exact test (Table 1). The statistical significance of differences between values of esophageal motility parameters obtained from whole-day monitoring and those from exercise tests using the nonparametric Wilcoxon test were estimated. Survival analysis was carried out. The Cox's F test in the Kaplan-Meier method for two and many groups and the Cox proportional hazard analysis were used.

RESULTS

Gradual exercise during a treadmill stress test, in comparison to the values obtained during the whole monitoring process (24 h) and daily activity period, provoked epGER in 8/63 (13%) subjects. This included four in whom neither acid reflux within the 24-h monitoring was found, nor motility disorders, such as a decrease in the percentage of peristaltic and effective contractions, a decrease in esophageal contraction amplitude, or an increase in the percentage of simultaneous and non-effective contractions (Table 2). In 14/63 (22%) patients, a percentage of simultaneous contractions during the treadmill stress test exceeded the arbitrarily established cut-off value of > 55%, according to the diffuse esophageal spasm (DES) definition by Stein *et al*[17]. This esophageal motor disorder was termed EPES. Patients with such an esophageal motility disorder had no special characteristics in comparison with the remaining subjects (Table 1). The appearance of EPES features was not predicted by the occurrence of chest pain during the 24-h esophageal function examination (P = 0.62), by *Helicobacter pylori* (*H. pylori*) infection (P = 0.42), or by a history of hypertension (P = 0.23) or diabetes (P = 0.23). However, an EPES diagnosis was significantly related to angina-like chest pain presence (P = 0.01) during the treadmill stress test (Table 1).

Moreover, in the subjects studied, erosive esophagitis occurred in eight (12%); features of gastroesophageal acid reflux in 24-h esophageal pH-metry during daily activity appeared in 13 (21%); in 24-h esophageal manometry, features of DES with a > 30% cut-off value percentage of simultaneous contractions[18] during daily activity were experienced by 17 (27%); and features of ineffective esophageal motility (IEM) were found in five (8%). The prevalence of the esophageal disturbances mentioned, in patients with CAD and without significant (> 50%) coronary artery narrowing, was similar; the exceptions being that features of IEM were not found in patients with CAD (Table 1). Moreover, neither patient group differed significantly in relation to the values of the majority of the demographic and clinical parameters or in non-invasive cardiac examinations (Table 1). Only CAD-EPES- patients had a significantly longer duration of the stress test than respective subjects with CAD (CAD+EPES-).

After the 2.7-year follow-up period, all subjects remained alive. Twenty-four patients (38%) were hospitalized because of acute coronary syndrome suspicion. Patients with an EPES diagnosis made up only 12.5% of this group. The percentage of patients with EPES who needed hospitalization in the follow-up period (3/14 = 21%) was two times lower than subjects without such

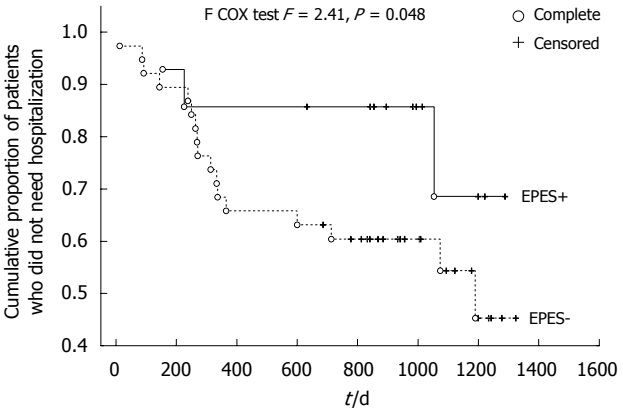

Figure 1 Comparison of two Kaplan-Meier curves in patients with and without signs of exercise-provoked esophageal spasm. EPES: Exercise-provoked esophageal spasm; EPES-: Patients, in whom the percentage of simultaneous contractions within the treadmill stress test did not exceed 55%; EPES+: Patients in whom the percentage of simultaneous contractions within the treadmill stress test exceeded 55%.

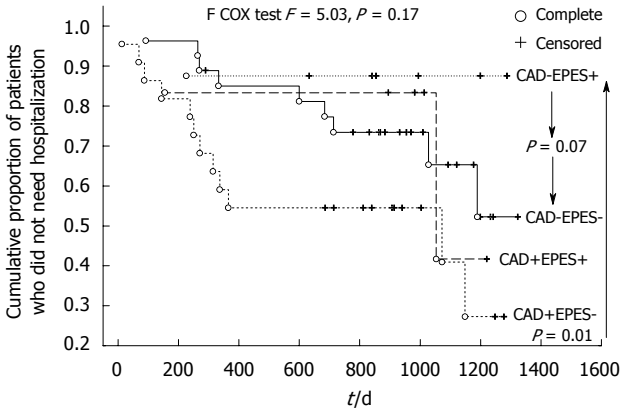

Figure 2 Comparison of four Kaplan-Meier curves in patients with and without signs of exercise-provoked esophageal spasm and a narrowing of coronary arteries > 50%. EPES: Exercise-provoked esophageal spasm; CAD: Coronary artery disease; CAD+EPES-: Patients with significant (> 50%) coronary artery narrowing in coronary angiography, in whom the percentage of simultaneous contractions within the treadmill stress test did not exceed 55%; CAD+EPES+: Patients with significant (> 50%) coronary artery narrowing in coronary angiography, in whom the percentage of simultaneous contractions within the treadmill stress test exceeded 55%; CAD-EPES-: Patients without significant coronary artery narrowing in coronary angiography, in whom the percentage of simultaneous contractions within the treadmill stress test did not exceed 55%; CAD-EPES+: Patients without significant coronary artery narrowing in coronary angiography, in whom the percentage of simultaneous contractions within the treadmill stress test exceeded 55%.

Table 3 Proportional hazard Cox regression model for the number of days to first hospitalization because of suspected acute coronary syndrome (χ^2 = 32.38, P = 0.039)

Independent variable	β	Standard error	*P*
Number of hospitalizations before gastrological diagnostic performance	0.01	0.06	0.87
Spontaneous chest pain during 24-h monitoring	1.05	0.56	0.06
Eradicative treatment recommendation due to *Helicobacter pylori* infection	-1.36	0.62	0.03
EPES	-2.38	0.93	0.01
epGER	-0.012	1.01	0.99
Significant (> 50%) coronary vessel narrowing	1.01	0.73	0.17
History of myocardial revascularization	-0.78	0.67	0.24
DUKE score	0.03	0.06	0.62
Gender (male/female)	-1.42	0.72	0.049
Smoking	0.06	1.38	0.96
Canadian Cardiovascular Society Classification	0.74	0.76	0.33
History of myocardial infarction	0.30	0.99	0.76
Hypertension	-0.47	0.96	0.63
Diabetes mellitus	-0.22	0.80	0.78
Age	0.085	0.04	0.047
BMI	0.10	0.09	0.27
WHR	-4.69	5.07	0.35
Fasting blood glucose	-0.074	0.05	0.11
LDL cholesterol	-0.015	0.01	0.18
Triglycerides	0.005	0.04	0.277

BMI: Body mass index; WHR: Waist to hip ratio; epGER: Exercise- provoked gastroesophageal reflux; EPES: Exercise-provoked esophageal spasm; LDL: Low-density lipoprotein; DUKE score = Duration of stress test according to Bruce protocol (min) - [5 × ST interval depression (mm)] - (4 × treadmill angina index).

a diagnosis (21/49 = 42%, $P > 0.05$). Patients with an EPES diagnosis also had a significantly longer event-free period and time to the first hospitalization episode within the follow-up period (Table 1). The favorable effect of an EPES diagnosis on the risk of hospitalization due to suspected acute coronary syndrome was also confirmed by the Kaplan-Meier survival analysis ($F = 2.41$, $P = 0.048$, Figure 1). Kaplan-Meier survival analysis was also performed (Figure 2) because the investigated group consisted of patients both with and without significant coronary artery narrowing and this variable plays a known role as a predictor of events in patients with recurrent chest pain. The common (four-way) effect of coronary artery narrowing and EPES presence did not reveal a statistically significant influence on the risk of hospitalization ($F = 5.03$, $P = 0.17$). However, CAD-EPES+ patients had a more favorable course of the Kaplan-Meier curve than CAD-EPES- patients (statistically borderline, $F = 2.56$, $P = 0.07$) and CAD+EPES- ($F = 6.89$, $P = 0.006$). The detailed Kaplan-Meier curve analysis shows that the differences mentioned had already appeared after an observation period of about 1 year.

A multi-factorial Cox proportional hazard analysis was also performed (Table 3) because hospitalization due to acute coronary syndrome suspicion may be an effect of many factors. It showed that in the researcher's group the independent significant variables which decreased the risk of the appearance of chest pain needing hospitalization were as follows: EPES, recommendation of eradicative triple therapy due to *H. pylori* infection, female gender and younger age (Table 3).

DISCUSSION

This preliminary observational investigation was undertaken to estimate the clinical usefulness of esophageal manometry examination during graded exercise, as a provocative test in the diagnosis of angina-like chest pain of sus-

pected noncardiac origin, both in patients with CAD and in subjects without significant coronary artery narrowing. As a criterion of clinical usefulness of such a diagnostic strategy, the risk of hospitalization due to the suspicion of acute coronary syndrome over a long follow-up period was assumed. The main observation of this study is that graded exercise during a treadmill test may induce esophageal motility disturbances leading to a decrease in esophageal mechanical clearance, expressed more in patients without CAD (Table 2). This particular kind of motility disorder, in which a percentage of simultaneous contractions exceed the cut-off value of 55%, was named EPES by the author. The prevalence of EPES in the studied group was rare (n = 14/63, 22%), but similar to the presence of the other esophageal disorders (GER, epGER, DES and IEM) in patients both with and without significant coronary artery narrowing (Table 1). The presence of this esophageal motility disorder was related to angina-like chest pain occurrence with the treadmill stress test. To my knowledge this is the first paper to present such a view of esophageal motility disorder.

The second important observation of this investigation is that patients with a diagnosis of EPES had an independently (Table 3) and significantly lower risk of hospitalization due to the suspicion of acute coronary syndrome in the 2.7-year long follow-up period (Figure 1). This independent effect, although confirmed by the multifactorial Cox proportional hazard analysis (Table 3), was less apparent when all subjects were divided into four groups in relation to the presence of EPES and CAD (Figure 2), although a more favorable trend for EPES was perceptible. These observations might have resulted from pharmacotherapy because calcium antagonists had been recommended in all patients with hypertensive esophageal disorders (Table 1). The reported effects of a calcium antagonist in the treatment of hypertensive esophageal motility disorders diagnosed using stationary manometry have been ambiguous. Some studies have shown a favorable outcome for this group of drugs; some have not confirmed this[1,5]. However, no reports concerning the usefulness of calcium antagonists in the treatment of IEM or the other esophageal motility disorders diagnosed on the basis of ambulatory 24-h manometry were found. Moreover, it is also possible that calcium channel blockers only have an influence on exercise-provoked esophageal motility disorder. It might also be that my observations of a good prognosis for patients with EPES treated with calcium antagonists resulted not from the effects of the medicine but from the diagnosis of an extra-cardiac and non-life-threatening source of their chest pain. Such an interpretation concurs with the results of a study by Spencer *et al*[19], who showed that patients with dysphagia or chest pain and a diagnosis of esophageal motility disorder (such as achalasia, diffuse esophageal spasm, "nutcracker" hypercontracting esophagus, and hypocontracting esophagus) reported clinical improvement in a 3-year follow-up. However, in the patients' opinion, the amelioration of their symptoms was not an effect of the recommended treatment but a belief in the benign character of their discomfort. Consideration of other than a drug-related mechanism of better prognosis in patients with EPES treated with calcium antagonists resulted from an open-label, uncontrolled study design. In this study the course of angina-like chest pain in patients with a diagnosis of EPES was also compared with patients for whom calcium antagonists were also recommended because of hypertensive esophageal motility disorders other than EPES (Table 1). However, in the latter patient group the recommendation of a calcium antagonist had no favorable effect on the risk of hospitalization.

The potential pathomechanism for EPES, which is a particular kind of IEM, is unknown. According to the results and conclusions of Adamek *et al*[20], it may be supposed that EPES could be a marker of general smooth muscle readiness for contractive reactions. This hypothesis would be confirmed by the coexistence of esophageal motility disorders with other vasospastic syndromes, such as migraine, Raynaud's phenomenon, Prinzmetal's angina, and hypertension and/or a hypertensive (exaggerated) reaction of blood pressure to exercise[21,22]. However, in the present study no relationship between EPES manometric features and the above-mentioned blood pressure reactions or signs of myocardial ischemia (significant ST interval depression) was found. In addition, no patients had a history of vasospasm during coronarography. The other possible explanations for the appearance of EPES features during the treadmill stress test, which occurred in only 14/63 (22%) of subjects, may be as follows: an individually related difference of exercise-related changes in sympathetic autonomic nervous system activity; different sensitivity of smooth muscle to noradrenalin; a decrease in vagally-mediated regulation of esophageal motility; the inhibition of nitric oxide synthase activity; as well as esophageal ischemia induced by a decrease in splanchnic blood flow during exercise observed in 50%-80% of subjects[23]. All these factors may also have an effect on the presence of visceral hypersensitivity and symptom intensity[18,24-27]. On the other hand, Tipnis *et al*[28] have shown that distension of the esophagus itself plays an important role in symptom modulation in patients with GERD. It seems that non-propulsive motility disorders, amongst which is EPES, may lead to similar esophageal conditions.

The third observation of my study is that patients with recurrent angina-like chest pain with CAD and without significant coronary artery narrowing were similar with regard to the majority of estimated demographic and clinical data, including parameters of esophageal manometry, especially those obtained from examination during the treadmill stress test (EPES) (Table 1). These observations corroborate data published by other authors, who have reported a similar prevalence of esophageal disorders in patients both with and without cardiovascular diseases[9,10]. However, some authors have also reported a lower percentage of diagnoses of esophageal disorders in patients with CAD than in individuals without CAD[11,12]. The clinical importance of this observation is that there is no clinical or demographic factor which could help to predict the origin of chest pain in respective patients, especially when they

have not responded to guided therapy. On the other hand, my results have confirmed the possibility of overlapping noncardiac and cardiac chest pain sources, and justified the common analysis of patients with and without significant coronary artery narrowing. A simple explanation for the coexistence of esophageal and cardiac disorders might be the actions of viscero-visceral and viscero-somatic reflexes, which may lead to a decrease in myocardial perfusion in response to a decrease in intraesophageal pH[24,26]. It might also relate to esophageal function disorders within myocardial ischemia[29] and/or to the increase of back or precordial muscle tension in response both to myocardial ischemia and esophageal function disorders[30].

My observations, like many others, have some limitations. Firstly, the number of patients in the studied group was small. However, in the PubMed database I could only find a few works concerning the gastroenterological aspects of chest pain which had a number of subjects greater than in the present study. However, the number of subjects in my study was enough to reach statistical significance in some important comparisons (Tables 2 and 3, Figure 1). The inclusion in the study of consecutive patients helped to avoid, or at least reduce, selection bias. Secondly, the follow-up period was relatively short but similar to that in the recent study by Eslick *et al*[31]. Thirdly, swallowing was not monitored during manometric examination, although this limitation seems only to carry importance in the definition of diffuse esophageal spasm. This resulted from the methodology (ambulatory motility monitoring) and the kind of manometric probe used (distal sensors only). In my opinion, the appearance of EPES in only 22% of individuals, all patients having been submitted to the same conditions on the treadmill, justified a new dysmotility diagnosis, independent of its primary, secondary or tertiary character. Fourthly, patients were recommended to take amlodipine or diltiazem in this study, depending on their resting heart rate. Such dual therapy might have influenced the results obtained, especially as a more favorable effect of the dihydropyridine class of calcium-channel blockers on vascular endothelial function in patients with coronary spastic angina has been reported[32]. Although the studied patient group did not present with vasospasm during the coronarography, this indicates a necessity to retry the investigation using another design in a homogeneous patient group. Fifthly, a similar conclusion applies to the lack of a placebo-controlled study design. However, in this study calcium antagonists were recommended in open-label design in all patients with hypertensive esophageal motility disorders (Table 1), not only those with EPES. Thus, patients without EPES and treated with a calcium antagonist should be recognized as a control group; this may even suggest that an improved prognosis in patients with EPES did not only result from a drug effect.

In conclusion, patients with recurrent angina-like chest pain non-responsive to treatment with PPIs, both with and without significant coronary artery narrowing, had a similar prevalence of potential noncardiac causes of precordial symptoms, including exercise-related esophageal motility disorders. However, patients with a diagnosis of EPES and a recommendation of a calcium antagonist showed significantly lower risk of hospitalization due to suspected acute coronary syndrome in the 2.7-year follow-up period than others, but this aspect needs further study.

COMMENTS

Background

Noncardiac chest pain (NCCP) due to esophageal motility disorders can occur both in patients with and without coronary artery disease (CAD). This symptom is frequently resistant to treatment with a recurrence rate of about 80%. NCCP significantly decreases patients' health-related quality of life and may predispose to invasive chest pain diagnostic procedures. However, the influence of diagnosis and treatment of esophageal motility disorders during the course of NCCP in patients both with and without CAD is still unclear. Moreover, the outcome of therapy with calcium channel inhibitors in patients with noncardiac chest pain and/or esophageal motility disorders is ambiguous.

Research frontiers

The results of this research were biased by the possibility of chest pain sources overlapping, including those of exercise-provoked angina-like chest pain. This fact should be considered both by cardiologists and gastroenterologists. The other limitations are listed in the article.

Innovations and breakthroughs

This study has shown that the use of exercise as a provocative test may not only help to distinguish cardiac and esophageal chest pain source, but may also offer the possibility of diagnosing exercise-provoked esophageal motility disorders having a favorable outcome with calcium antagonists. Moreover, it was found that calcium antagonists might not show a favorable effect in patients with all hypertensive esophageal motility abnormalities because their efficacy may be limited only to patients with exercise-provoked esophageal dysmotility. This investigation also confirmed a similar prevalence of esophageal disorders in patients both with and without CAD.

Applications

The results of this investigation should be the premise for further studies on the use of exercise as a provocative test in NCCP diagnosis and the application of calcium channel inhibitors in the treatment of esophageal motility disorders and NCCP. The outcome may be a change in the diagnostic and therapeutic strategy for patients with recurrent chest pain of suspected noncardiac origin.

Terminology

Ischemic heart disease is an effect of the imbalance between blood supply and myocardial demand. CAD is a form of heart disease in which myocardial ischemia is due to coronary artery narrowing. Esophageal motility disorder is a condition in which uncoordinated esophageal contractions with increased or decreased pressure amplitude occur. Gastroesophageal reflux disease is a disorder resulting from regurgitation of stomach content into the esophagus.

Peer review

The introduction is adequate. The materials and methods are really well written and specific. The clinical experiment has been well thought out and conducted. Conclusions are supported by data, and discussion addressed specific points.

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BRIEF ARTICLE

High-dose-rate intraluminal brachytherapy during preoperative chemoradiation for locally advanced rectal cancers

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Abstract

AIM: To determine the feasibility and safety of high dose rate intraluminal brachytherapy (HDR-ILBT) boost during preoperative chemoradiation for rectal cancer.

METHODS: Between 2008 and 2009, thirty-six patients with locally advanced rectal cancer (≥ T3 or N+), were treated initially with concurrent capecitabine (825 mg/m^2 oral twice daily) and pelvic external beam radiotherapy (EBRT) (45 Gy in 25 fractions), then were randomized to group A; HDR-ILBT group (n = 17) to receive 5.5-7 Gy × 2 to gross tumor volume (GTV) and group B; EBRT group (n = 19) to receive 5.4 Gy × 3 fractions to GTV with EBRT. All patients underwent total mesorectal excision.

RESULTS: Grade 3 acute toxicities were registered in 12 patients (70.6%) in group A and in 8 (42.1%) in group B. Complete pathologic response of T stage (ypT0) in group A was registered in 10 patients (58.8%) and in group B, 3 patients (15.8%) had ypT0 (P < 0.0001). Sphincter preservation was reported in 6/9 patients (66.7%) in group A and in 5/10 patients (50%) in group B (P < 0.01). Overall radiological response was 68.15% and 66.04% in Group A and B, respectively. During a median follow up of 18 mo, late grade 1 and 2 sequelae were registered in 3 patients (17.6%) and 4 patients (21.1%) in the groups A and B, respectively.

CONCLUSION: HDR-ILBT was found to be effective dose escalation technique in preoperative chemoradiation for rectal cancers, with higher response rates, downstaging and with manageable acute toxicities.

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Key words: High dose rate; Intraluminal brachytherapy boost; Locally advanced rectal cancer; Preoperative chemoradiation

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INTRODUCTION

The incidence of rectal cancer in Pakistan is similar to those in other Asian countries, but much lower than in the developed countries. At present, the risk is equal in both sexes. However a 41% rise in incidence was noted in Pakistani males during the period 1995-1999, which may indicate a higher risk in males in the future[1]. Most rectal cancers present at advanced stages, and are not amenable to upfront curative surgery. Recent prospective randomized studies with large sample sizes and long-term follow up have reported that preoperative chemoradiation was superior to postoperative chemoradiation in terms of local control, feasibility and toxicity in patients with locally advanced rectal cancer[2,3].

For patients with ⩾ T2 or N+ disease, preoperative chemoradiation is given in two phases; initially external beam radiation therapy (EBRT) is given to cover the pelvic nodes along with the primary tumor followed by a dose escalated boost which is given to the gross tumor volume (GTV) with either EBRT, contact X ray therapy or rarely high-dose-rate intraluminal brachytherapy (HDR-ILBT)[4-6].

HDR-ILBT due to its advantage of rapid fall-off of radiation dose allows the delivery of a high tumor dose while sparing normal structures such as normal rectal mucosa and small bowel[7,8]. Additionally HDR-ILBT may reduce overall radiation treatment time and the waiting period for radiation, especially in busy radiation centers. However, there is limited data available regarding the non-inferiority of HDR-ILBT with EBRT as compared to EBRT alone[9,10]. Kaufman *et al*[9] suggested HDR-ILBT as a boost along with external beam radiation after treating 27 patients with persistant and recurrent rectal, sigmoid and anal cancers.

We attempted to evaluate the feasibility and safety of HDR-ILBT as a boost during preoperative chemoradiation for locally advanced rectal cancer, and to determine whether HDR-ILBT has an advantage in terms of achieving higher pathologic response rates and sphincter preservation.

MATERIALS AND METHODS

Eligibility

Patients referred to our department between November 2008 and October 2009 were selected when they met the following eligibility criteria: (1) histologically proven rectal adenocarcinoma; (2) distal margin of tumor located within 10 cm of the anal verge on endoscopy; (3) T stage ⩾ T3 or nodes positive on preoperative imaging [computed tomography (CT), magnetic resonance imaging (MRI)] and M0; (4) Eastern Cooperative Oncology Group performance status 0-2; and (5) normal hematological parameters (hemoglobin ⩾ 10 g/dL, white blood cell ⩾ 4000/mm^3, absolute neutrophil count ⩾ 1500/mm^3 and platelets ⩾ 100000/mm^3), normal hepatic parameters (serum bilirubin level ⩽ 1.5 mg/dL and liver transaminase levels ⩽ 3 times upper normal limit) and normal renal function (serum creatinine level ⩽ 1.5 mg/dL). Patients who had received prior chemotherapy or pelvic radiotherapy or with poor functional status and severe comorbidities were excluded. The treatment protocol is represented in flow chart in Figure 1.

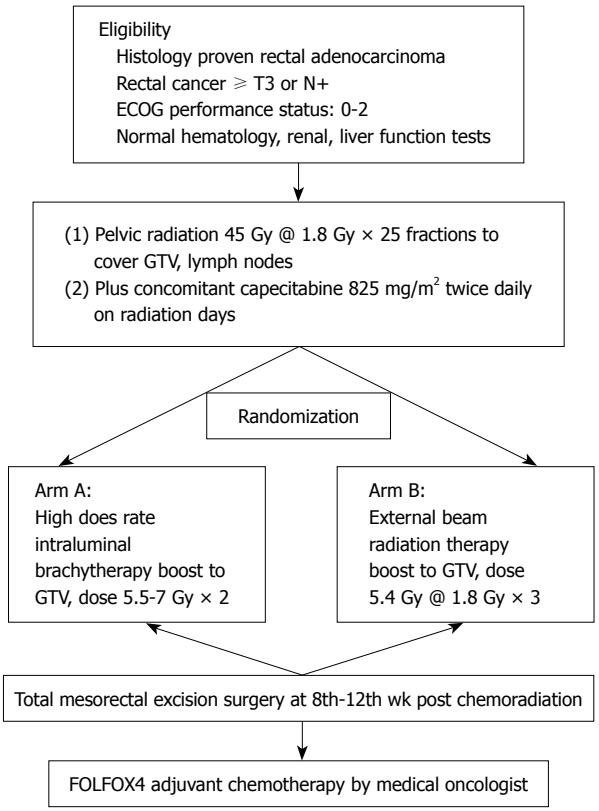

Figure 1 Treatment algorithm for study protocol. ECOG: Eastern Cooperative Oncology Group; GTV: Gross tumor volume.

Treatment protocol

Radiotherapy: Preoperative radiotherapy was delivered using a high-energy multi-leaf collimator linear accelerator (15 MV). All patients were virtually simulated in the prone position using a Siemens® SOMATOM emotions 6 CT scanner, with a device to displace the small bowel (belly board). The whole pelvis was treated with the three field technique and up to 45 Gy in 25 fractions over 5 wk; The superior border was at the L5-S1 interspace, and the lower border was kept at least 3 cm below the tumor. The lateral borders of the anteroposterior-posteroanterior fields were defined 1 cm away from lymph nodes using vessels as surrogate markers (Figure 2). Lateral portals covered the full sacrum and coccyx with a margin; anteriorly they were 3 cm from the sacral promontory.

After the nature of HDR-ILBT was fully explained, patients were classified into two groups: group A; boost to GTV with HDR-ILB and group B; boost to GTV with

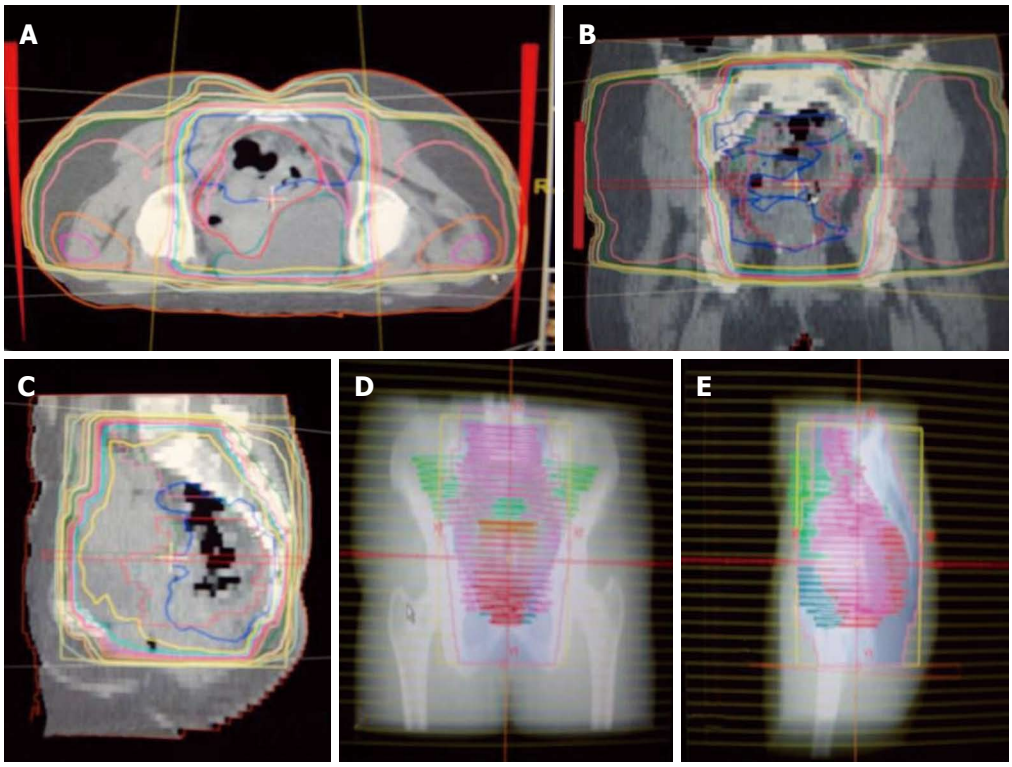

Figure 2 External beam radiation treatment techniques used for rectal cancer. A: Patient in prone position on computed tomography simulation; B, C: Treatment plans were made following coronal and sagittal views of multi-planar reconstructions showing isodose distribution; D, E: Resultant reconstructed radiographs showing tumor, lymph nodes and radiation beams in the anteroposterior and lateral sides.

EBRT as the control group, according to their own choice as to whether to receive the therapy. The study was performed with the approval of the institutional ethics committee. Written informed consent was obtained from all patients before treatment.

HDR-ILB technique: HDR brachytherapy was given in two sessions at 20 and 40 Gy of EBRT. In a dedicated room, where the patients were kept in the lithotomy position under analgesia, the oncologist inserted an in-house built rectal catheter after per rectal and endoscopic localization of the gross tumor. Catheter placement was confirmed with plain radiographs of the pelvis taken with a 12 inch C-arm unit (OEC 9800Plus; GE Medical Systems). After the procedure was completed, patients were transported to the CT simulator. 3 mm slice thickness images were obtained and were transferred *via* digital Imaging and Communications in Medicine to the Flexiplan brachytherapy planning system version 2.2. All organs (GTV, normal rectum, bladder, prostate, uterus, and vagina) were contoured. The apparatus used for HDR-ILBT was a Flexitron® remote afterloading unit with an iridium-192 (Ir^{192}) stepping source. Dwell positions were activated at 2.5 mm along each needle. Dwell times were optimized using a reverse planning optimization algorithm. The prescribed dose was 5.5-7 Gy at 1 cm of the rectal catheter in each session (Figure 3).

Chemotherapy: Oral capecitabine was given at 825 mg/m^2 bid for the duration of radiotherapy with the initial dose

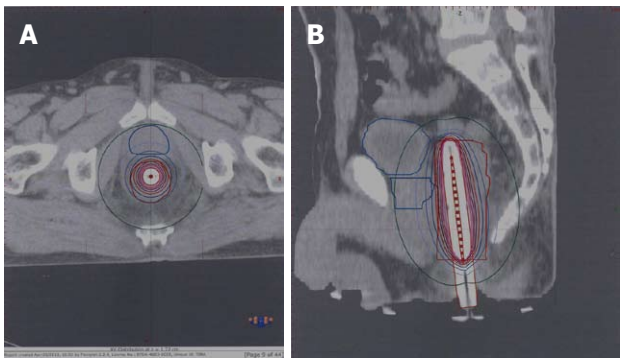

Figure 3 High dose rate intraluminal brachytherapy with single channel catheter with interchangeable shields (A) axial and (B) sagittal views.

starting 1 h before radiotherapy. The drug was given during radiation days only (5 d/wk). Dose modifications were given if a patient experienced Grade 2 hematologic toxicities, and capecitabine was stopped until these toxicities resolved. For Grade 2 or greater non-hematologic toxicities, the drug was reduced to 50% of the initial dose. If toxicities recurred, capecitabine was stopped until they resolved. Radiotherapy side effects were managed as per departmental protocols.

Surgery: After the completion of chemoradiation, patients underwent assessment (repeat CT/MRI, endoscopy, ± exploratory laparoscopy) for surgery at the 8th and 10th wk. The choice of procedure (low anterior resection or abdominoperineal resection) was at the discretion of the surgeon.

Postoperative chemotherapy: The choice of chemotherapy was at the discretion of the medical oncologist.

Study endpoints

Radiologic and pathologic response rates: At the 8th and 10th wk after chemoradiation, the radiologic response was measured by comparing pre- and post-chemoradiation imaging (CT or MRI). The volume reduction rate was calculated by the following formula:

Tumor volume reduction rate = (pre-chemoradiation tumor volume - post-chemoradiation tumor volume) × 100/pre-chemoradiation tumor volume[11].

Following surgery, pathologic tumor staging was determined according to the TNM classification system by the International Union against Cancer and the American Joint Committee on Cancer. Downstaging was applied for T stage and was defined as "yp", where "y" was after chemoradiation and "p" for postoperative pathologic examination. All resected specimens were evaluated for pathologic response with careful inspection of tumor, mesorectal fat and circumferential margins. The pathologic complete response of T stage (ypT0) was defined as the absence of cancer cells in resected specimens.

Toxicity profile: Adverse events were graded according to National Cancer Institute Common Toxicity Criteria version 2.0 and were recorded weekly during follow up. Hematology and serum chemistry was checked on a weekly basis and after completion of chemoradiation at 4 and 8 wk.

Statistical analysis

Pathologic complete response of T stage (yp T0) was considered a binary variable and was scored as 0 or 1 based on the presence of tumor cells. This study design was planned using Simon's optimal two-stage design[12]. According to this design, in the first stage to document ⩾ 2 ypT0, 19 patients were required, otherwise the study would be closed prematurely. The descriptive data (mean, median, range and frequency) were calculated using SPSS version 16.0. The response rates and toxicities were summarized with 95% CI. Comparisons between the two groups were performed with paired-sample *t* tests for metric data and chi-square tests for frequencies. For all tests, a *P* value less than 0.05 was considered statistically significant.

RESULTS

A total of 36 patients were considered eligible for the study. Patient characteristics in each treatment group are described in Table 1. There were no differences between the two groups with regard to mean age, gender, baseline TNM stage, site of primary tumor and performance scale. The study population was predominantly young males and the majority of patients had tumor grade T3N+.

Table 1 Characteristics of the study patients *n* (%)

Variable	Group A (intraluminal brachytherapy) (*n* = 17)	Group B (control arm) (*n* = 19)
Median age (yr)	34.7 (range 17-55)	34.7 (range 17-55)
Gender		
Male	13 (76.5)	14 (73.7)
Women	4 (23.5)	5 (26.3)
Site of primary tumor		
Upper rectum	8 (47.1)	9 (47.4)
Lower rectum	9 (52.9)	10 (52.6)
Clinical/radiological stage		
T2 N+	1 (5.9)	1 (5.3)
T3 N0	2 (11.8)	3 (15.8)
T3 N+	7 (41.1)	8 (42.1)
T4 N0	5 (29.4)	6 (31.5)
T4 N+	2 (11.8)	1 (5.3)
Performance status (ECOG)		
0	13 (76.5)	12 (63.2)
1	4 (23.5)	7 (36.8)

ECOG: Eastern Cooperative Oncology Group.

HDR-ILBT

A total of 34 procedures were successfully performed, with a technical success rate of 98.0%. The total dose in these 17 patients was 11-14 Gy (dose given each session was 5.5-7 Gy). The procedure was well tolerated with the exception of one patient who developed per rectal bleeding within 2 h of the procedure. The bleeding was stopped after securing hemostasis without the need for blood transfusion.

No treatment related deaths or life-threatening events were seen. Twelve patients (70.6%) had grade 3 rectal pain. Other grade 3 acute toxicities were diarrhea in 7 patients (41.2%), and nausea and vomiting in 3 patients (17.6%) which were more frequent when compared to group B patients (Table 2). Late toxicities in the HDR-ILBT group were mild grade 1 and 2; no grade 3 late toxicities were seen.

EBRT

All 19 patients tolerated EBRT very well with a success rate of 100%. Grade 3 nausea and vomiting was seen in 5 patients (26.3%), which was significantly higher than the HDR-ILBT group (17.6%) with a *P* value of 0.02. However, grade 3 diarrhea and rectal pain were less common when compared with the HDR-ILBT arm. Wound complications were slightly higher (15.8%) in this group when compared with the HDR-ILBR group (11.8%).

Radiologic response

A CT and MRI volumetry evaluation was carried out in 36 patients. The mean pre-chemoradiation tumor volume was 21.9 cm^3 (18.30-30, SD 3.7) in group A and 22.3 cm^3 (19.3-30, SD 3.4) in group B. The mean post-chemoradiation tumor volume was 6.78 cm^3 (5.3-10.3, SD 1.7) in group A and 7.5 cm^3 (5.9-10.5, SD 1.6) in group B. The median tumor volume reduction rate was 68.15% and 66.04% in group A and B, respectively (*P* value not significant).

Pathologic response

Pathologic response data were available for all 36 patients

Table 2 Toxicity profiles (grade 3 or worse) in group A and group B *n* (%)

Type of toxicity	Group A (HDR-ILBT)	Group B (EBRT boost)	*P* value
Hematologic			0.3
Leucopenia	2 (11.7)	2 (10.5)	
Neutropenia	2 (11.7)	2 (10.5)	
Thrombocytopenia	1 (5.9)	1 (5.3)	
Non-hematologic			
Hand-foot syndrome	1 (5.9)	1 (5.3)	
Nausea/vomiting	3 (17.6)	5 (26.3)	0.02
Diarrhea	7 (41.2)	5 (26.3)	0.001
Rectal pain	12 (70.6)	4 (21.1)	0.001
Wound complications	2 (11.8)	3 (15.8)	
Cystitis	2 (11.8)	3 (15.8)	

Group A: High dose rate intraluminal brachytherapy boost; Group B: External beam radiation therapy boost. HDR-ILBT: High dose rate intraluminal brachytherapy; EBRT: External beam radiotherapy.

Table 3 Pathologic response in both groups *n* (%)

Pathologic stage	Group A (HDR-ILBT) (*n* = 17)	Group B (control arm) (*n* = 19)	*P* value
yp T stage			
ypT0	10 (58.8)	3 (15.8)	0.0001
ypT1	3 (17.6)	6 (31.6)	
ypT2	1 (5.9)	4 (21)	
ypT3	2 (11.8)	5 (26.3)	
ypT4	1 (5.9)	1 (5.3)	
yp N stage			
ypN0	6 (60)	5 (50)	0.02
ypN+	4 (30)	5 (50)	

Group A: High dose rate intraluminal brachytherapy boost; Group B: External beam radiation therapy boost. HDR-ILBT: High dose rate intraluminal brachytherapy.

who underwent surgery. The pathologic response results are shown in Table 3. Complete pathologic response of T stage (ypT0) in group A was registered in 10 patients (58.8%) and in group B, 3 patients (15.8%) had ypT0 ($P < 0.0001$). Sphincter preservation was reported in 12 patients (70.6%) in group A and in 11 patients (57.9%) in group B ($P = 0.04$). When we compared the pathologic stage after chemoradiation and the clinical stage at the time of chemoradiation, overall downstaging for T stages was found in 55.5% and in 55% for N+ stages.

DISCUSSION

High dose rate intraluminal brachytherapy is a well-known treatment modality for gynecologic, esophagus, lung, biliary tract, and nasopharyngeal malignancies[13-17]. The major advantage of HDR-ILBT over external beam irradiation is its rapid fall-off; a high dose can be delivered to the tumor without significantly affecting the adjacent normal tissues.

HDR-ILBT for advanced or inoperable tumors of the rectum has been used both palliatively and to dose escalate after chemoradiation for curative treatment[18]. For palliative relief, recurrent or inoperable rectal cancers, HDR-ILBT has been used worldwide[19-21], however, there is limited data available regarding the use of HDR-ILBT as a boost to GTV along with EBRT during preoperative chemoradiation for rectal cancers (Table 4). Our study showed higher sphincter preservation rates than in all the trials published so far and comparable ypT0 rates. Better pathological response following HDR-ILBT can easily be explained by higher radiation doses to tumor and shorter distances between the radiation source and tumor. One criticism which may arise is that no difference in radiological response was observed between the two groups. Looking at the differences in pathological response, we believe that radiation-induced trauma and fibrosis might be responsible for no radiological response being observed. In the HDR-ILBT arm, a higher incidence of grade 3 rectal toxicity was seen, especially rectal pain followed by diarrhea. We saw only one case with per rectal bleeding which was successfully managed. However, late toxicities were mild and manageable. In addition, more nausea and vomiting and wound complications in the control arm (EBRT) can be explained by greater irradiated bowel, skin and subcutaneous volumes by external beam irradiation.

Our study had a few limitations; first, the primary endpoint was limited to pathologic response rate and the toxicity profile. This study did not determine the survival benefit at the time of analysis, however, further follow up of these patients will show the impact of HDR-ILBT on disease outcome. Our sample size was small, with more advanced unresectable stages and capecitabine was given as a radiosensitizer only during radiation days, rather than continuously[25]. The smaller sample size is justified by poor referral to tertiary care centers, lack of multidisciplinary approaches and lack of patient education especially in developing countries[26]. In our study, we used a single channel catheter with interchangeable shields, which gave better dose homogeneity in the tumor volume and minimal dose to normal rectal mucosa. This type of catheter has been proved to achieve better dose homogeneity as compared to multi-channel catheters[27]. Another finding was that the study population was composed of predominantly young males. One potential cause of the higher incidence of rectal cancer in the Indo-Pak region could be the number of inter-cousin marriages in this region. However, further research by epidemiologists, oncologists and geneticists is required.

In conclusion, the results of our single center experience were similar or better than previously published studies, albeit with higher but manageable gastrointestinal toxicities. High dose rate intraluminal brachytherapy was found to be more convenient, had satisfactory response rates and can be safely used as a tool to boost the gross tumor volume during preoperative chemoradiation. However, a multicenter randomized trial is warranted to evaluate long-term local control and survival benefit.

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Table 4 Selected trials of intraluminal brachytherapy during preoperative chemoradiation or as preoperative monotherapy in patients with locally advanced rectal cancer

Study, period of study (type of study)	No. of patients	Follow up (mo)	Treatment, protocol	Sphincter preservation rates	ypT0	Local recurrence rate	Distant metastasis	Acute toxicity (ileitis, proctitis), all grades	Disease free survival
Yanagi *et al*[6], 1986-1995	Arm A: 96	60	Arm A: MDR-ILBT→surgery	Arm A: 72%		Arm A: 8%	Arm A: 23%	38%	Arm A: 72%
(retrospective)	Arm B: 19		ArmB: HDR-ILBT→surgery	Arm B: 63%	-	Arm B: 5%	Arm B: 16%	74%	Arm B: 68%
	Arm C: 115		Arm C: surgery alone	Arm C: 42%		Arm C: 21%	Arm C: 17%		Arm C: 65%
Kusunoki *et al*[10], 1986-1995 (case series)	Arm A: 59	5-108	Arm A: MDR-ILBT→surgery	Arm A: 74%				Perforation Arm A: 0%	
	Arm B: 65		Arm B: HDR-ILBT→surgery	Arm B: 63%	-	-	-	Arm B: 11%	-
Vuong *et al*[22], 1998-2001 (case series)	49	29 (16-48)	HDR-ILBT→surgery→chemoradiation	-	64%	2%	4.10%	Grade 2 proctitis 100% Grade 4 dermatitis 4%	-
Ishikawa *et al*[23], 1988-1997 (case series)	41	79.2	EBRT 30 Gy→HDR-ILBT 10 Gy × 4→surgery	-	-	15%	10%	61%	71.80%
Jakobsen *et al*[24] (case series)	50	Not mentioned	CRT 45 Gy→HDR-ILBT boost→surgery	-	27%	-	-	30%	-
Present study	36	18 (5-22)	Arm A: CRT 45 Gy→ILBT boost 5.5-7 Gy × 2→surgery	Arm A: 66.7%	Arm A: 58.8%		N/A	Arm A: 70.6%	-
			Arm B: CRT 45 Gy→EBRT boost 5.4 Gy→surgery	Arm B: 50%	Arm B: 15.80%	N/A		Arm B: 42.1%	

MDR: Moderate dose rate; HDR-ILBT: High dose rate intraluminal brachytherapy; EBRT: External beam radiotherapy; CRT: Chemoradiation; ypT0: Post chemoradiation pathologic T0 stage; N/A: Not available.

for his constant support, and for providing the state-of-the-art radiation oncology department which is offering its services poor patients with cancer with dignity.

COMMENTS

Background

Preoperative chemoradiation is now the standard of care for locally advanced rectal cancers. Radiotherapy in these patients is given by external beam radiation therapy through the shrinking field technique, initially targeting the primary tumor and lymph nodes followed by a boost to the gross residual tumor. High dose rate intraluminal brachytherapy (HDR-ILBT) has been incorporated at various centers to treat recurrent and persistant rectal cancer. This is the first randomized controlled study in which intraluminal brachytherapy was given as a boost along with external beam radiation.

Research frontiers

Tunio *et al* used high dose rate intraluminal brachytherapy during preoperative chemoradiation as a boost to the gross residual tumor.

Innovations and breakthroughs

This study showed a complete pathologic response rate of 58.8% in patients who were treated with high dose rate intraluminal brachytherapy along with external beam radiation compared with a complete pathologic response rate of 15.8% in patients who were treated with external beam radiation alone. The intraluminal brachytherapy group had more acute side effects (rectal pain and diarrhea) but these were successfully managed. Chronic side effects were minimal and mild.

Applications

The incidence of rectal cancer is increasing worldwide. High dose rate intraluminal brachytherapy during curative chemoradiation can provide better treatment outcomes in terms of downstaging and sphincter preservation, preventing many patients having colostomy bags.

Terminology

Intraluminal (intra = inside, luminal = lumen), brachytherapy (brachium = hand, therapy = treatment) is a type of radiation therapy in which the radiation is given by introducing a radioactive source into the lumen, e.g. cervix, bowels, trachea, biliary tract or rectum.

Peer review

The aim of the present study was to determine whether HDR-ILBT as boost during preoperative chemoradiation for advanced rectal cancer is feasible, safe as well as offer higher pathologic response rates and sphincter preservation.

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BRIEF ARTICLE

Clinical significance of *Helicobacter pylori cagA* and *iceA* genotype status

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Abstract

AIM: To study the presence of *Helicobacter pylori* (*H. pylori*) virulence factors and clinical outcome in *H. pylori* infected patients.

METHODS: A prospective analysis of ninety nine *H. pylori*-positive patients who underwent endoscopy in our Endoscopy suite were included in this study. DNA was isolated from antral biopsy samples and the presence of *cagA*, *iceA*, and *iceA2* genotypes were determined by polymerase chain reaction and a reverse hybridization technique. Screening for *H. pylori* infection was performed in all patients using the rapid urease test (CLO-Test).

RESULTS: From a total of 326 patients who underwent endoscopy for upper gastrointestinal symptoms, 99 patients were determined to be *H. pylori*-positive. Peptic ulceration was seen in 33 patients (33%). The main virulence strain observed in this cohort was the *cagA* gene isolated in 43 patients. cagA was associated with peptic ulcer pathology in 39.5% (17/43) and in 28% (16/56) of non-ulcer patients. IceA1 was present in 29 patients (29%) and iceA2 in 15 patients (15%). Ulcer pathology was seen in 39% (11/29) of patients with iceA1, while 31% (22/70) had normal findings. The corresponding values for iceA2 were 33% (5/15) and 33% (28/84), respectively.

CONCLUSION: Virulence factors were not common in our cohort. The incidence of factors cagA, iceA1 and iceA2 were very low although variations were noted in different ethnic groups.

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Key words: Ethnicity; *Helicobacter pylori*; Peptic ulcer disease; Virulence factors

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral, gram-negative microaerophilic bacterium that causes chronic inflammation of gastric mucosa in more than half of the population worldwide. It is a major cause of peptic ulcer (PU) disease and a recognized risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The lifetime risk of an *H. pylori*-infected individual developing peptic ulcer disease (PUD) is estimated to be one in six.

The relationship between *H. pylori* genotype and its association with clinical outcome is not fully understood. The predominant *H. pylori strain* found in geographic locations differ with regard to genomic structures. Genetic diversity in *H. pylori* strains may affect the function and antigenicity of virulence factors associated with bacterial infection and, ultimately disease outcome[1].

H. pylori produces a number of virulence factors that are essential for colonization of the stomach and survival in the hostile gastric environment. In addition to urease, which plays an important role in the neutralization of gastric acid secretion and vacuolating cytotoxin, which induces vacuolar degeneration of various epithelial cell lines, there are a few other important factors. These factors are the gene products encoded by the cag pathogenicity island, which causes up-regulation of cytokines; iceA, a homologue of a gene for restriction endonuclease, induced by contact with gastric epithelium; and OipA, a pro-inflammatory protein that contributes to interleukin-8 induction. It has been proposed that *cagA*[1] and *iceA*[2] genes are markers and can identify patients with peptic ulcers.

Studies have indicated that *H. pylori* infection is common in Malaysia as in other developing countries. Most reports in Malaysia have focused on the prevalence and clinical patterns of gastroduodenal disease, detection of *H. pylori* infection, and effectiveness (or ineffectiveness) of anti-*H. pylori* therapies in the local population. Only a few small studies have provided information on the genotypes of the Malaysian *H. pylori* strains.

The aim of our study was to characterize *H. pylori* strains isolated from Malaysian patients and to determine if the genotypes implicated in patients with disease in the West are similar to those in the Malaysian population.

MATERIALS AND METHODS

Patients and samples

Patients found to be positive for *H. pylori* from those undergoing endoscopy at the Endoscopy Unit of Hospital Ampuan Afzan Kuantan were selected. This study was approved both by the Research and Ethical committees. Informed consent was obtained from each patient prior to the study. A questionnaire on demography was completed.

Gastric and duodenal pathology were identified at endoscopy. Gastritis was defined as macroscopically identifiable inflammation (antral gastritis or pangastritis) with no peptic ulcers, gastric cancer or any esophageal diseases (e.g. gastro-esophageal reflux disease and esophageal cancer). These patients were grouped as non-ulcer dyspepsia (NUD). Patients who had definite erosions or ulcers were grouped as PUD.

Two sets of gastric biopsy specimens were obtained from the antrum in all patients and one set was tested for *H. pylori* using the Rapid Urease test, CLO-test (Ballard Medical Products, USA) and the other specimen was selected for DNA extraction. We felt that CLO-test was the most suitable investigation to screen for *H. pylori* as it is quick, simple and inexpensive with sensitivity and specificity comparable to culture, histology and polymerase chain reaction (PCR)[3].

Genotyping

The biopsy tissue was stored at 4℃ until DNA extraction. DNA was isolated from the biopsy tissue by the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) using the tissue protocol outlined in the manufacturer's instructions.

The DNA yield in the eluate was obtained by measuring its absorbance at 260 nm. The reading should fall between 0.1 and 1.0. The DNA purity was obtained by calculating the $A_{260\ nm}$:$A_{280\ nm}$ ratio. Ideally the ratio should be ⩾ 1.7-1.9. The ratios of our DNA extracts were 1.7-1.9.

GeneAmp® PCR system 9700 (PE Applied Biosystem) was used for molecular analysis. The PCR protocol using *Taq* PCR Master Mix (Qiagen, Germany) was followed.

The amplification cycles for cagA, iceA1, and iceA2 consisted of an initial denaturation of target DNA at 94℃ for 1 min and then denaturation at 94℃ for 1 min, primer annealing at 48℃ or 58℃ (iceA1 and iceA2) for 1 min and extension at 72℃ for 1 min (35 cycles for cagA and 40 cycles for iceA1 and A2). The final extension was another cycle lasting 15 min. The primers used to amplify the targeted genes are summarized in Table 1. A negative control (without template DNA) was included in each experiment.

Agarose Gel Electrophoresis was used to separate and purify the extracted DNA. DNA bands were visualized under BIO-RAD UV transilluminator 2000 (Bio-Rad, UK).

RESULTS

From a total of 326 endoscopies carried out for upper gastrointestinal symptoms during the study period, 99 (30%, 99/326) were found to be CLO-test positive. All specimens were analyzed using the PCR assay.

Of the ninety nine patients, 33 patients were diagnosed with PUD (12 gastric ulcers and 21 duodenal ulcers), while 66 were categorized as NUD.

As shown in Table 2, the *cagA* gene was isolated in only 43 patients (43%). An association between *cagA* and peptic ulcer pathology was noted in 39.5% (17/43) and with NUD in 28% (16/56). This was not statistically significant.

iceA1 was present in 29 patients (29%) and *iceA2* in 15 patients (15%). Ulcer pathology was seen in 39% (11/29) of patients with *iceA1*, while 31% (22/70) had normal findings. The corresponding values for *iceA2* were 33% (5/15) and 33% (28/84), respectively. Again no statistical significance was noted.

A combination of *cagA* and *iceA1* was observed in 13 isolates and a combination of *cagA* and *iceA2* was noted in 4 patients. Only two patients had a combination of iceA1 and iceA2. A total of 5 isolates were positive for all three virulence factors as shown in Figure 1. There was no significant difference noted between the combinations and clinical outcome.

Table 1 Polymerase chain reaction primers for amplification of *cagA*, *iceA1* and *iceA2* genes

Amplified gene	Primer destination	Sequence of primer	Size of PCR product (bp)	Ref.
cagA	D008 F	ATAATGCTAAATTAGACAACTTGAGCGA	297	[4,5]
	R008 R	TTAGAATAATCAACAAACATCACGCCAT		
iceA1	*iceA1* F	GTGTTTTTAACCAAAGTATC	247	[6]
	iceA1 R	CTATAGCCASTYTCTTTGCA		
iceA2	*iceA2* F	GTTGGGTATATCACAATTTAT	229 or 334	[1]
	iceA2 R	TTRCCCTATTTTCTAGTAGGT		

PCR: Polymerase chain reaction; F: Forward primer; R: Reverse primer.

Table 2 Genotype and virulence factors in relation to clinical conditions *n* (%)

Clinical outcome	Starin genotypes			Total
	cagA	*iceA1*	*iceA2*	
PUD	17 (51.5)	11 (33.3)	5 (15.1)	33 (33.3)
NUD	26 (39.3)	18 (27.2)	10 (15.1)	66 (66.6)
Total	43 (43.3)	29 (29.2)	15 (15.1)	99 (100)

n: No. of *Helicobacter pylori* positive strains with the given characteristics; PUD: Peptic ulcer disease; NUD: Non ulcer dyspepsia.

Table 3 Distribution of virulence factors *cagA*, *iceA1* and *iceA2* among different ethnic groups in the study sample *n* (%)

Ethnic group	Strain genotypes			Total
	cagA	*iceA1*	*iceA2*	
Malays	12 (37)	5 (15)	2 (6)	32
Chinese	21 (48)	17 (39)	9 (20)	43
Indians	7 (38)	4 (22)	2 (11)	18
Others	3 (50)	3 (50)	2 (33)	6

n: Total No. of patients of the respective ethnicity.

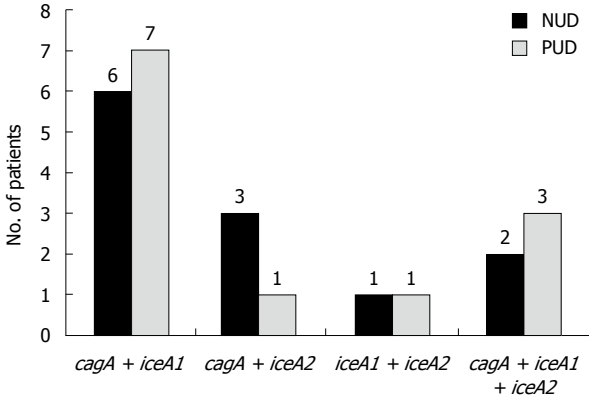

Figure 1 Patients with a combination of virulence factors and the relation to non ulcer dyspepsia and peptic ulcer disease in the study sample. NUD: Non ulcer dyspepsia; PUD: Peptic ulcer disease.

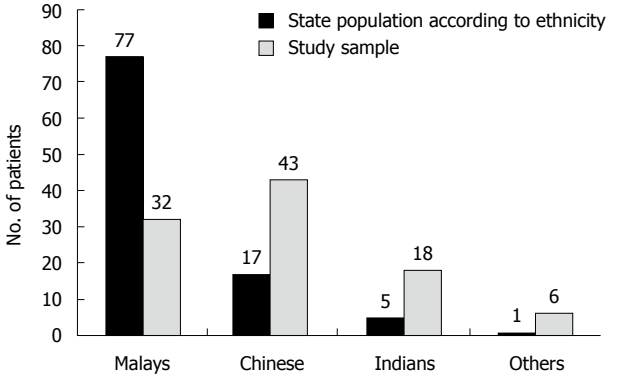

Figure 2 Distribution of ethnicity in our cohort. This shows the population of the state of Pahang according to ethnicity and the detection of *Helicobacter pylori* infection among the different groups in our cohort.

Malaysia has a unique population consisting of three main ethnic groups namely Malays, Chinese and Indians. The other minority groups are categorized as others. As shown in Figure 2, the distribution of our cohort according to their ethnicity and also the distribution of these same groups in the state population highlight the diverse nature of the prevalence of *H. pylori* among the different ethnic groups.

Table 3 shows the variable distribution of the virulence factors among the different ethnic groups. The overall rate is low when compared to other regional studies. This was very evident especially in the Malay patients.

DISCUSSION

The presence of *H. pylori* in the gastric mucosa cannot be considered a disease in itself but as a potential risk factor for the development of upper gastrointestinal tract diseases. It is estimated that about 10% of these individuals will subsequently develop PUD and a smaller percentage of about 1%-2% will develop gastric malignancy[7]. The International Agency for Research on Cancer and the World Health Organization in 1994 concluded that *H. pylori* has a causal link with gastric carcinogenesis and classified it as a Group 1 or definite carcinogen in humans.

Two of the three major ethnic groups in Malaysia, the Chinese and Indians are migrant populations and have been in Malaysia for nearly three generations. The prevalence of *H. pylori* varies among the different ethnic groups. Several studies have demonstrated a high prevalence ranging from 68%-75% in the Indian community, 45%-66.6% in the Chinese and a lower prevalence of 8%-43.3% among Malays[8,9]. According to the last census in 2000, Malays and other indigenous groups (Bumiputras) constitute 58%; Chinese, 24%; persons of Indian descent,

8%; and other groups, 10%[10]. The state of Pahang where these patients were recruited has an ethnic distribution of 77% Malays, 17% Chinese and 5% Indian. The ethnic distribution of *H. pylori* patients recruited from Pahang to our study is shown in Figure 2. Studies from the West show that the prevalence of *H. pylori* is often considerably higher among first and second-generation immigrants[11,12]. Similar reasons may be attributed to the higher incidence in Chinese and Indian populations in Malaysia. The lower rate of *H. pylori* infection in Malay patients in our cohort is similar to other studies in Malaysia. There is no clear explanation for this but may reflect improvements in the standards of household hygiene with a clear shift in this group to the middle income category. Studies in the United States of America show socioeconomic status and household hygiene during childhood as being very significant factors for the variation in prevalence of *H. pylori* infection in different races.

It has been postulated that the functional diversity of *cagA* may have an important relationship with disease outcome. According to Yamaoka *et al*[13], more than 90% of *H. pylori* strains are *cagA* positive in East Asian countries, irrespective of clinical presentation. This was not the case in our study. *cagA* was positive in only 43% of our samples but was associated with peptic ulcer pathology in only 39.5% (17/43) of patients compared to 28% (16/56) of non-ulcer patients. This was not statistically significant. Eradication failure was also significantly higher in *cagA* strain-positive patients (cure rate 73%) as compared to *cagA*-negative (cure rate 84%) in a meta-analysis by Suzuki *et al*[14].

The other virulence factor studied was the *iceA* gene. *iceA* gene ("induced by contact with epithelium") which has two allelic variants (*iceA1* and *iceA2*). Studies suggest an association between the *iceA* variant and PUD. According to Yamaoka *et al*[6] *iceA1* was the predominant subtype in an east Asian population, while the *iceA2* subtype was predominant in Columbia and the USA. Conflicting data has emerged from other parts of the world. In an analysis of *iceA* alleles from *H. pylori* strains among Finnish and African patients, the presence of *iceA* was significantly less in the former (35%) than the latter group (93%)[15]. In another study in Germany involving 141 *H. pylori* patients, the *iceA* gene was detected in 98% of *H. pylori* isolates (138 of 141)[16]. Similar results were reported by groups from Turkey, where 74.7% were positive for *iceA1* and 25.3% for *iceA2,* and in Shanghai where *iceA1* and *iceA2* were found in 74.5% and 15.6%, respectively[17,18]. Our study showed a completely different picture. The corresponding values in our cohort for *iceA1* and *iceA2* were 29% and 15%, respectively. iceA1 was predominant as in other east Asian populations but was very low compared to the other studies. Data from Thailand, which is close to Malaysia, reported higher levels in a study involving 112 *H. pylori* isolates. The positive rates for *cagA, iceA1* and *iceA2* were 98.2%, 45.5% and 33.1%, respectively[19]. The reason for the low values in our study may be due to the reduced incidence of *H. pylori* infection among the major ethnic group, the Malays. The Malay community had positive rates of only 15% and 6% for iceA1 and iceA2, respectively. This trend was also noted in the other communities in our study (Table 3).

Is the presence of peptic ulcer related to the virulence factors? We were able ascertain peptic ulcer pathology in only 39% (17/43) of *cagA*, 37% (11/29) of *iceA1* and 33% (5/15) of *iceA2* isolates. Momenah *et al*[20] from Saudi Arabia reported much higher values. Their study revealed that 100% of ulcer cases were infected with *iceA1,* and *iceA2* was present in 94.6% of their gastritis and in 90.9% of normal patients. Caner *et al*[21] also reported similar conflicting findings in a study involving a total of 46 patients. Isolates from 20 patients with chronic gastritis (66.6%) were *iceA2*-positive, while *iceA1* was predominant in those with duodenal ulcers (68.8%).

As shown in Figure 2, combinations of *cagA* with *iceA1* or *iceA2* were not significantly different among the NUD and the PUD groups, which is in concordance with results from other Asian countries[11].

In conclusion we feel that the prevalence of *H. pylori* infection in Malaysia is lower than that in most countries in Southeast Asia. This may be partly due to the consistently lower incidence reported in the Malay community. The Chinese and Indian communities both have high a incidence of *H. pylori* infection but not as high as those noted in mainland China or India. This trend is similar to studies from the West that show the prevalence of *H. pylori* being persistently higher among first and second-generation immigrants[11,22]. The Chinese and Indian cohorts in our study were at least third-generation immigrants.

Of the virulence factors studied, cagA was noted to be present in 43% of patients which was much lower than most other countries in the region. A recent study from Malaysia[23] showed the occurrence of cagA diversity in the same population, where most of the isolates from Chinese patients carried East Asian cagA type and most of the isolates from Indians and Malays carried the Western cagA type. This may explain the lower rates seen here. The prevalence of iceA1 and iceA2 were very low and there were no significant differences noted between these virulence factors and any pathology either individually or in combination.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) produces a number of virulence factors that are essential for colonization of the stomach and survival in the hostile gastric environment. Studies have shown considerable inconsistencies with regard to the presence of virulence factors and their associations, depending on the population or geographic origin. The authors' aim was to determine the presence of these strains and their relationship with clinical outcome in a multi-ethnic cohort.

Research frontiers

The population of Malaysia is unique in that it comprises three major ethnic groups: Malays, Chinese and Indians. Studies have shown a low prevalence of *H. pylori* infection among the Malay community while Indians have the highest, however, the Chinese community have the highest rate of peptic ulcer pathology. Does the distribution of diverse strains among the groups have any relation to this observation?

Innovations and breakthroughs

The rate of *H. pylori* infection in Malaysia is lower than most countries in South-

east Asia. This may be partly due to the consistently lower incidence reported in the Malay community. The Chinese and Indian communities being third-generation immigrants have a high incidence but not as high as those noted in India or China. This is a trend noted in studies from the West which shows a persistently higher prevalence of *H. pylori* infection among first- and second-generation immigrants.

Applications

By creating awareness of the inconsistent distribution of *H. pylori* strains in this multi-ethnic group, this study may represent a change in strategy that is needed to address the management of dyspeptic symptoms in this part of the world. A large multicenter study to confirm these observations at national level is required in Malaysia.

Peer review

In this manuscript, Amjad *et al* reported their studies in the presence of *H. pylori* virulence factors (cagA and iceA) and clinical outcome in Malaysian patients. The authors found that the *H. pylori* isolates from their patients carried an overall low rate of *cagA* and *iceA* genes as compared to other geographical regions including Southeast Asia. cagA was present in less than half the patients and the presence of iceA1 and A2 were not significantly related to any pathology.

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BRIEF ARTICLE

p73 G4C14 to A4T14 polymorphism is associated with colorectal cancer risk and survival

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Abstract

AIM: To analyze the association between the p73 G4C14-to-A4T14 polymorphism (a.k.a., the GC/AT variation) and colorectal cancer risk and survival in the Korean population, and to evaluate the relationships between p73 polymorphism and the p73 protein expression or clinicopathological characteristics of colorectal cancer.

METHODS: Three hundred and eighty-three histologically confirmed cases and 469 healthy controls, recruited at one teaching hospital in Pusan, Korea from 2001 and 2007, were genotyped for p73 G4C14-to-A4T14 by PCR with confronting two-pair primers (PCR-CTPP) and the expression profile of p73 in cancer tissues (n = 383) was analyzed by immunohistochemistry.

RESULTS: Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression model adjusted for age and gender. Compared with the GC/GC genotypes, the GC/AT and AT/AT genotypes were significantly associated with colorectal cancer risk (GC/AT *vs* GC/GC: OR = 1.46, 95% CI: 1.10-1.94; AT/AT *vs* GC/GC: 1.72, 0.98-3.03; P_{trend} = 0.01). When stratified by age and gender, the association was restricted to those less than 60 years of age (GC/AT or AT/AT *vs* GC/GC: 2.22, 1.39-3.55) and male (GC/AT or AT/AT *vs* GC/GC: 1.91, 1.31-2.77). The expression of p73 was associated with invasion depth (P = 0.003) and advanced Duke's stage (P = 0.06) of colorectal cancer. The patients with the GC/GC genotype were associated with worse survival compared with those with the other genotypes (P = 0.02). However, no significant relationship was observed between the p73 G4C14-to-A4T14 polymorphism and p73 protein expression in cancer tissues.

CONCLUSION: Our results suggest that the p73 GC/AT polymorphism is associated with an increased colorectal cancer risk and survival in the Korean population.

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Key words: p73 G4C14 to A4T14 polymorphism; Colorectal cancer

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INTRODUCTION

Colorectal cancer is a multigenetic disease whereby biologically relevant single nucleotide polymorphisms might play roles as genetic susceptibility factors. One of the candidates is the p73 gene, which is structurally similar to p53 and localized to the 1p36 chromosomal region[1]. Functionally, p73 activates the promoters of several p53-responsive genes participating in cell-cycle control, DNA repair, and apoptosis and inhibits cell growth in a p53-like manner by inducing apoptosis or G_1 cell-cycle arrest[2,3]. Increased p73 protein expression has been found in human malignancies associated with p53 mutations[4,5], suggesting a role of p73 in compensating for the loss of p53 function[4-6]. However, overexpression of wild-type p73 might have some p53-independent functions either as an oncogene or as a tumor suppressor gene in human tumorigenesis[7,8].

It is not known whether the alteration of p73 expression has any genetic basis such as sequence variations or polymorphisms. The two completely linked single nucleotide polymorphisms at positions 4 (G→A) and 14 (C→T) affect p73 function by altering gene expression[1]. This dinucleotide polymorphism lies just upstream of the initiating AUG in exon 2, a region that could form a stem-loop structure and modulate susceptibility to cancer[1,9,10]. There are several studies indicating that subjects with the p73 GC/AT polymorphism might have an increased risk for certain types of cancers, including colorectal cancer[11-13]. Other studies reported that overexpression of the p73 protein has been seen in benign and malignant tumors, including colorectal cancer, when compared with the matched normal tissues[14]. In addition, p73 overexpression was correlated with a poor prognosis in colorectal cancer[14], hepatocellular carcinoma[15], and breast cancer[16] in several studies. However, the genotype-phenotype relationship between the p73 polymorphism and p73 protein expression in colorectal cancer has not yet been studied.

In this context, we hypothesized that the p73 GC/AT polymorphism plays an important role in colorectal carcinogenesis and progression of colorectal cancer in the Korean population. We also evaluated the relationship between the p73 polymorphism and p73 protein expression.

MATERIALS AND METHODS

Study population and tissue samples

Three hundred and eighty-three colorectal cancer cases and 469 cancer-free controls were recruited from the Dong-A University Medical Center between January 2001 and December 2007. All cancer cases were histopathologically confirmed and had no preoperative chemotherapy or radiotherapy. The clinical records and pathological reports were also obtained for cases. The HE-stained slides were reviewed in each case to confirm the original diagnosis, which was based on the WHO classification[17]. Postoperative pathological staging was determined according to the Dukes' classification system for colorectal cancer[18]. The control subjects were randomly selected from a pool of non-cancer individuals of Busan city residents. These subjects were matched to cases by age (± 5 years) and area of residence.

This study was approved by the institutional review board and written informed consent was obtained from all subjects.

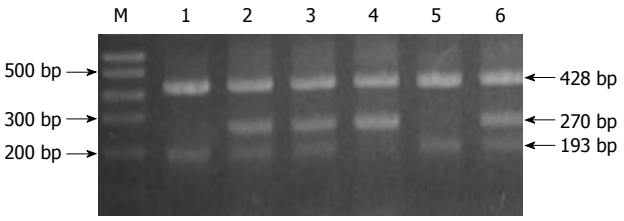

Figure 1 Detection of the p73 genotype by polymerase chain reaction with confronting two-pair primers. Lane M: 100-bp DNA ladder; Lanes 1 and 5: GC/GC genotype; Lanes 2, 3 and 6: GC/AT genotype; Lane 4: AT/AT genotype.

Genotyping

Immediately after surgical resection of colorectal cancer, tumor tissues and adjacent normal colon tissues were sampled by a pathologist and stored at -80℃. The genomic DNA was extracted from normal colorectal tissue (cancer cases) and blood samples (controls) using a Wizard genomic DNA purification kit (Promega, USA), according to the manufacturer's instructions.

DNA amplification and genotyping of the p73 GC/AT polymorphism were performed by the method of the polymerase chain reaction with confronting two-pair primers (PCR-CTPP). DNA was amplified with the primers: 5'CCACGGATGGGTCTGATCC (F1) and 5'GGCCTCCAAGGGCAGCTT (R1) for the A allele, and 5'CCTTCCTTCCTGCAGAGCG (F2) and 5'TTAGCCCAGCGAAGGTGG (R2) for the G allele. Allele specific bases are underlined. PCR was performed in a volume of 20 μL containing 100 ng of DNA template, 0.2 mmol/L each deoxynucleotide triphosphate, 1 × PCR buffer (50 mmol/L KCl, 10 mmol/L Tris HCl, and 0.1% Triton X-100), 1.5 mmol/L $MgCl_2$, 1 U of *Taq* polymerase (Sigma-Aldrich Biotechnology) and 10 pmol of each of four primers. For amplification, an initial denaturation step at 95℃ for 10 min was followed by 35 cycles of 95℃ for 1 min, 62℃ for 45 seconds, and 72℃ for 1 min, and a final extension step at 72℃ for 5 min. The amplified DNA was visualized on a 2% agarose gel with ethidium bromide staining. The p73 G4A polymorphism was genotyped as a 193 base pair band for the G allele, a 270 base pair band for the A allele, and a 428 base pair common band (GenBank: AL 136528, Figure 1). The genotyping by PCR-CTPP analysis was confirmed

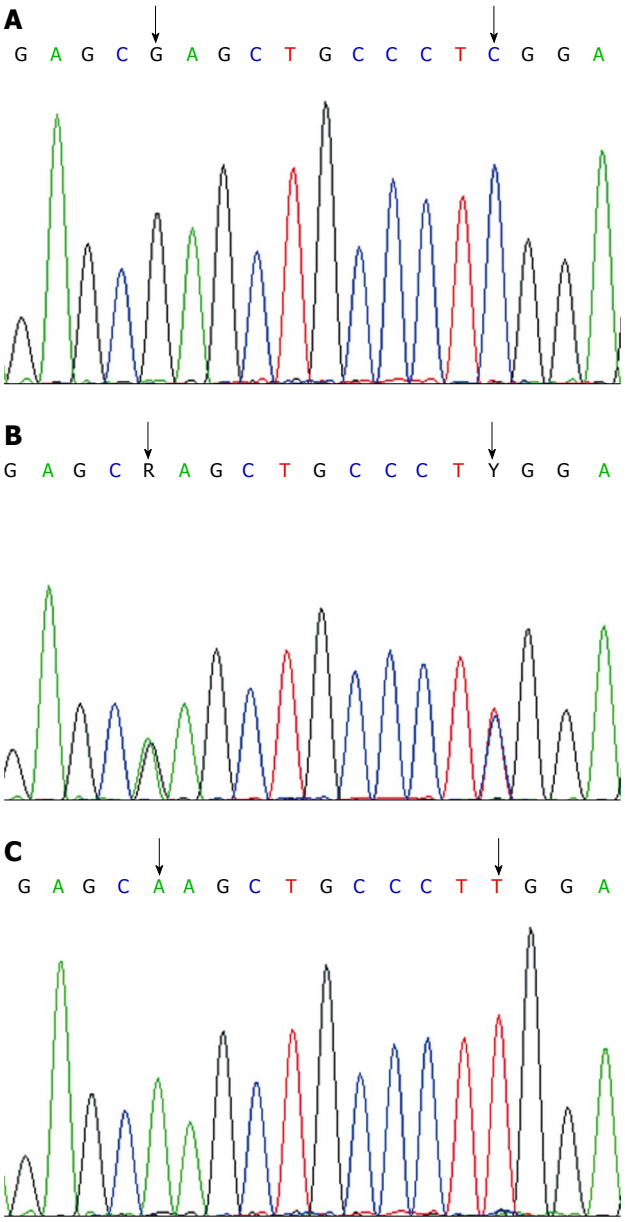

Figure 2 GC/GC genotype (A), GC/AT genotype (B), AT/AT genotype (C) confirmed by sequencing.

by DNA sequencing analysis; the results of PCR-CTPP genotyping and sequencing analysis were completely concordant (Figure 2). The genotype distributions of controls were in agreement with Hardy-Weinberg equilibrium ($P = 0.71$).

Immunohistochemistry

Immunohistochemical study was performed for p73 protein expression with tissue microarray (TMA) slides prepared by the avidin-biotin-peroxidase complex method. Deparaffinization of all the sections was performed through a series of xylene baths, and rehydration was performed with a series of graded alcohol solutions. To enhance immunoreactivity, microwave antigen retrieval was performed at 750 W for 30 min in Tris-EDTA buffer (pH 9.0). After blocking endogenous peroxidase activity with 5% hydrogen peroxidase for 10 min, primary antibody incubation was performed for 1 h at room temperature. The primary antibody was a mouse monoclonal antibody directed against p73 (abcam, Cambridge, UK) used in a 1:100 dilution. An Envision Chem Kit (DakoCytomation, Carpinteria, CA, USA) was used for the secondary antibody at room temperature for 30 min. After washing the tissue samples in Tris-buffered saline for 10 min, 3,3'-diaminobenzidine was used as a chromogen, and Mayer's hematoxylin counterstain was applied.

All the slides were evaluated without knowledge of any of the clinicopathological data. p73 immunoreactivity was defined by a nuclear staining pattern of the lesional tissue with a minimal background. The percentage of immunoreactive tumor cells was categorized into four groups: 0 (0%), 1 (1%-10%), 2 (11%-50%), and 3 (> 50%). The staining intensity was also categorized into four groups: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). A final score was obtained for each case by multiplying the percentage and the intensity score. Finally, tumors with multiplied scores exceeding 4 (i.e. tumors with a moderate and strong intensity of > 10% of the tumor cells) were recorded as positive immunoreactivity to p73; all the other scores were considered negative.

Statistical analysis

Colorectal cancer risks were estimated as the odds ratios (OR) and 95% confidence intervals (CI) calculated by an unconditional logistic regression model adjusted for age and gender. χ^2 tests were used to assess the relationship between the immunoreactivity of p73 protein and the clinicopathological characteristics of colorectal cancer patients and p73 genotypes. In survival analysis, survival curves were computed according to the Kaplan-Meier method. All statistical analysis was performed with the Statistical Package Service Solution software (SPSS for Windows, Standard version 14.0, Chicago, IL, USA).

RESULTS

The p73 G4C14 to A4T14 polymorphism and colorectal cancer risk

Due to frequency matching, the median ages were similar between cases (60.4 ± 11.6 years) and controls (60.2 ± 6.2 years) (data not shown).

The genotype frequencies of the p73 G4C14 to A4T14 polymorphism among the controls and cases are shown in Table 1. The frequency of the p73 genotypes in the colorectal cancer patients (GC/GC 47.8%, GC/AT 44.6%, AT/AT 7.6%) was significantly different from that in the control groups (GC/GC 57.8%, GC/AT 36.9%, AT/AT 5.3%). When the GC/GC genotype was used as the reference, the GC/AT and the AT/AT genotypes were significantly associated with the risk for colorectal cancer (OR = 1.46, 95% CI: 1.10-1.94; and OR = 1.72, 95% CI: 0.98-3.03, respectively; $P_{trend} = 0.01$). In a dominant model, the combined variant genotype (GC/AT or

Table 1 Distribution of the *p73* G4C14 to A4T14 polymorphism and colorectal cancer risk *n* (%)

p73 genotype	Cases (*n* = 383)	Controls (*n* = 469)	OR (95% CI)[1]
GC/GC	183 (47.8)	271 (57.8)	1.00 (reference)
GC/AT	171 (44.6)	173 (36.9)	1.46 (1.10-1.94)
AT/AT	29 (7.6)	25 (5.3)	1.72 (0.98-3.03)
GC/AT or AT/AT	200 (52.2)	198 (42.2)	1.50 (1.14-1.96)
P_{trend}			0.01

[1]Adjusted for age and gender.

Table 2 Stratified analysis by age and gender for the association between *p73* G4C14 to A4T14 polymorphism and colorectal cancer risk

Variables	*p73* genotype		Odds ratio of GC/AT + AT/AT OR (95% CI)[1]
	GC/GC (case/control)	GC/AT + AT/AT (case/control)	
Age (yr)			
< 60	70/81	96/50	2.22 (1.39-3.55)
⩾ 60	113/190	104/148	1.18 (0.84-1.66)
Gender			
Male	88/153	114/104	1.91 (1.31-2.77)
Female	95/118	86/94	1.14 (0.76-1.69)

[1]Adjusted for age and gender.

AT/AT) increased the risk by 1.5-fold compared with the GC/GC genotype (OR = 1.50, 95% CI: 1.14-1.96). Stratified analysis showed that the association was restricted to patients less than 60 years of age (GC/AT or AT/AT *vs* GC/GC: 2.22, 1.39-3.55) and male (GC/AT or AT/AT *vs* GC/GC: 1.91, 1.31-2.77) (Table 2).

When various clinicopathological characteristics of the colorectal cancer patients were compared by the p73 GC/AT genotypes, p73 G4C14 to A4T14 polymorphism was correlated with age ($P = 0.003$), but there were no significant associations of p73 polymorphism with gender, histological type, invasion depth, lymph node metastasis, and Dukes' stage (each $P > 0.05$) (data not shown).

Relationship between the p73 G4C14 to A4T14 polymorphism and p73 expression

p73 immunoreactivity was detected in 252 (65.8%) out of the 383 colorectal cancer tissues (Table 3). The proportion of positive p73 expression was higher among those with GC/AT or AT/AT genotypes (54.0%) than that among those with GC/GC genotype (46.0%) ($P = 0.40$). Although there was no statistical significance, AT/AT genotype (6.0%) showed a tendency towards lower expression of the p73 protein expression than those with GC/GC genotype (46.0%) and GC/AT genotype (48.0%) when stratified by three genotypes ($P = 0.09$).

Among various clinicopathological characteristics, invasion depth ($P = 0.003$) and advanced Duke's stage ($P = 0.06$) of colorectal cancer were correlated with p73 expression. There were no significant associations of p73 protein expression with age, gender, histological type, and lymph node metastasis (each $P > 0.05$, Table 4, Figure 3).

Table 3 The relationship between p73 protein expression and genotypes in 383 colorectal cancer patients *n* (%)

Genotype	Cases	p73 protein expression		*P*
		Positive (*n* = 252)	Negative (*n* = 131)	
GC/GC	183	116 (46.0)	67 (51.1)	0.09
GC/AT	171	121 (48.0)	50 (38.2)	
AT/AT	29	15 (6.0)	14 (10.7)	
P_{trend}				0.40
GC/GC	183	116 (46.0)	67 (51.1)	
GC/AT+AT/AT	200	136 (54.0)	64 (48.9)	

Table 4 The relationship between p73 protein expression and clinicopathological characteristics in 383 colorectal cancer patients *n* (%)

Clinicopathological characteristics	No. of cases	p73 protein expression		*P*
		Positive (*n* = 252)	Negative (*n* = 131)	
Age (yr)				0.45
< 60	166	113 (68.1)	53 (31.9)	
⩾ 60	217	139 (64.1)	78 (35.9)	
Gender				1.00
Male	203	134 (66.0)	69 (34.0)	
Female	180	118 (65.6)	62 (34.4)	
Histological type				0.19
Well	256	169 (66.0)	87 (34.0)	
Moderate	102	64 (62.7)	38 (37.3)	
Poorly	12	7 (58.3)	5 (41.7)	
Mucinous	13	12 (92.3)	1 (7.7)	
Invasion depth				0.003
Mucosa	3	1 (33.3)	2 (66.7)	
Submucosa	9	9 (100)	0 (0)	
Muscle	47	39 (83.0)	8 (17.0)	
Subserosa	324	203 (62.7)	121 (37.3)	
Lymph node metastasis				0.67
Positive	175	113 (64.6)	62 (35.4)	
Negative	208	139 (66.8)	69 (33.2)	
Dukes' stage				0.06
A	3	1 (33.3)	2 (66.7)	
B1	43	36 (83.7)	7 (16.3)	
B2	162	102 (63.0)	60 (37.0)	
C1	13	10 (76.9)	3 (23.1)	
C2	148	92 (62.2)	56 (37.8)	
D	14	11 (78.6)	3 (21.4)	

The p73 G4C14 to A4T14 polymorphism and colorectal cancer survival

The patients with GC/GC genotype were associated with worse survival compared with those with the other genotypes ($P = 0.02$, Figure 4). The 5-year overall survival (OS) was 64.1% among those with GC/GC genotype and 76.3% among those with GC/AT or AT/AT genotypes.

DISCUSSION

In our study, the p73 GC/AT polymorphism was associat-

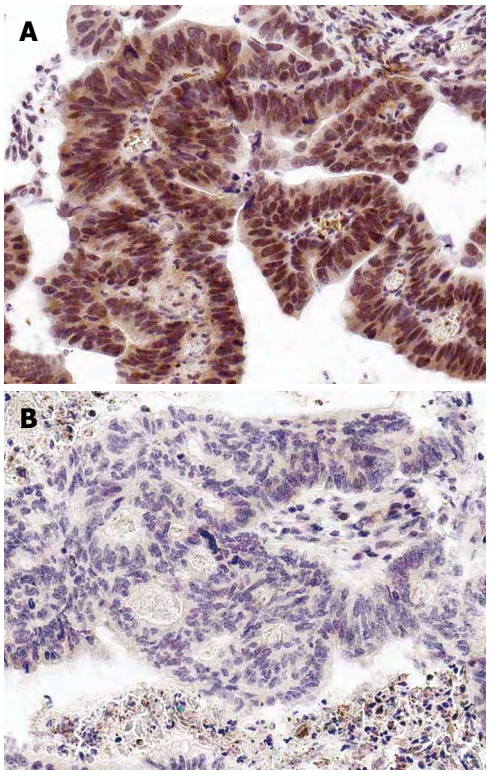

Figure 3 Immunohistochemical analysis of p73 in colorectal cancer tissues (immunohistochemistry, × 200). A: An adenocarcinoma with the GC/GC genotype revealed positive nuclear immunoreactivity for p73; B: An adenocarcinoma with AT/AT genotype revealed negative immunoreactivity for p73.

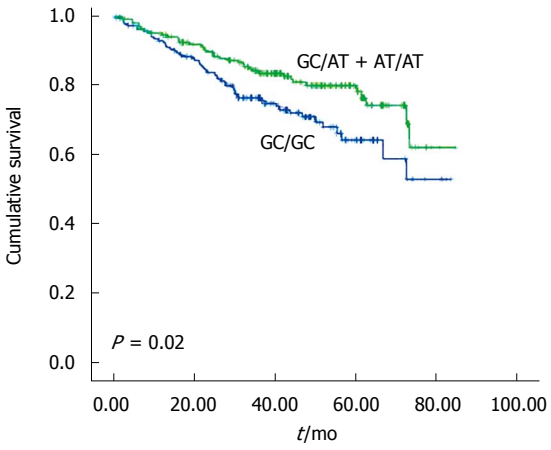

Figure 4 The p73 G4C14 to A4T14 polymorphism and colorectal cancer survival. Kaplan-Meier analysis of overall survival for 383 colorectal cancer patients shows that the patients with the GC/GC genotype are associated with worse survival compared with those with other genotypes (P = 0.02).

ed with an increased risk of colorectal cancer. When stratified by age and gender, male subjects less than 60 years of age with p73 GC/AT polymorphisms were at an increased risk of colorectal cancer. We suggest that the p73 GC/AT polymorphisms, which is associated with an early onset of less than 60, might play a more important role in the development of colorectal cancer among younger patients.

The association of the p73 GC/AT polymorphism with cancer risk has been investigated in a variety of cancers[11-13,19,20]. There are several studies indicating that subjects with the p73 GC/AT polymorphism might have an increased risk for head and neck cancer[12] and lung cancer in an American population[13]. Pfeifer *et al*[11] found a relationship of the AT/AT genotype with high risk for colorectal cancer in a Swedish population, which is consistent with our results. Other studies, however, reported that the p73 GC/AT polymorphism was associated with a significantly decreased risk for lung cancer in a Chinese population[19] and esophageal cancer in an Irish population[20]. A few studies even failed to find any correlation between the p73 GC/AT polymorphism and risk for lung cancers in Korean and Japanese populations[21,22]. These inconsistent findings suggest that the p73 polymorphism might have different roles in various cancer types and ethnic populations.

The mechanism whereby the p73 polymorphism influences colorectal cancer development remains to be elucidated. One study has shown that the GC to AT change might lead to formation of a stem-loop structure, which could influence p73 translation efficiency[1]. Another study suggested that the p73 GC/AT polymorphism has a critical role in the development or progression of early cancerous lesions, possibly due to alteration in expression of the p73 protein, perhaps due to alternative splicing[20]. Another study has reported that the GC/AT polymorphism is in linkage disequilibrium with other functional polymorphisms that affect either the expression or activity levels of proteins or enzymes involved in tumorigenesis[19]. However, we note that no significant relationship between the p73G4C14-to-A4T14 polymorphism and p73 protein expression was found in our study. The exact relationship between p73 polymorphism and p73 protein expression might not have been detected due to limitations of this study such as small number of AT/AT groups, relatively small and heterogeneous histological and stage subgroups, and the retrospective study design.

In our study, the expression of p73 was associated with invasion depth and advanced Duke's stage of colorectal cancer. These results suggest that p73 protein has an important role in colorectal cancer progression. p73 overexpression has been found in various types of cancer, including breast[23], lung[7,8], colorectal[24], and liver[25]. These reports indicated that p73 might be involved in the development of cancers. p73 was initially suggested to be a tumor suppressor gene[1]. However, the result that p73 knockout mice do not develop spontaneous tumors[26] and the p73 gene is rarely mutated in a human cancers[16], suggesting that the activation of a silent allele or overexpression of p73, rather than an impairment of tumor suppressor function, might contribute to tumor development.

To explain the role and mechanism of p73 protein expression, this study investigated the associations between p73 polymorphism and p73 protein expression. There was no statistical significance, when the positive p73 expression in cases with GC/GC genotype (46.0%), GC/AT genotype (48.0%), and AT/AT genotype (6.0%) were

stratified by three genotypes. Sun[14] found that overexpression of p73 protein was a valuable prognostic factor to predict poor outcome for patients with colorectal cancer. Tannapfel *et al*[15] found that patients with p73 positive tumors had a poorer prognosis than those with p73 negative tumors in hepatocellular carcinoma patients. In this study, the patients with GC/GC genotype were associated with worse survival compared with those with the other genotypes.

In conclusion, this study provides the first indication that the p73 G4C14 to A4T14 polymorphism is significantly associated with an increased colorectal cancer risk in a Korean population. Moreover, our study suggests an association between the p73 GC/AT polymorphisms and men with early-onset colorectal cancer. There was a significant association of p73 protein expression with invasion depth, which suggests that p73 immunoreactivity may be a useful pathological marker for colorectal cancer progression. However, patients with the GC/GC genotype were significantly associated with worse survival than those with GC/AT polymorphism. Future molecular epidemiologic studies are needed to elucidate the relationship between p73 polymorphism, its related genes, such as members of the p53 family, and p73 protein expression in colorectal cancer development, and to define whether the association of p73 protein expression may independently occurs of p73 polymorphism or not.

COMMENTS

Background

Colorectal cancer is a multigenetic disease whereby biologically relevant single nucleotide polymorphisms might play role as a genetic susceptibility factors. As one of the p53 tumor suppressor family, p73 is known to inhibit cell growth in a p53-like manner, by inducing apoptosis or cell cycle arrest. However, overexpression of wild-type p73 might have some p53-independent functions either as an oncogene or as a tumor suppressor gene in human tumorigenesis.

Research frontiers

There are several studies indicating that subjects with the p73 GC/AT polymorphism might have an increased risk for certain types of cancers, including colorectal cancer. However, the genotype-phenotype relationship between p73 polymorphism and p73 protein expression in colorectal cancer has not yet been studied. In this study, patients with the GC/GC genotype were associated with worse survival compared with those with the other genotypes. However, no significant relationship was observed between the p73 G4C14-to-A4T14 polymorphism and p73 protein expression in colorectal cancer.

Innovations and breakthroughs

This study provides the first evidence that the p73 G4C14 to A4T14 polymorphism is significantly associated with an increased colorectal cancer risk in the Korean population. Moreover, our study suggests an association between the p73 GC/AT polymorphisms and men with early-onset colorectal cancer. There was a significant association of p73 protein expression with invasion depth, which suggests that p73 protein has a certain important role in colorectal cancer progression.

Application

To define whether the association of p73 protein expression occurs independently of the p73 polymorphism or not, further study is required. This study provides a valuable prognostic factor to predict poor outcome for patients with colorectal cancer.

Peer review

The author examined the *p73* G4C14 to A4T14 polymorphism and *p73* protein expression. The p73 GC/AT polymorphism is associated with an increased colorectal cancer risk and survival in the Korean population. The expression of p73 was associated with invasion depth and advanced Duke's stage of colorectal cancer. The results are interesting and might provide a useful pathological marker for colorectal cancer progression.

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BRIEF ARTICLE

Antacid effects of Chinese herbal prescriptions assessed by a modified artificial stomach model

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Abstract

AIM: To assess the antacid effects of the tonic Chinese herbal prescriptions, Si-Jun-Zi-Tang (SJZT) and Shen-Ling-Bai-Zhu-San (SLBZS).

METHODS: Decoctions of the tonic Chinese herbal prescriptions, SJZT and SLBZS, were prepared according to Chinese original documents. The pH of the prescription decoctions and their neutralizing effects on artificial gastric acids were determined and compared with water and the active controls, sodium bicarbonate and colloidal aluminum phosphate. A modified model of Vatier's artificial stomach was used to determine the duration of consistent neutralization effect on artificial gastric acids. The neutralization capacity *in vitro* was determined with the titration method of Fordtran's model.

RESULTS: The results showed that both SJZT and SLBZS have antacid effects *in vitro*. Compared with the water group, SJZT and SLBZS were found to possess significant gastric acid neutralizing effects. The duration for consistent neutralization of SLBZS was significantly longer than that of water. Also, SLBZS and SJZT exhibited significant antacid capacities compared to water.

CONCLUSION: SJZT and SLBZS were consistently active in the artificial stomach model and are suggested to have antacid effects similar to the active control drugs.

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Key words: Traditional Chinese medicines; Antacid; Acid neutralizing capacity; Artificial stomach

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INTRODUCTION

Peptic ulcer (PU) is one of the most common gastrointestinal disorders[1-3]. The goals of treatment for PU are to relieve pain, enhance ulcer healing, prevent complications, and prevent ulcer recurrence. Current drug therapy for PU is oriented primarily toward neutralizing (e.g. antacids)[4-6] or reducing the amount of acid secreted (e.g. H_2 receptor antagonists, proton pump inhibitors)[7,8] or protecting the gastric mucosa from the effects of acid (e.g. sucralfate)[9]. As the role of *Helicobacter pylori* is becoming better understood, treatment with antibiotics is becoming an important part of PU therapy and recurrence prevention[10].

Although effectiveness can be obtained with these

clinical drugs, their potential side effects and drug interactions represent a major problem in therapy[11]. Moreover, newer drugs introduced for the treatment of PU are expensive and the cost-effectiveness is also an important consideration[12]. Clinically, many people cannot use chemosynthetic drugs because of the potential side effects. Therefore, traditional herbal drugs possessing fewer side effects should be investigated as an ideal alternative for the treatment of PU.

Traditional Chinese medicine has been clinically used in China for thousands of years for the treatment of many chronic diseases. Si-Jun-Zi-Tang (SJZT) is a basic tonic prescription consisting of six herb components: *Panax ginseng*, *Atractylodes ovata*, *Poria cocos*, *Glycyrrhiza uralensis*, *Zingiber officinale*, and *Zizyphus jujube*. Shen-Ling-Bai-Zhu-San (SLBZS) is also an energy tonic prescription consisting of eleven herb components: *Atractylodes ovata*, *Poria cocos*, *Panax ginseng*, *Glycyrrhiza uralensis*, *Dioscorea batatas*, *Dolichos lablab*, *Coix lachryma-jobi*, *Amomum xanthioides*, *Platycodon grandiflorum*, *Nelumbo nucifera* and *Citrus erythrosa*. SJZT and SLBZS, the common Chinese herbal prescriptions tonifying the spleen and stomach, are traditionally used for the treatment of gastrointestinal diseases in oriental countries. Their anti-ulcer activities have also been mentioned in recent research.

The secretion of gastric acid (HCl) is intimately related to PU disease. Antacids heal ulcers through elimination of gastric acid by neutralization and have been used in the treatment of PU for many years. Common antacid preparations include sodium bicarbonate (SB), calcium carbonate, and salts of aluminum and magnesium. Since some people cannot use chemosynthetic drugs because of their side effects, the tonic Chinese herbal prescriptions SJZT and SLBZS should be considered as an alternative for the treatment of PU. To reveal the anti-ulcer effects of SJZT and SLBZS, therefore, the present study aimed to assess their antacid effects on gastric acid neutralization compared with water and the active controls, SB and colloidal aluminum phosphate (CAP). The antacid effects were assessed *in vitro* using the titration method of Fordtran's model[13], and the modified model of Vatier's artificial stomach[14-17] was utilized to determine the effects on gastric acid secretion and emptying.

MATERIALS AND METHODS

Preparation of the Chinese prescriptions

All the Chinese herbal materials used in the study were purchased from the Shun-yuan Herbal Pharmacy, Taipei and authenticated at Taipei Medical University. The prescription SJZT contains the following six herbal components: *Panax ginseng*, *Atractylodes ovata*, *Poria cocos*, *Glycyrrhiza uralensis*, *Zingiber officinale*, and *Zizyphus jujube* (6:6:6:3:3:2). The prescription SLBZS contains the following eleven herbal components: *Atractylodes ovata*, *Poria cocos*, *Panax ginseng*, *Glycyrrhiza uralensis*, *Dioscorea batatas*, *Dolichos lablab*, *Coix lachryma-jobi*, *Amomum xanthioides*, *Platycodon grandiflorum*, *Nelumbo nucifera* and *Citrus erythrosa* (3:3:3:3:3:2.3:1.5:1.5:1.5:1.5:1.5). The prescriptions were prepared according to Chinese original documents. Prepared daily quantities of SJZT and SLBZS weighed 26 g and 24.8 g, respectively. The herb materials weighed as a daily quantity were extracted twice by refluxing with boiling water (1:20, w/v) for 1 h. The decoction was filtered and then concentrated to 270 mL. Concentrations of SJZT and SLBZS decoctions were 96.30 mg/mL and 91.85 mg/mL, respectively. The volume of each test dose was 90 mL.

Chemicals and reagents

Sodium chloride and pepsin were purchased from Sigma (St. Louis, MO, USA) and 1 mol/L hydrochloric acid was obtained from Merck (Darmstadt, Germany). SB and CAP, the active control drugs, were purchased from Astar Pharmaceutical Co. Ltd (Taipei, Taiwan).

Instruments

The experimental instruments consisted of an adjustable electrode stand, a series flatbed recorder, a micro tubing pump, a standard pH meter, a stirrer/hot plate, and a series multi-functional temperature controller.

Preparation of artificial gastric acid and saline

Two grams of salt and 3.2 mg of pepsin enzymes were dissolved in 500 mL water; 7.0 mL hydrochloric acid and adequate water were added to make a 1000 mL solution of the artificial gastric acid at pH 1.20. Nine grams of sodium chloride were dissolved in adequate water to make 1000 mL saline.

pH determination of the prescription decoction

One dose of prescription decoction (90 mL) was used for the pH determination at temperatures ranging from 25℃ to 37℃. The pH values of the control solutions, SB and CAP, were also determined for comparison.

Determination of the neutralizing effects on artificial gastric acids

Ninety milliliters of each test solution was added to 100 mL artificial gastric juices at pH 1.2. The pH values were determined to examine the neutralizing effect.

Determination of the duration of consistent neutralization effect on artificial gastric acids using the modified model of Vatier's artificial stomach

As previously described, the apparatus is made up of three elements: a pH recording system (R), a stomach (S) and a peristaltic pump (P). The stomach is made up of three portions, S1, S2 and S3. S1 is a reservoir (container), S2 models the secretory flux (F-IN), and S3 models the gastric emptying flux (F-OUT).

Ninety milliliters of each test sample was added to 100 mL of artificial gastric juice at pH 1.2 in the container of the artificial stomach at 37℃ and continuously stirred (30 rpm) with a 2.5-cm magnetic stirring apparatus. Artificial gastric juice at pH 1.2 was pumped at 3 mL/min into the container of the artificial stomach, and it was pumped out at 3 mL/min at the same time. A pH meter

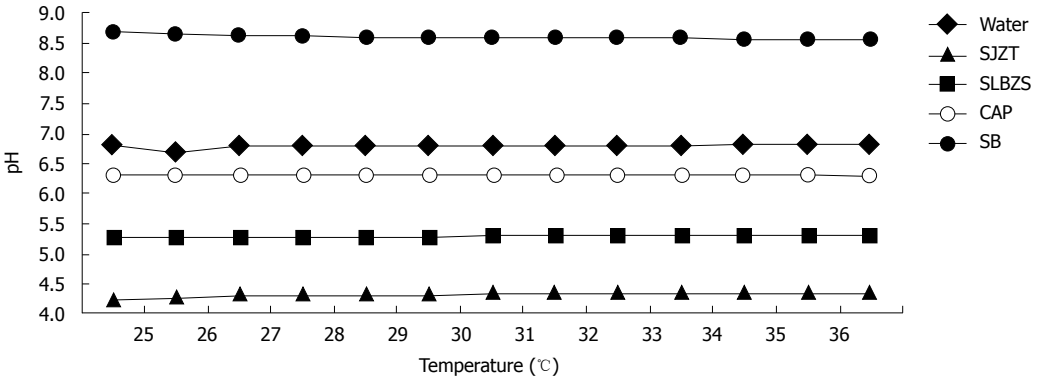

Figure 1 pH values of test samples determined at temperatures from 25℃ to 37℃. SJZT: Si-Jun-Zi-Tang; SLBZS: Shen-Ling-Bai-Zhu-San; CAP: Colloidal aluminum phosphate; SB: Sodium bicarbonate.

was connected to continuously monitor the changes of pH in the container of the artificial stomach. The duration of neutralization effect was determined when the pH value was returned to its initial value (pH 1.2).

Determination of the neutralization capacity in vitro using the titration method of Fordtran's model

Ninety milliliters of the test sample was placed in a 250-mL beaker and warmed to 37℃. A magnetic stirrer was continuously run at 30 rpm to imitate the stomach movements. The test samples were titrated with artificial gastric juice to the end point of pH 3. The consumed volume (V) of artificial gastric juice was measured. The total consumed hydrogen ion (mmol) was measured as 0.063096 (mmol/mL) × V (mL).

Statistical analysis

Experimental data were expressed as mean ± SD. Comparisons between groups were analyzed by unpaired Student's *t*-test. The differences were considered to be statistically significant when $P < 0.05$.

RESULTS

pH values of the tested solutions at temperatures ranging from 25℃ to 37℃

The pH values of the SJZT and SLBZS solutions at temperatures from 25℃ to 37℃ ranged from 4.25 to 4.34 and 5.25 to 5.30, respectively. The pH values of water, CAP and SB solutions at temperatures from 25℃ to 37℃ ranged from 6.79 to 6.81, 6.32 to 6.27 and 8.70 to 8.56, respectively. The results indicated that temperature did not affect pH significantly (Figure 1).

Neutralizing effects on artificial gastric acids

When 90 mL of the test solution was added to 100 mL of the artificial gastric juice (pH 1.2), the pH values of SJZT and SLBZS solutions were found to be 1.57 ± 0.04 and 1.76 ± 0.03, respectively. The pH values of water, CAP and SB solutions were 1.44 ± 0.03, 2.16 ± 0.03 and 1.83 ± 0.03, respectively. This result shows that the neutralizing capacity of CAP was better than those of SB, SLBZS and SJZT. The neutralizing capacity of SLBZS was similar to that of SB (Table 1).

Table 1 pH values as 90 mL of the test solution was added to 100 mL of artificial gastric juices

Drug	pH value
Water	1.44 ± 0.03
SJZT	1.57 ± 0.04[a]
SLBZS	1.76 ± 0.03[b]
CAP	2.16 ± 0.03[b]
SB	1.83 ± 0.03[b]

Data are presented as mean ± SD ($n = 6$). [a]$P < 0.05$, [b]$P < 0.01$ *vs* water group. SJZT: Si-Jun-Zi-Tang; SLBZS: Shen-Ling-Bai-Zhu-San; CAP: Colloidal aluminum phosphate; SB: Sodium bicarbonate.

Table 2 Duration of antacid effect for consistent neutralization of gastric acids

Drug	Time (min)
Water	87 ± 6
SJZT	123 ± 24
SLBZS	127 ± 5[a]
CAP	172 ± 3[b]
SB	121 ± 14

Data are presented as mean ± SD ($n = 6$). [a]$P < 0.05$, [b]$P < 0.01$ *vs* water group. SJZT: Si-Jun-Zi-Tang; SLBZS: Shen-Ling-Bai-Zhu-San; CAP: Colloidal aluminum phosphate; SB: Sodium bicarbonate.

Duration of consistent neutralization effect on artificial gastric acids

The durations for consistent neutralizing effects of SJZT and SLBZS solutions were 123 ± 24 min and 127 ± 5 min, respectively. Those of water, CAP and SB solutions were 87 ± 6, 172 ± 3 and 121 ± 14 min, respectively. The action duration of CAP was the longest, followed by the SLBZS and SJZT (Table 2, Figure 2).

Physical antacid potency (neutralization capacity) in vitro

The consumed volumes of artificial gastric juices to titrate to pH 3.0 for water, SJZT, SLBZS, CAP and SB solutions were 1.2 ± 0.17, 22.4 ± 1.10, 33.3 ± 0.85, 58.9

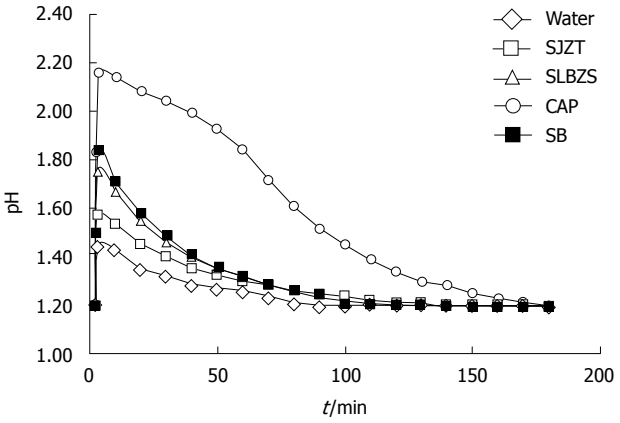

Figure 2 Duration of antacid effect for consistent neutralization of gastric acids. Ninety mililiters of test sample was added to 100 mL of artificial gastric juices at pH 1.2 in an artificial stomach. The duration was determined as the pH value was returned to 1.2. SJZT: Si-Jun-Zi-Tang; SLBZS: Shen-Ling-Bai-Zhu-San; CAP: Colloidal aluminum phosphate; SB: Sodium bicarbonate.

Table 3 Consumed volume of artificial gastric juice and hydrogen ion (mmol) in the titration of 90 mL test samples with pH 1.2 artificial gastric juice to the end point of pH 3

Drug	Consumed volume of artificial gastric juice (mL)	mmol of H^+
Water	1.2 ± 0.17	0.08 ± 0.01
SJZT	22.4 ± 1.10[b]	1.41 ± 0.07[b]
SLBZS	33.3 ± 0.85[b]	2.10 ± 0.05[b]
CAP	58.9 ± 0.79[b]	3.72 ± 0.05[b]
SB	69.2 ± 0.96[b]	4.37 ± 0.06[b]

Data are presented as mean ± SD (n = 6). [b]P < 0.01 *vs* water group. SJZT: Si-Jun-Zi-Tang; SLBZS: Shen-Ling-Bai-Zhu-San; CAP: Colloidal aluminum phosphate; SB: Sodium bicarbonate.

± 0.79 and 69.2 ± 0.96 mL, respectively. The consumed hydrogen ions were 0.08 ± 0.01, 1.41 ± 0.07, 2.10 ± 0.05, 3.72 ± 0.05 and 4.37 ± 0.06 mmol, respectively (Table 3). The active controls, SB and CAP, exhibited significant antacid potency. Simultaneously, neutralization capacities of the tonic Chinese herbal prescriptions, SJZT, SLBZS, were better than that of water.

DISCUSSION

There have recently been extraordinary advances in the understanding of the pathophysiology and treatment of PU[1-3]. It is generally accepted that it results from an imbalance between gastric aggressive factors and maintenance of the mucosal integrity through an endogenous defense mechanism[5]. The main aggressive factors reported for several decades are acid and pepsin. Therefore, PU disease has been predominantly treated with antacids, H_2 receptor antagonists and proton pump inhibitors. Among these, antacids have been widely used in the treatment of ulcer disease for many years.

Antacids heal ulcers through elimination of gastric acid by neutralization; however, they do not decrease the volume of gastric secretions. Different antacid drugs vary markedly in their *in vivo* and *in vitro* potency, and that should be taken into account when antacids are prescribed. On the other hand, potency is not the only factor that should be considered in the choice of an antacid. Cost, taste, salt content, bowel habit, and side effects are also important.

Although antacids have been used frequently, their side effects and drug interactions represent a major clinical problem. Consequently, traditional Chinese herbal medicines have recently generated increasing interest for the treatment of ulcer disease. The common Chinese herbal prescriptions nourishing the spleen and stomach, SJZT and SLBZS, are traditionally used for the treatment of gastrointestinal diseases in oriental countries. Therefore, the present study applied the titration method of Fordtran's model and the modified model of Vatier's artificial stomach, which mimic the regular physiological functioning of a human stomach, to explore the antacid effects of the tonic Chinese herbal prescriptions, SJZT and SLBZS.

In the present study, the Chinese herbal prescriptions SJZT and SLBZS were found to have antacid effects *in vitro*. Compared with the water group, all the treatments including SJZT, SLBZS, CAP and SB were shown to possess significant gastric acid neutralizing effects (Table 1). With regard to the duration for consistent neutralization of gastric acids, the neutralization duration of CAP and SLBZS were significantly longer than that of water (Table 2). Also, SB, CAP, SLBZS and SJZT exhibited significant antacid capacities compared to water (Table 3). According to these findings, the Chinese tonic prescriptions, SJZT and SLBZS, are suggested to have antacid effects similar to CAP and SB.

Efficacious, intensive antacid therapy is often unacceptable because of the common side effects, especially altered bowel functions. Aluminum salts may cause constipation and magnesium salts cause diarrhea. SB should be avoided even though it is a potent neutralizer of acid because it contains significant amounts of sodium and may alter the systemic pH. In addition, antacid drug interactions have been frequently reported and this is a problem worthy of being noticed. The most clinically significant interactions occur with ferrous sulfate, tetracycline and quinolone antibiotics. Other interactions are potentially significant because they involve drugs with narrow therapeutic ranges. Considering the side effects and interactions of antacids, the traditional Chinese prescriptions possessing fewer side effects should be looked to as an alternative for the treatment of PU.

In traditional Chinese medicine, the spleen and stomach are the principal organs in tonification and are responsible for receiving, digesting and transforming food and drink into *Qi*, *Blood* and *Body Fluids*[18]. *Qi* is a type of refined *Essence* produced by the internal organs. *Qi insufficiency* may manifest in specific organs. For instance, *Spleen-Qi* deficiency manifests as a poor appetite, abdominal distension, loose stools or diarrhea, a feeling of heaviness in the limbs and fatigue[18,19]. The herbs that tonify the *Qi* are very effective for improving the general condition and treating general weakness. Traditionally, the Chinese herbal prescrip-

tions tonifying the spleen and stomach are widely used for the treatment of gastrointestinal diseases in oriental countries[20]. SJZT and SLBZS are common Chinese herbal prescriptions tonifying the spleen and stomach. As described above, SJZT consists of six herbal medicines and SLBZS consists of eleven herb components. Among these, *Panax ginseng*, *Atractylodes ovata*, *Poria cocos* and *Glycyrrhiza uralensis* are the major herbs in SJZT and SLBZS. Since these herbs are commonly used for tonifying *Qi* in clinical practice, further studies for the identification of the active components and to elucidate their mode of action are in progress.

In conclusion, the Chinese herbal prescriptions SJZT and SLBZS are consistently active in the artificial stomach model. There may be a potential benefit in offering these as an alternative for the treatment of PU. The mechanism of antacid activity by SJZT and SLBZS is not proven from the present results and requires further investigations.

COMMENTS

Background

Antacids, H_2 receptor antagonists and proton pump inhibitors are the predominant drug therapies for the treatment of peptic ulcer (PU). Although effectiveness can be obtained with these drugs, their potential side effects and drug interactions represent a major problem in therapy. Traditional Chinese herbal medicines have recently generated increasing interest for the treatment of ulcer disease.

Research frontiers

Si-Jun-Zi-Tang (SJZT) and Shen-Ling-Bai-Zhu-San (SLBZS), the common Chinese herbal prescriptions tonifying the spleen and stomach, are traditionally used for the treatment of gastrointestinal diseases in oriental countries. The present study aimed to assess the antacid effects of SJZT and SLBZS on gastric acid neutralization compared with water and the active controls. The antacid effects were assessed *in vitro* using the titration method of Fordtran's model, and the modified model of Vatier's artificial stomach was utilized to determine the effects on gastric acid secretion and emptying.

Innovations and breakthroughs

Clinical experience indicates the Chinese herbal prescriptions tonifying the spleen and stomach are traditionally used for the treatment of gastrointestinal diseases in oriental countries. Their anti-ulcer effects needed to be proven by scientific studies. This research is the first study using a modified artificial stomach model to reveal the antacid effects of SJZT and SLBZS. The related findings might contribute to clinical applications in future.

Applications

The study highlighted the antacid activities of SJZT and SLBZS in the artificial stomach model. There may be a potential benefit in offering an alternative for the treatment of PU. Further investigations for the identification of active components and to elucidate the mechanism of action are in progress.

Terminology

SJZT is a Chinese prescription consisting of six herb components: *Panax ginseng*, *Atractylodes ovata*, *Poria cocos*, *Glycyrrhiza uralensis*, *Zingiber officinale*, and *Zizyphus jujube*. SLBZS is a Chinese prescription consisting of eleven herb components: *Atractylodes ovata*, *Poria cocos*, *Panax ginseng*, *Glycyrrhiza uralensis*, *Dioscorea batatas*, *Dolichos lablab*, *Coix lachryma-jobi*, *Amomum xanthioides*, *Platycodon grandiflorum*, *Nelumbo nucifera* and *Citrus erythrosa*.

Peer review

Antacid effects of Chinese herbal prescriptions assessed by a modified artificial stomach model. This paper comprises an antacid and anti-ulcer study on two Chinese herbal preparations evaluated in an artificial stomach model. The study design is simple and easy to understand and useful in evaluating antacid studies. It is a neat and good study.

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BRIEF ARTICLE

Evaluation of cross-reactive antibody response to HVR1 in chronic hepatitis C

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Abstract

AIM: To evaluate the presence and cross-reactive antibodies against hypervariable region 1 (HVR1) in hepatitis C virus (HCV) infected patients and its relationship with the progression of the disease.

METHODS: Sixteen representative HVR1 proteins selected from a unique set of 1600 natural sequences were used to semiquantitate the cross-reactivity of HVR1 antibodies in the sera of HCV patients. Fifty-five chronic HCV patients including 23 with asymptomatic mild hepatitis, 18 with chronic hepatitis and 16 with liver cirrhosis patients were studied.

RESULTS: The degree of the cross-reactivity of anti-HVR1 antibodies in 23 patients with mild asymptomatic hepatitis was 3.09 ± 2.68, which was significantly lower than in those with chronic hepatitis (5.44 ± 3.93, $P < 0.05$) and liver cirrhosis (7.44 ± 3.90, $P < 0.01$). No correlation was observed between the broadness of the cross-reactivity anti-HVR1 antibodies and patient's age, infection time, serum alanine aminotransferase activity, or serum HCV-RNA concentration. It was the breath of cross-reactivity rather than the presence of anti-HVR1 antibody in HCV sera that was associated with the progression of liver disease.

CONCLUSION: The broadly cross-reactive HVR1 antibodies generated in natural HCV patients can not neutralize the virus, which results in persistent infection in patients with chronic hepatitis.

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Key words: Hepatitis C virus; Hypervariable region 1; Cross-reactivity

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INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of

post-transfusional and sporadic non-A, non-B hepatitis. HCV infection is persistent in over 70% of cases and may result in various forms of chronic hepatitis and other liver diseases, ranging from asymptomatic mild chronic hepatitis to cirrhosis and hepatocellular carcinoma[1].

HCV is a RNA virus that replicates with a high rate of mutation. The maximal variation is confined to a short sequence of the second envelope glycoprotein (E2), and has been designated the hypervariable region 1 (HVR1)[2]. It is accepted that the immune escape is the main mechanisms responsible for HCV persistence. It indicated that the viral and humoral responses may be two important factors in pathogenesis of hepatitis C[3,4]. The virological studies reveal that HCV circulates within an infected host as a heterogeneous viral population containing genetically distinct, but closely related variants, known as quasispecies[5,6]. The diversity of quasispecies and its relationship with the progression of liver disease and interferon treatment were largely studied by single-strand conformation polymorphism or sequencing of HVR1[7-9]. However, for the high antigenic variability of HVR1, the humoral response against the HVR1 could not be easily characterized in chronic HCV infection.

Early observations have suggested that the HVR1 is a critical neutralization domain. Antibodies to HVR1 in human serum have been shown to block viral attachment and protect chimpanzees from HCV infection[10,11]. The recent development of model systems, including retroviral HCV pseudotypes (HCVpp) and recombinant cell-culture derived infectious virions (HCVcc), has established the neutralization of HVR1 antibodies[12]. In viral infections, the appearance of neutralizing antibody is usually a prognostic marker which coincides with the onset of recovery from the disease and viral elimination from the circulation. Although an early anti-HVR1 response is associated with self-limiting acute infection[13], anti-HVR1 antibodies are frequently produced in the majority of chronically infected individuals and appear to coexist with the HVR1 variants[14]. In most instances, the sera of HCV infected patients are frequently cross-reactive with unrelated HVR1 sequences[15,16]. However, the cross-reactive nature of anti-HVR1 responses and the relationship between the cross-reactivity and the progression of hepatitis are largely unknown.

The HCVpp and HCVcc could characterize the neutralizing antibodies in HCV infection, but could not be used to detect antibodies to the hypervariable region conveniently. In this report, we used a protein microarray immobilized with a set of HVR1 proteins selected by alignments to study the humoral anti-HVR1 response in 57 cases of chronic HCV infections with different clinical course. Our findings suggest that the broadly cross-reactive antibody responses against HVR1 are associated with the progression of liver disease with chronic HCV infection, and this may contribute to the better understanding of HCV natural infection and its prognosis.

MATERIALS AND METHODS

Patients

All of the patients from the rural area of Zhao County in Hebei Province with a history of plasmapheresis before 1990 were first diagnosed as having HCV infection in 1993. Fifty-seven of them were followed up till 2007 and were seropositive for anti-HCV antibodies by a third-generation enzyme-linked immunosorbent assay (ELISA) technique (HCV ELISA 3.0, Ortho Diagnostic Systems, Raritan, NJ). Serum HCV-RNA was measured by quantitative completive reverse transcription polymerase chain reaction analysis (Amplicor, Roche Diagnostic Systems, Inc., Branchburg, NJ) according to the manufacturer's protocol. According to the results of ultrasound (US) evaluation and clinical symptoms, 23 of them had asymptomatic hepatitis, 18 chronic hepatitis and 16 liver cirrhosis. The US parameters, including the assessment of liver surface, liver parenchyma, hepatic vessel, spleen index, were used to define the severity of the liver disease[17,18]. The clinical symptoms, including fatigue, nausea, abdominal, pain, anorexia, itching, dark urine and extra hepatic manifestations, were used to define the asymptomatic and chronic hepatitis. The duration of the infection was calculated from the time of plasmapheresis. No antiviral treatment was given until blood sampling, and none of them was infected with hepatitis A or B viruses, and human immunodeficiency virus. The study protocol was approved by the committee of ethics of the authors' institution.

Selection and preparation of a representative set of HVR1 antigens

The 1600 HVR1 sequences from Genbank were ranked according to the results of multiple sequence alignments using Biosun molecular biology software[19,20]. The representative HVR1 sequence with properties of highest similarity in each group was selected according to their pairs (SP) scores.

The representative HVR1 sequences were modified considering the *Escherichia coli* (*E. coli*) favorable codon usage. The full-length genes were synthesized chemically and cloned into the *Bam*H I -*Eco*R I sites of the expression vector pGEX4T-2. All the genes were expressed as fusion protein with glutathione transferases (GST) in *E. coli*. Then the recombinant HVR1 antigens were purified by GST resin.

ELISA

Microplates were coated with 0.3 μg recombinant HVR1 antigen in 100 mmol/L phosphate buffer (pH 7.4) by incubation overnight at 4℃. The plates were blocked with the phosphate buffer containing 0.2% BSA at 4℃ for 3 h, and incubated with 100 μL of the serum sample 1:10 diluted with a sample buffer (100 mmol/L sodium phosphate buffer pH 7.5 containing 10% goat serum and 0.05% Tween) at 37℃ for 1 h. After being washed for five times with 100 mmol/L sodium phosphate buffer (pH 7.5) containing 0.05% Tween, the plate was incubated for 30 min at 37℃ with 1:25000 diluted horseradish peroxidase-conjugated monoclonal antibody against human IgG. After washing, the reaction was visualized in the substrate buffer (50 mmol/L sodium phosphate-citric acid buffer,

pH 5.0, containing 0.4 mg/mL tetramethylbenzidine and 0.4 μL/mL of 30% hydrogen peroxide). The reaction was stopped by adding 50 μL of 2 mol/L sulfuric acid, and the absorbance was measured in a microplate ELISA reader at 450 nm.

Preparation of HVR1 protein microarrays

All of the recombinant HVR1 antigens were diluted in spotting buffer (CapitalBio Corporation), and were printed onto the silylanized slides (CapitalBio Corporation) by a SmartArrayer™ 48 microarray printer. The microarray included all of selected HVR1 antigens printed in three replicates with IL-1 and human IgG as negative and positive control, respectively. The arrays were blocked with blocking buffer [phosphate buffered saline (PBS) containing 2% BSA] at room temperature for 3 h, and were air dried.

Serological analysis of anti-HVR1 antibody cross-reactivity by microarray

The arrays were covered with the sera of HCV infected patients at 1/10 in the blocking buffer for 40 min at 37℃ and washed 3 times with PBS Tween-20 (PBST) solution dissolved in PBS to a final concentration of 0.05%. After rinsed twice with PBS, the slides were incubated in Cy3 conjugated protein A for 30 min at 37℃ and washed 3 times with PBST solution. The slides were finally air dried under short centrifugation and examined in a LuxScan™ 10K microarray scanner (CapitalBio Corporation). The fluorescence intensities were determined by the software taken by the scanner.

Statistical analysis

The number of HVR1 antigen reacted with the HCV sera on the chip was used to define the range of cross-reactive responses for each sample. Qualitative data were analyzed using the χ^2 test or the Fisher's exact test when necessary. Quantitative values were compared using the Student's *t* test, and the Kruska Wallis test when necessary. *P* values lower than 0.05 were considered significant. All statistical calculations were performed using the SPSS for Windows, version 6.0 software package.

RESULTS

Selection of HVR1 sequences representing the variability of natural isolates

A total of 1600 HVR1 sequences were collected from Genebank to construct the database by Biosun software. The duplicated sequences were removed from the database to obtain a unique set of 843 natural HVR1 sequences. Thirty HVR1 sequences were selected from the database according to the results of multiple sequence alignment using Biosun software. All were cloned and expressed in *E. coli*, and the 30 HVR1 was successively selected according to their immunological reactivity tested by ELISA.

To evaluate the cross-reactivity of HCV sera, we need the representative HVR1 sequences which display not only the sequence diversity but also immunological variability. The non-overlapping reactivity of the representative HVR1 with the sera of the in-house panel was emphasized in this selection. As a result, 16 representative peptides (Figure 1) were selected from the 30 HVR1 with the cross-reactivity frequency ranging from 20% to 30% with the in-house panel (Figure 2). The 16 representative HVR1s could react with 32/34 sera in the panel and span the overall reactivity of the above 30 HVR1.

```
01  E T H V T G G S A A H T A S G I A S L F S P G A K Q N
02  N - - - - - - A - - R - - A - L - G - - T - - - R - -
03  - - - T - - - - V - R A T - - V V - - - N - - - - - -
04  T - Y I - - - - - - Q N - R - L - - - - - T - - Q - K
05  T - Y T - - - - - - R A - Q - L T G - - - I - - R - -
06  S - - - - - - A S - - - T R Q V - - - - - - - V L R K
07  N - Y - - - - A - G - - M A - F - N F L A - - - - - -
08  S - - - - - - A - - - - T - - L T G - - I S - P S - K
09  Q - T - S - - K - - Q - T - - F V K - - - - - - Q - K
10  - - - T - - - - - - R Q T R - F - - F - D Q - P S - D
11  S - - - - - - - - Q - R D T R - L V - - - T L - P S - K
12  D - - T V - - A T - - - T R - L T - - - T - - P S - K
13  - - Y T - - - A - - R A - Y - L T - F L - V - P - - D
14  T - - T S - - A - - F N T H - F T - - - - T - P R - R
15  G - R - - - - T - G Y - T Q R F T T F - A - - P T - K
16  T - Y T - - A Q M G R G I T - F S N - - N L - S Q - K
```

Figure 1 The amino acid sequences of the 16 representative hypervariable region 1 proteins. Sequences are shown with a single-letter amino acid code, and dashes indicate residues identical at that position with the reference sequence above.

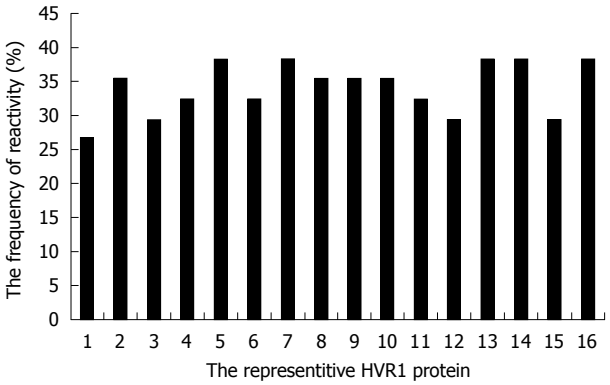

Figure 2 The frequency of reactivity of each representative hypervariable region 1 protein with in-house panel. HVR1: Hypervariable region 1.

The 16 representative HVR1 sequences were aligned further as shown in Table 1 and compared with the consensus pattern of 843 HVR1 sequences. The residues of 16 representative HVR1 sequences accounted for 90.4% (median value) of the observed variability of the consensus pattern of 843 HVR1 sequences (Figure 3). This indicated that the 16 representative sequences we selected could cover most of the natural variability of HVR1 at least from the amino acid residue sequence.

Serological analysis of anti-HVR1 antibodies in chronic HCV patients

The microarray with 16 HVR1 immobilized as antigens was used to evaluate the cross-reactivity of anti-HVR1 an-

Table 1 Percentages of amino acid sequence variation in 16 selected hypervariable region 1 (%)

	Percentages of amino acid sequence variation														
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	51.9	40.7	29.6	48.1	40.7	40.7	44.4	40.7	29.6	40.7	70.4	59.3	48.1	48.1	37.0
2	-	44.4	51.9	51.9	55.6	66.7	44.4	44.4	51.9	48.1	63.0	59.3	40.7	48.1	44.4
3		-	33.3	51.9	66.7	59.3	51.9	48.1	40.7	55.6	55.6	63.0	55.6	37.0	33.3
4			-	44.4	48.1	37.0	51.9	37.0	44.4	48.1	66.7	59.3	44.4	51.9	44.4
5				-	44.4	70.4	37.0	33.3	40.7	51.9	59.3	59.3	33.3	55.6	44.4
6					-	59.3	55.6	40.7	51.9	51.9	74.0	59.3	40.7	66.7	51.9
7						-	59.3	55.6	59.3	55.6	63.0	48.1	63.0	51.9	59.3
8							-	48.1	40.7	59.3	63.0	55.6	44.4	40.7	48.1
9								-	51.9	66.7	44.4	48.1	29.6	51.9	51.9
10									-	48.1	51.9	66.7	48.1	48.1	51.9
11										-	63.0	51.9	51.9	66.7	44.4
12											-	66.7	66.7	66.7	55.6
13												-	55.6	63.0	66.7
14													-	44.4	51.9
15														-	48.1

The Percentages of amino acid sequence variation are based upon manual calculation. Symbols for the hepatitis C virus isolates are the same as those of Figure 1.

83.6%	97.1%	86.7%	93.1%	87.5%	97.4%	94.5%	83.4%	90.8%	91.7%	86.2%	80.2%	87.1%	82.7%	81.7%	95.2%	95.1%	90.6%	84.7%	97.7%	88.9%	81.4%	99.2%	97.1%	88.6%	98.6%	90.4%
E	T	H	V	T	G	G	S	A	A	R	T	T	S	G	F	A	S	L	F	S	P	G	A	S	Q	N
T	N	Y	T	S	A	A	A	V	G	H	A	A	R	R	L	T	G	F	L	T	L	R	P	K	R	K
S	H	R	I	V	R	S	T	Q	S	Q	N	V	A	S	I	V	N	I	S	N	S	A	S	R	H	D
G	P	T	A	M		D	Q	T	V	Y	S	I	Y	T	V	S	K	M	V	A	R	E	V	Q	P	S
N	I	K	L	I		E	V	S	T	S	G	M	Q	Q	N	L	T	V	N	D	T	Q	G	A	S	R
D	A	Q	S	L			R	P	E	F	D	R	H	A	S	G	R	P	I	R	Q	S	R	T	G	T
H	M	V	R	D			E	I	R	K	Q	L	F	K	M	N	H	T	A	Q	F		I	N	E	Q
R	V	L	M	N			N	M		D	L	H	L	V	P	I	A	H	G	I	A		T	L	A	P
Q	K	I	H	A			K	E		A	H	F	T	H	D	Y	Q	R		P	M		K	H	K	E
A		S	G	F			G	L		P	F		K	P	G	H	D	S			L	I			V		H
K		F					L	H		M	V		G	M	R	M	C	K			V	V			P		L
V		P					M	C		G	R		N	N		F	Y				F	Y			M		G
Y		N					I	D		L	E		P	D		R	F				H	K			I		V
I		M					D	G		E	Y		I	G							G	G					
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Figure 3 Consensus patterns of the 843 natural variants of the hepatitis C virus hypervariable region 1 sequences used in this work. Residues are listed in decreasing order of observed frequency from top to bottom. The residues in shade are present in 16 representative hypervariable region 1 proteins, and the total frequency of the shaded resides was expressed as percentages on the top of the consensus pattern.

tibodies, and GST and human IgG were used as negative and positive controls, respectively (Figure 4A). The value of the cross-reactive response for each sample was evaluated according to the number of HVR1 antigens reacted with the HCV sera (Figure 4B).

Relationship between the cross-reactivity antibodies to HVR1 and disease severity

Considering the size of the sample, we devised a microarray to evaluate the broadness of serological reactivity to 16 HVR1 selected antigens. The basal features of patients and the results of the microarray are summarized in Table 2. The value of the cross-reactivity observed in individual cases did not correlate with patient's age (r = 0.0063, P > 0.05), infection time (r = 0.14, P > 0.05),

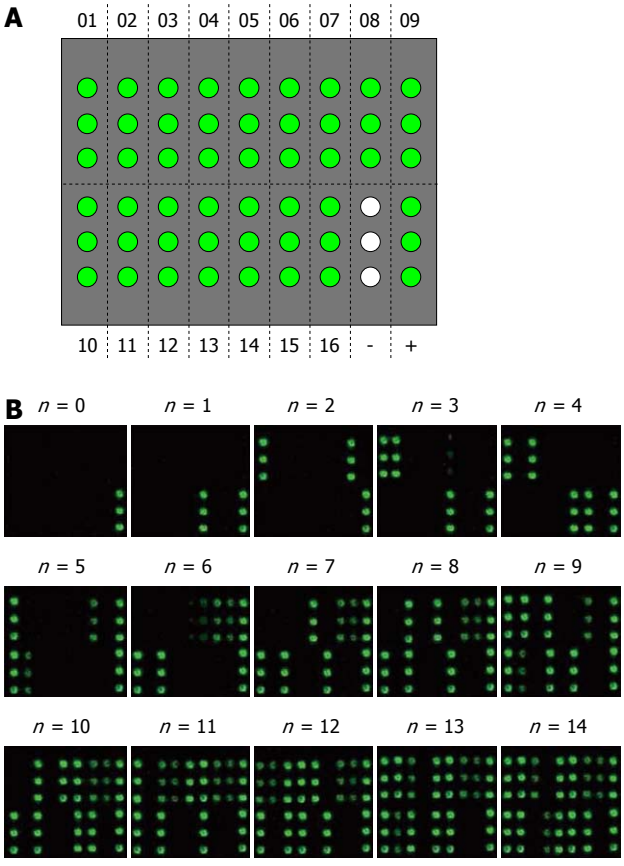

Figure 4 Evaluation of the cross-reactivity of anti-hypervariable region 1 antibodies in sera of chronic hepatitis C virus patients. A: Microarray patterns of 16 representative hypervariable region 1 (HVR1) proteins. The representative HVR1 proteins, negative and positive controls were printed in three replicates on the microarray; B: The value of cross-reactive responses for each sample. The number under the picture represents the value of cross-reactivity evaluated by the sum of HVR1 antigens reacted with hepatitis C virus sera.

serum alanine aminotransferase activity (r = 0.181, P > 0.05), or serum HCV-RNA concentration (r = 0.125, P

Table 2 Basal features and cross-reactivity of hypervariable region 1 antibodies determined by cross-reactivity chip in patients with genotype 1b hepatitis C virus chronic infection according to the severity of the underlying liver diseases

	Mild (n = 23)	Moderate (n = 18)	Hepatic cirrhosis (n = 16)
Age (yr)	44.5 ± 7.3	47.2 ± 10.8	45.4 ± 8.0
Time of infection	16.2 ± 4.3	18.1 ± 4.3	17.8 ± 5.7
Sex (M/F)	9/14	7/11	11/5
ALT	42.3 ± 48.1	39.4 ± 43.2	94.1 ± 167.6
HCV-RNA (copes × 10^3)	2189 ± 4562	4153 ± 11564	1582 ± 1676
Cross-reactivity	3.09 ± 2.68[a,d]	5.44 ± 3.93	7.44 ± 3.90
Positive for anti-HVR1	21	18	16

Quantitative data are expressed as mean ± SD. The value of cross-reactivity of hypervariable region 1 (HVR1) antibodies is determined by the number of representative HVR1 proteins reacted with the hepatitis C virus (HCV) sera. [a]$P < 0.05$ *vs* patients with moderate hepatitis; [d]$P < 0.01$ *vs* patients with liver cirrhosis. ALT: Alanine transaminase.

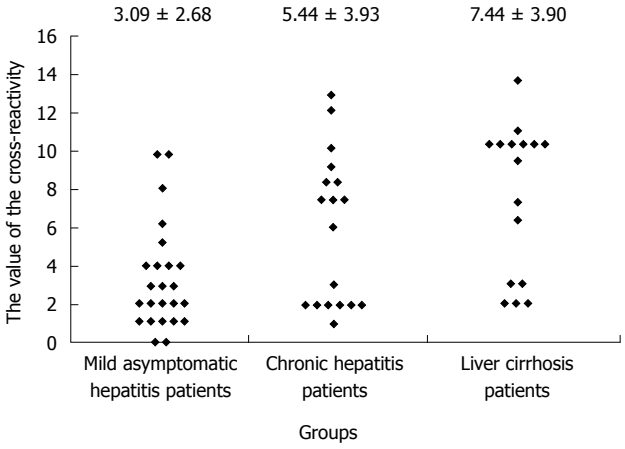

Figure 5 Relationship between the value of the cross-reactivity of hypervariable region 1 antibodies of the hepatitis C virus sera, as determined by the number of representative hypervariable region 1 proteins, and the severity of liver disease in patients with chronic hepatitis C virus infection.

> 0.05). No differences related to sex were found (5.93 ± 4.18 in men and 4.7 ± 3.54 in women, $P > 0.05$).

The degree of the cross-reactivity of anti-HVR1 antibodies (Figure 5) in 23 patients with mild chronic hepatitis was 3.09 ± 2.68, which was significantly different from that in those with severe hepatitis (5.44 ± 3.93, $P < 0.05$) and liver cirrhosis (7.44 ± 3.90, $P < 0.01$).

The appearance of HVR1 antibodies was defined positive when the serum could react with more than 1 HVR1 antigen (include 1). In the sera of 23 mild chronic patients, 21 were anti-HVR1 positive, and all were anti-HVR1 positive in moderate hepatitis and liver cirrhosis patients (Table 2). The appearance of HVR1 antibodies was found similar in the three groups of patients ($P > 0.05$).

DISCUSSION

It is well known that the HCV infection is persistent in up to 85% of cases and may result in mild chronic hepatitis, cirrhosis and hepatocellular carcinoma. There are no obvious serologic features that can work as a prognostic marker although Zibert *et al*[11] found that early appearance of anti-HVR1 antibodies within the first 6 mo is associated with self-limited HCV infections. In this study, all the patients were not at the early stage, and had the disease for at least 10 years. We found that anti-HVR1 antibodies were widely produced in chronic patients, and there was no significant difference in mild hepatitis, moderate hepatitis and liver cirrhosis. That means that the anti-HVR1 antibodies could not be used in prognostic and turnover studies of chronic HCV infection, which was coincided with other studies[14,21].

It has been reported that the anti-HVR1 antibodies in HCV infected individuals could react with more than one variant of HVR1[15,16,22]. In this study, 16 representative HVR1 antigens distributed homogeneously were used to evaluate the cross-reactivity of HVR1 antibodies. Our data suggest that the cross-reactivity of HVR1 antibodies in moderate hepatitis and liver cirrhosis is broader than that in mild chronic hepatitis. Mondelli *et al*[23] found that the heterogeneity of cross-reactive antibodies was significantly higher in patients with chronic hepatitis than in those with acute hepatitis. This suggested that it is the broadly cross-reactivity of HVR1 antibodies that were associated with the progression of liver disease and could be a new marker in prognostic study of chronic HCV infection, but not the appearance of anti-HVR1 antibodies.

In our studies, the patients selected owned similar background with plasmapheresis transmitted, genotype 1b, without any previous treatment and at the same geographical area. Considering the invasion and complications of the liver biopsy[24], US was used to assess the severity of hepatitis C. For the limitation of US determination, the patients were divided into asymptomatic hepatitis, chronic hepatitis and liver cirrhosis group[17,18]. To elevate the accuracy of the US, the patients with indeterminate US sign were excluded from our studies. In addition, the clinical symptoms were considered to assess the severity of hepatitis C. The patients with no more than one clinical symptom were defined as having the asymptomatic hepatitis. This shows that the chronic hepatitis group in our study consisted of moderate and severe liver diseases. Further studies will focus on the patients with liver biopsy to give detailed information about the relationship between the cross-reactivity antibodies of HVR1 with the severity of hepatitis C.

HCV exists in the bloodstream of infected individual as quasispecies, and quasispecies nature of HCV may confer important biological properties to the virus, including immunologic escape, viral persistence, and resistance to antiviral agents[25,26]. There was greater nucleotide sequence diversity between HCV variants in isolates from patients with more advanced liver diseases[27,28]. Our data indicate that the broadly cross-reactivity of anti-HVR1 antibodies was associated with the advanced liver disease, and was immune reaction responding to heterogeneity of the HCV quasispecies at certain time in chronic patients.

Using the sequential serum samples and HCVpp model, HANA verified that the mutation of HVR1 resulted in loss of recognition of the cognate antibody response and escape from antibody-mediated neutralization in chronic patients[29]. It is the presence of the immune pressure and mutation of quasispecies which lead to the persistence of HCV.

It is mentioned that HVR1 domains of HCV appear to contain a neutralizing epitope[10-12]. It is important to acquire broadly cross-reactive HVR1 antibodies for the development of HCV vaccines[30]. Obviously, cross-reactive HVR1 antibodies in chronic patients failed to clear viral infection in our studies. Recently, interfering mechanism of neutralizing antibodies was established using two different antibodies against E1. That means that the presence of an abundance of neutralizing antibodies will interfere with the neutralizing activity to HCV[31]. The same mechanism may exist for the HVR1 antibodies.

With the 16 representative HVR1 antigens, we developed an assay to evaluate cross-reactivity antibodies against HVR1 and discussed the humoral anti-HVR1 response and its relationship with the severity of the liver disease with chronic HCV infection. Our study may contribute to the better understanding of the knowledge of immune response of natural HCV infection, which will benefit the study of HCV vaccines based on the HVR1 and HCV prognosis.

COMMENTS

Background

Although an early anti-hypervariable region 1 (HVR1) response is associated with self-limiting acute infection, anti-HVR1 antibodies are frequently produced in the majority of chronically infected individuals and appear to coexist with the HVR1 variants. In most instances, the sera of hepatitis C virus (HCV) infected patients are frequently cross-reactive with unrelated HVR1 sequences. However, the cross-reactive nature of anti-HVR1 responses and the relationship between the cross-reactivity and the progression of hepatitis are largely undefined.

Research frontiers

HCV is a RNA virus that replicates with a high rate of mutation. The virological studies revealed that HCV circulates within an infected host as a heterogeneous viral population containing genetically distinct, but closely related variants, known as quasispecies. The diversity of quasispecies and its relationship with the progression of liver disease and interferon treatment were largely studied by single-strand conformation polymorphism or sequencing of HVR1. However, for the high antigenic variability of HVR1, the humoral response against the HVR1 could not be easily characterized in chronic HCV infection. HVR1 domains of HCV appear to contain a neutralizing epitope. To acquire broadly cross-reactive HVR1 antibodies is important for the development of HCV vaccine. Recently, interfering mechanism of neutralizing antibodies was established using two different antibodies against E1.

Innovations and breakthroughs

For the high antigenic variability of HVR1, the humoral response against the HVR1 could not be easily characterized in chronic HCV infection. In this report, the authors used a protein microarray immobilized with a set of HVR1 proteins selected by alignments to study the humoral anti-HVR1 response in chronic HCV infections with different clinical courses.

Applications

The findings suggest that the broadly cross-reactive antibodies response against HVR1 is associated with the progression of liver disease with chronic HCV infection, and this may contribute to the better understanding of HCV natural infection and its prognosis.

Peer review

This is an interesting study designed to evaluate the presence of cross-reactive antibodies against the HVR1 in HCV infected patients and its relationship with disease progression.

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BRIEF ARTICLE

Diabetes mellitus and hepatocellular carcinoma: Comparison of Chinese patients with and without HBV-related cirrhosis

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Abstract

AIM: To determine the role of diabetes mellitus (DM) and other associated factors in Chinese hepatocellular carcinoma (HCC) patients with cirrhosis, compared with those HCC patients without cirrhosis, in the single setting of hepatitis B virus (HBV) infection, after other known concomitant diseases were excluded.

METHODS: A total of 482 patients, treated at the China-Japan Friendship Hospital, Ministry of Health (Beijing, China), in the period January 2003 to June 2009, and with a hospital discharge diagnosis of HCC, were included. Demographic, clinical, laboratory, metabolic and instrumental features were analyzed.

RESULTS: Of the total, 310 patients were diagnosed with HBV infection and, following the inclusion and exclusion criteria, 224 were analyzed, including 122 patients (54.5%) with cirrhosis (the case group) and 102 patients without cirrhosis (the control group). Twenty-seven patients (12.1%) were diabetic, including 19 in the case group and 8 in the control group (19/122 = 15.6% *vs* 8/102 = 7.8%, $P = 0.077$). Thirty-one possible relevant parameters were compared by univariate analysis, and 9 variables were selected for multivariable analysis, including DM ($P = 0.077$), past history of HBV infection ($P = 0.005$), total bilirubin ($P < 0.001$), albumin level ($P < 0.001$), international normalized ratio (INR) ($P < 0.001$), alanine aminotransferase ($P = 0.050$), platelet ($P < 0.001$), total cholesterol ($P = 0.047$), and LDL cholesterol ($P = 0.002$) levels. Diabetes showed a statistical difference by multivariable analysis [odds ratio (OR) 4.88, 95% confidence interval (CI): 1.08-21.99, $P = 0.039$], although no significant difference was found in univariate analysis. In addition, three cirrhosis-related parameters remained statistically different, including INR (OR 117.14, 95% CI: 4.19-3272.28, $P = 0.005$), albumin (OR 0.89, 95% CI: 0.80-0.99, $P = 0.027$), and platelet count (OR 0.992, 95% CI: 0.987-0.999, $P = 0.002$).

CONCLUSION: Besides the three cirrhosis-related parameters, DM was found to be the sole independent factor associated with HCC in patients with HBV-related cirrhosis, compared with those without cirrhosis.

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Key words: Diabetes mellitus; Hepatocellular carcinoma; Hepatitis B virus; Cirrhosis; Chinese patients

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Gao C, Zhao HC, Li JT, Yao SK. Diabetes mellitus and hepatocellular carcinoma: Comparison of Chinese patients with and without HBV-related cirrhosis. *World J Gastroenterol* 2010; 16(35): 4467-4475 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i35/4467.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i35.4467

INTRODUCTION

Hepatocellular carcinoma (HCC) is a worldwide malignancy, and the incidence rate has increased significantly over the past two decades in China, Japan, the USA and other countries[1-3]. The reason for this increase in HCC has not yet been explained clearly, although more than 50% of this increase has been attributed to hepatitis virus or alcoholic liver disease, especially to hepatitis C virus (HCV)[1,4]. Risk factors for HCC that have been identified include hepatitis B virus (HBV), HCV, cirrhosis, heavy alcohol consumption, non-alcoholic steatohepatitis (NASH), aflatoxin exposure, increasing age, male sex, and positive family history; however, in 15%-50% of HCC patients no specific risk factor has been found[4-6].

Diabetes mellitus (DM) has been put forward as a potential risk factor for HCC by some studies; however, no consensus has been reached about the "true" role of DM in HCC, at least as a "true" independent risk factor in patients with HBV infection, for example, whether DM itself directly predisposes to HCC[1]. Earlier epidemiologic studies showed no association between HCC and DM[7,8], but some recent studies have identified DM as a risk factor for HCC[4,5,9,10], especially two cohort studies conducted in Sweden and the USA[5,11]. In the Swedish cohort study, 153 852 patients diagnosed with diabetes were identified by use of the Swedish In-patient Register. The observations showed that patients with DM were at increased risk of developing primary liver cancers[11]. This conclusion was supported by another subsequent study conducted in the USA[5].

However, some limitations in these studies, which would influence the interpretation of the results and conclusion, could not be omitted[1]. The first was that the studies were performed by use of specific medical systems and large samples were extracted using ICD-9 codes and computers. The special medical systems functioned for specific people and the study population could not be regarded as representative of the overall population. The second was that the number of studies was very limited and performed in the USA or European countries, not in the areas with higher incidence of HCC, such as China and other Asian countries. The third was that the values of the odds ratio (OR) or relative risk (RR) were very much lower, indicating no strong relationship between DM and HCC, which could be modified by other possible risk factors; for example, some factors related to hepatitis or cirrhosis. The fourth was that healthy or non-liver cancer patients served as controls in almost all of the studies, and thus the possible influencing factors which were related to carcinoma could not be adjusted easily. No information was available about the role of DM in HCC patients with cirrhosis, when HCC patients without cirrhosis served as controls.

Moreover, one recent study which was conducted in the Taiwan province of China showed that neither DM nor being overweight were risk factors for HCC in this area, which has a higher incidence of HCC on the basis of a series of community-based cross-sectional and case-controlled studies[12]. These aforementioned issues indicate that it is not appropriate to draw a final conclusion about the relationship between DM and HCC before related questions are resolved and further related issues should be clarified by future research. Our study was designed to determine: (1) the role of DM and other associated factors in Chinese HCC patients with liver cirrhosis, compared with HCC patients without cirrhosis, in the single setting of HBV infection, after other known concomitant diseases were excluded; (2) the role of some factors associated with the metabolic syndrome in these patients, including body weight, height, serum glucose, blood pressure and blood lipid levels; and (3) any relationships of DM, past history of HBV infection and cirrhosis in these Chinese HCC patients; for example, the influence of courses of HBV infection and DM.

MATERIALS AND METHODS

Study population

All patients treated at our hospital (China-Japan Friendship Hospital, Ministry of Health, Beijing, China) in the period January 2003 to June 2009, and with a hospital discharge diagnosis of HCC, were included. Those patients fulfilling the diagnostic criteria, inclusion and exclusion criteria were analyzed. HBV infection was defined as serum hepatitis B surface antigen (HBsAg)-positive for at least 6 mo or HBsAg-positive when diagnosed with HCC. Patients fulfilling the following criteria were excluded: (1) confirmed diagnosis of HCC for more than 15 d or had been treated at entry; (2) duration of DM was less than 1 year before confirmed diagnosis of HCC; (3) confirmed HCV, hepatitis D virus or human immunodeficiency virus infection; (4) heavy alcohol consumption (> 80 g/d in males and > 40 g/d in females for more than 10 years); (5) confirmed exposure to *Aspergillus flavus*, confirmed diagnosis of drug- or poison-induced liver damage; (6) presence of other malignancies; and (7) presence of hemachromatosis, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and beriberoid diseases or allergic disorder. The Human Research Ethics Committee at the China-Japan Friendship Hospital approved the study and our study was carried out in accordance with the Helsinki Declaration.

Subject determinations

The diagnosis of HCC was histologically confirmed by needle biopsy or based on the findings of typical radiological features in at least two image examinations, includ-

ing ultrasound scan (US), contrast-enhanced dynamic computerized tomography (CT), magnetic resonance imaging (MRI), and hepatic angiography (showing tumor stain), or by a single positive imaging technique associated with serum α-fetoprotein (AFP) level > 400 ng/mL[13]. Liver cirrhosis was diagnosed based on the histological findings of needle biopsy, or the findings of typical radiological features in at least two image examinations (a non-homogenous hepatic texture or surface, rarefied hepatic central vein, an enlarged caudate lobe, splenomegaly, or collateral veins), or by a single image technique associated with typical manifestations of decompensated liver function and portal hypertension (jaundice, spider angiomata, caput medusae, palmar erythema, anorexia, fatigue, weight loss, anemia, leucopenia, thrombocytopenia, increased liver enzymes, lowered albumin, elevated serum bilirubin or prolonged prothrombin time)[6]. The definition of DM was a fasting plasma glucose level of 126 mg/dL or greater on at least two occasions, plasma glucose of 200 mg/dL or greater at 2 h for a 75-g oral glucose tolerance test (OGTT), or the need for insulin or an oral hypoglycemic drug to control glucose levels. These data were re-evaluated carefully by at least two authors.

Clinical and laboratory parameters

The possibly relevant demographic, clinical, laboratory, metabolic and instrumental features of patients were recorded, including age, sex, past history of HBV infection, history of cirrhosis and diabetes, body weight, height, systolic blood pressure (SBP), diastolic blood pressure (DBP), smoking, alcohol consumption, presence of hepatitis B e antigen (HBeAg), hepatitis B virus load (HBV-DNA), AFP, blood glucose, total bilirubin (TBil), albumin, international normalized ratio (INR), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), platelet count, triglycerides (TG), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, creatinine (Cr) and blood urea nitrogen (BUN).

These data were obtained when patients were diagnosed with HCC, and data obtained 15 d before or after diagnosis of HCC were excluded. If the same patient was admitted to our hospital for more than one time, the data from the first time of hospitalization were recorded; if multiple values for one parameter could be obtained from one hospitalization, the first measured or detected value was regarded as the final value. Patients having missing values which would play some role for statistical results would be excluded from the study population. Body-mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Overweight was defined as BMI ≥ 23 kg/m^2, and obesity BMI ≥ 25 kg/m^2, according to the Asian and Chinese criteria. Hypertension was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg, and mean artery pressure (MAP) was calculated as 1/3 SBP plus 2/3 DBP. Child-Pugh score were calculated based on 5 associated parameters, including ascites, hepatic encephalopathy, TBil, serum albumin and INR.

Information regarding smoking and alcohol consumption was obtained from the medical records; those smoking less than one pack of cigarettes in a week were regarded as non-smoking; those whose alcohol consumption was < 140 g/wk in males and < 70 g/wk in females were regarded as non-alcohol use; those having stopped smoking or alcohol use for more than 5 years were also considered as non-smoking or non-alcohol use. The findings from physical examinations and image techniques, including ultrasound scan, contrast-enhanced dynamic CT, MRI, and hepatic angiography, were re-assessed carefully for clinical classification, clinical stage and tumor nodus metastasis (TNM) stage. The criteria for classification or stages were derived from the anticancer committee of China and the International Union against Cancer. For the clinical classification, massive-type HCC was defined as the diameter of carcinoma ≥ 5 cm; nodular-type HCC was defined as the diameter < 5 cm; small-type HCC was defined as the diameter < 3 cm for one single or two nodules.

Statistic analysis

In one case-control study conducted in the USA, 823 patients were included and the incidence rate of DM was 33%. However, among the HCC patients, 34% had HCV infection and 47% had alcoholic cirrhosis[14]. Considering the effect of HCV and alcohol intake on diabetes and cirrhosis, we assessed that the incidence rate of DM in HCC patients with HBV-related cirrhosis should be reduced to less than 30%. Our deduction was supported by two studies performed in Japan[15] and Taiwan (China)[16]. These studies showed that 40 (16%) of 245 HCC patients and 92 (15.8%) of 581 patients were diabetic. Taken together[1,9], we assumed that the incidence rate of DM in HCC patients with HBV-related cirrhosis would be 15%, whereas the rate in patients without cirrhosis would be 5%. With 80% power and at a 5% significance level, we calculated a sample size of 187 patients would be needed (94 in each group).

The analysis was performed using SPSS for Windows, version 13.0 (SPSS, Chicago, IL, USA). For continuous variables with normal curve distribution, mean ± SD was described and Independent-Samples *t*-test was used. For continuous variables with skewed distribution, the medians and inter-quartile ranges were described and Mann-Whitney non-parametric *U*-test was used. For the categorical variables, the numbers and proportions of patients in each group were described, and Pearson χ^2 test, continuity correction χ^2 tests or Fisher's exact test were used. $P < 0.05$ was considered statistically significant and selected for multivariable analysis, including DM and ALT with marginal differences.

Based on the results from univariable analysis, nine variables were included in the unconditional logistic regression analysis, including DM, presence of past history of HBV infection, TBil, albumin, INR, ALT, platelet count, total cholesterol, and LDL cholesterol. For better understanding of the role of Child-Pugh score, TBil, albumin and INR were included in the multivariable analy-

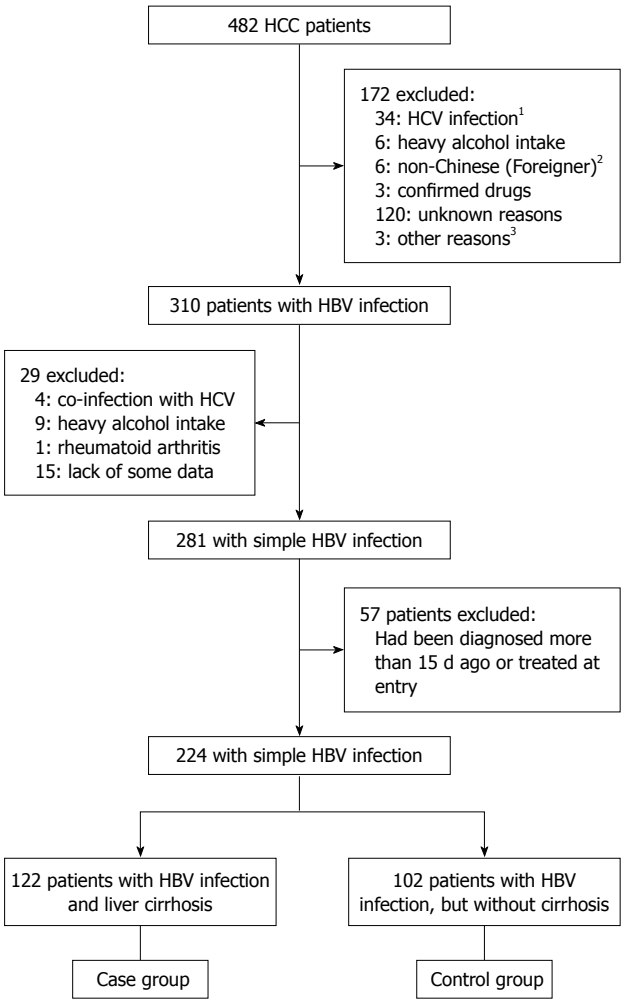

Figure 1 Patient selection in our study. [1]Two had heavy alcohol intake among the 34 patients with hepatitis C virus (HCV) infection; [2]Six foreigners included: three had HCV infection, two had hepatitis B virus (HBV) infection, and no specific reason for the remaining patient; [3]Reasons included: Bechterew's spondylitis, autoimmune hepatitis and primary biliary cirrhosis for the three patients. HCC: Hepatocellular carcinoma.

sis as 3 independent variables, and then Child-Pugh score was excluded for dependence of variables. We expressed results as odds ratios (ORs) and their 95% confidence intervals (CIs). For all tests, $P < 0.05$ was considered statistically significant and all P values quoted are two-sided.

RESULTS

Baseline characteristics of patients

A total of 482 HCC patients were treated at our hospital in the study period and 258 were excluded for the following reasons (Figure 1): 38, HCV infection (4 co-infection with HBV); 15, heavy alcohol consumption (9 in the HBV infection group); 120, unknown reasons (cryptogenic); 6, non-Chinese (foreigners); 3, confirmed drug- or poison-induced liver damage; 57, confirmed diagnosis of HCC for more than 15 d or had been treated at entry; 15, lack of some data which influenced the statistical analysis; and 4, other reasons. Of the total, 310 patients were diagnosed with HBV infection and 224 patients were subsequently analyzed, including 122 patients (54.5%) with cirrhosis (the case group) and 102 patients without cirrhosis (the control group).

Table 1 Demographic, clinical, laboratory, metabolic and instrumental features of 224 hepatocellular carcinoma patients *n* (%)

Variable	HCC patients (n = 224)[1]
Mean age (yr)	54.6 ± 10.7
Male sex	190 (84.8)
Liver cirrhosis	122 (54.5)
Diabetes mellitus	27 (12.1)
Overweight or obesity[a]	95 (58.6)
Hypertension	59 (26.3)
Body weight (kg)	68.8 ± 11.0
Body height[a] (cm)	169.4 ± 6.4
Mean body mass index[a] (kg/m^2)	23.9 ± 3.4
Systolic blood pressure (mmHg)	128 ± 16
Diastolic blood pressure (mmHg)	79 ± 9
Mean artery pressure (mmHg)	95 ± 10
Past history of HBV infection	173 (77.2)
Smoking	99 (44.2)
Alcohol intake	69 (30.8)
Positive for HBeAg[b]	52 (26.1)
HBV-DNA[c], Log$_{10}$	5.32 ± 1.40
AFP[d], > 400 ng/mL	110 (50.9)
Child-Pugh Score[2]	6 (5-8)
Blood Glucose (mmol/L)	5.88 ± 2.22
Total Bilirubin[2] (mg/L)	13 (8-23)
Albumin level (g/L)	36.6 ± 6.3
International normalized ratio	1.22 ± 0.24
Alanine aminotransferase[2] (U/L)	53 (34-83)
γ-glutamyl transferase[2] (U/L)	113 (58-254)
Platelet count[2] (× 10^9/L)	125 (81-183)
Triglycerides[e] (mmol/L)	1.02 ± 0.52
Total cholesterol[e] (mmol/L)	4.27 ± 1.33
HDL cholesterol[f] (mmol/L)	0.93 ± 0.44
LDL cholesterol[f] (mmol/L)	2.44 ± 1.06
Creatinine[2] (mg/dL)	0.9 (0.8-1.0)
Blood urea nitrogen[2] (mmol/L)	4.98 (3.92-6.28)

[1]Plus-minus value indicates mean ± SD; [2]Median (inter-quartile range, Q1-Q3). Data were available in [a]162, [b]199, [c]57, [d]216, [e]148 and [f]132 patients. AFP: α-fetoprotein; HBV: Hepatitis B virus; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

Table 1 shows the demographic, clinical, laboratory, metabolic and instrumental features of the 224 HCC patients. Of them, 27 patients (12.1%) were diabetic [12 patients had been excluded because they were diagnosed at the same time of diagnosis of HCC in 10 patients and diagnosed for less than 1 year before the diagnosis of HCC in 2 patients, (27 + 12)/224 = 17.4%]; 173 patients (77.2%) had confirmed past history of HBV infection, and pathological diagnosis from needle biopsy was obtained from 32 patients (18/122 = 14.8% *vs* 14/102 = 13.7%, $P > 0.05$). The mean age of the 224 HCC patients was 54.6 (± 10.7) years old and 190 patients (84.8%) were male sex. Ninety-five patients (95/162 = 58.6%) were overweight or obese, and 59 patients (26.3%) had hypertension.

The clinical classification, clinical stage and TNM stage are shown in Table 2. Regarding the clinical classification, massive-type HCC (134, 59.8%) and nodular-type HCC (61, 27.2%) were the two major types, which

Table 2 Comparison of clinical classification, clinical stage and tumor nodus metastasis stage of hepatocellular carcinoma patients with cirrhosis (*n* = 122) and patients without cirrhosis (*n* = 102) *n* (%)

	Total patients (*n* = 224)	Patients with LC (*n* = 122)	Patients without LC (*n* = 102)	*P* value[1]
Clinical classification				
Massive-type	134 (59.8)	52 (42.6)	82 (80.4)	< 0.001
Nodular-type	61 (27.2)	49 (40.2)	12 (11.8)	< 0.001
Small-type	15 (6.7)	13 (10.7)	2 (2.0)	0.010
Diffuse-type	14 (6.3)	8 (6.6)	6 (5.9)	0.835
Clinical Stage				
Stage Ⅰ	15 (6.7)	9 (7.4)	6 (5.9)	0.656
Stage Ⅱ	95 (42.4)	44 (36.1)	51 (50.0)	0.036
Stage Ⅲ	114 (50.9)	69 (56.6)	45 (44.1)	0.064
TNM stage				
Stage Ⅰ	11 (4.9)	8 (6.6)	3 (2.9)	0.212
Stage Ⅱ	43 (19.2)	34 (27.9)	9 (8.8)	< 0.001
Stage Ⅲ	76 (33.9)	30 (24.6)	46 (45.1)	0.001
Stage Ⅳ	94 (42.0)	50 (41.0)	44 (43.1)	0.745
T Stage				
Stage 1-2	81 (36.2)	61 (50.0)	20 (19.6)	< 0.001
Stage 3-4	143 (63.8)	61 (50.0)	82 (80.4)	-
N Stage				
Stage 0	194 (86.6)	110 (90.2)	84 (82.4)	0.087
Stage 1	30 (13.4)	12 (9.8)	18 (17.6)	-
M stage				
Stage 0	130 (58.0)	72 (59.0)	58 (56.9)	0.745
Stage 1	94 (42.0)	50 (41.0)	44 (43.1)	-

[1]Comparison of the 122 hepatocellular caecinoma (HCC) patients with hepatitis B virus-related cirrhosis and 102 HCC patients without cirrhosis, using Pearson χ^2 tests because the total number of cases was more than 40 and no cells had an expected count less than 5. TNM: Tumor nodus metastasis; LC: Liver cirrhosis.

accounted for nearly 90% of the total patients. The massive-type HCC was more likely to appear in HCC patients without cirrhosis (52/122 = 42.6% *vs* 82/102 = 80.4%, $P < 0.001$), whereas the nodular-type HCC was more likely to appear in the group with cirrhosis (49/122 = 40.2% *vs* 12/102 = 11.8%, $P < 0.001$). Regarding the clinical stage, a statistical difference between groups was only found for stage Ⅱ (44/122 = 36.1% *vs* 51/102 = 50.0%, $P = 0.036$). Regarding TNM stage, a difference was observed for T stage, whereas no differences were shown for N stage or M stage.

Diabetes and other associated factors for HCC with HBV-related cirrhosis

Of the 27 diabetic patients, 19 patients were in the case group and 8 patients in the control group (19/122 = 15.6% *vs* 8/102 = 7.8%, $P = 0.077$): not significant, but a marginal difference was shown for DM by Pearson Chi-Square test. In univariable analysis (Table 3), compared with HCC patients without cirrhosis, HCC patients with cirrhosis had a higher percentage of past history of HBV infection ($P = 0.005$), higher Child-Pugh score ($P < 0.001$), higher total biliribin ($P < 0.001$), lower albumin level ($P < 0.001$), prolonged prothrombin time/INR ($P < 0.001$), lower platelet count ($P < 0.001$), lower total cholesterol ($P = 0.047$), and lower LDL cholesterol ($P = 0.002$). In addition, a marginal statistical difference was found for the comparison of serum ALT level ($P = 0.050$). No statistical differences were shown for the other 21 variables.

Based on the results from univariable analysis, nine variables were included in the unconditional logistic regression analysis, including DM, presence of past history of HBV infection, TBil, albumin, INR, ALT, platelet count, total cholesterol, and LDL cholesterol levels. For better understanding of the role of Child-Pugh score, TBil, albumin and INR were included as three independent variables and Child-Pugh score was excluded for dependence of variables. In multivariable analysis (Table 4), diabetes mellitus (OR 4.88, 95% CI: 1.08-21.99, $P = 0.039$), INR (OR 117.14, 95% CI: 4.19-3272.28, $P = 0.005$), albumin level (OR 0.89, 95% CI: 0.80-0.99, $P = 0.027$), and platelet count (OR 0.992, 95% CI: 0.987-0.999, $P = 0.002$) showed statistical differences, whereas the other five variables did not differ significantly.

Metabolic syndrome associated factors for HCC with HBV-related cirrhosis

Factors associated with metabolic syndrome in our study included DM, overweight or obesity, hypertension, triglycerides, and HDL cholesterol. Because waist circumference measurements could not be obtained, total cholesterol and LDL cholesterol levels were included in the final analysis. DM has been described and will be omitted in this section. Univariable analysis (Table 3) showed that significant differences were found for the total cholesterol and LDL cholesterol levels ($P = 0.047$ and $P = 0.002$, respectively). Multivariable analysis (Table 4) showed no variables remained statistically different, indicating that the statistical differences in the univariable analysis were probably modified by the presence of diabetes mellitus and/or the factors associated with HBV-related cirrhosis (INR, platelet count and albumin).

Some relationships of DM, HBV Infection and cirrhosis in HCC

Our study was designed to determine the role of DM and other associated factors in Chinese HCC patients with liver cirrhosis, compared with those HCC patients without cirrhosis, in the setting of HBV infection. Next, we further demonstrated some relationships between DM, HBV infection and cirrhosis in the HCC patients. Of the 224 HCC patients, 173 patients had a confirmed past history of HBV infection (103/122 = 84.4% *vs* 70/102 = 68.6%, $P = 0.005$), indicating the presence of past history of HBV infection was more likely to occur in those HCC patients with hepatic cirrhosis. Only a marginal statistical difference ($P = 0.06$) was observed when the courses of HBV infection were compared between the two groups. However, no significant difference ($P = 0.798$) was found after the 51 patients without past history of HBV infection were excluded. Of the 27 diabetics, 19 patients (70.4%) were in the case group, and 14 of the 19 patients (73.7%) were diagnosed with DM before the diagnosis of cirrhosis, whereas the remaining 5 patients were diagnosed after

Table 3 Univariate analysis of factors associated with hepatocellular carcinoma and cirrhosis *n* (%)

Variable	Patients with LC[1] (*n* = 122)	Patients without LC[1] (*n* = 102)	*P* value[2]
Mean age (yr)	54.7 ± 10.0	54.6 ± 11.4	0.909
Male sex	106 (86.9)	84 (82.4)	0.346
Diabetes mellitus	19 (15.6)	8 (7.8)	0.077
Overweight or obesity[a]	54 (60.7)	41 (56.2)	0.562
Hypertension	33 (27.0)	26 (25.5)	0.792
Body weight (kg)	69.7 ± 11.4	67.7 ± 10.4	0.168
Body height[a] (cm)	169.4 ± 6.2	169.5 ± 6.6	0.912
Mean body mass index[a] (kg/m^2)	24.1 ± 3.6	23.7 ± 3.1	0.441
Systolic blood pressure (mmHg)	128 ± 15	130 ± 16	0.323
Diastolic blood pressure (mmHg)	78 ± 9	79 ± 9	0.255
Mean artery pressure (mmHg)	95 ± 10	96 ± 10	0.243
Past history of HBV infection	103 (84.4)	70 (68.6)	0.005
Smoking	57 (46.7)	42 (41.2)	0.405
Alcohol intake	43 (35.2)	26 (25.5)	0.115
Positive for HBeAg[b]	32 (29.4)	20 (22.2)	0.254
HBV-DNA[c], Log10	5.35 ± 1.37	5.26 ± 1.51	0.829
AFP[d], > 400 ng/mL	59 (50.9)	51 (51.0)	0.984
Child-Pugh Score[3]	7 (6-10)	5 (5-6)	< 0.001
Blood glucose (mmol/L)	5.99 ± 2.44	5.73 ± 1.93	0.362
Total bilirubin[3] (mg/L)	17 (9-31)	10 (8-17)	< 0.001
Albumin level (g/L)	34.8 ± 6.3	38.8 ± 5.6	< 0.001
International normalized ratio	1.31 ± 0.27	1.12 ± 0.15	< 0.001
Alanine aminotransferase[3] (U/L)	58 (37-90)	45 (30-81)	0.050
γ glutamyl transferase[3] (U/L)	110 (52-257)	117 (61-257)	0.816
Platelet count[3] (× 10^9/L)	94 (61-152)	162 (115-217)	< 0.001
Triglycerides[e] (mmol/L)	1.02 ± 0.54	1.02 ± 0.49	0.995
Total cholesterol[e] (mmol/L)	4.08 ± 1.44	4.53 ± 1.13	0.047
HDL cholesterol[f] (mmol/L)	0.89 ± 0.48	0.99 ± 0.38	0.152
LDL cholesterol[f] (mmol/L)	2.21 ± 1.10	2.78 ± 0.92	0.002
Creatinine[3] (mg/dL)	0.9 (0.8-1.0)	0.9 (0.8-1.0)	0.839
Blood urea nitrogen[3] (mmol/L)	4.95 (3.85-6.32)	5.02 (3.96-6.19)	0.927

[1]Plus-minus value indicates mean ± SD; [2]Comparison of the two groups, using independent-samples *t*-test, Mann-Whitney non-parametric *U*-test or Pearson χ^2 tests, respectively, depending on the categories of variables and different conditions. $P < 0.05$ was considered statistically significant and selected for multivariable analysis, including diabetes mellitus (DM) and alanine aminotransferase (ALT) with marginal differences; [3]Median (inter-quartile range, Q1-Q3). Data were available in [a]162 (89 + 73), [b]199 (109 + 90), [c]57 (41 + 16), [d]216 (116 + 100), [e]148 (87 + 61) and [f]132 (79 + 53) patients. In the brackets, the numbers before the plus sign indicate the available cases in the group of patients with cirrhosis, whereas the latter indicate the cases in another group of patients without cirrhosis. AFP: α-fetoprotein; HBV: Hepatitis B virus; HDL: High-density lipoprotein; LC: Liver cirrhosis; LDL: Low-density lipoprotein.

Table 4 Multivariate analysis of factors associated with hepatocellular carcinoma and cirrhosis

Variable[1]	Adjusted OR	95% CI	*P* value
Diabetes mellitus	4.88	1.08-21.99	0.039
INR	117.14	4.19-3272.28	0.005
Albumin level	0.89	0.80-0.99	0.027
Platelet count	0.992	0.987-0.997	0.002

[1]Nine variables were included in multivariable analysis, including diabetes, presence of past history of hepatitis B virus infection, total bilirubin, albumin, international normalized ratio, alanine aminotransferase, platelet count, total cholesterol, and low-density lipoprotein cholesterol. For better understanding of the role of Child-Pugh score, TBil, albumin and international normalized ratio (INR) were included as three independent variables and Child-Pugh score was excluded for dependence of variables. OR: Odds ratios; CI: Confidence interval.

cirrhosis. When the courses of DM between the 2 groups were compared in these 27 patients, no significant difference ($P = 0.389$) was found.

DISCUSSION

No consensus has been reached about the role of DM in HCC, at least as a "true" independent risk factor in patients with HBV infection, for example, whether DM itself directly predisposes to HCC. Our study provided some answers for this question and showed: (1) besides the 3 cirrhosis-related parameters (INR, albumin and platelet count), DM was found to be the sole factor associated with HCC in Chinese patients with cirrhosis, compared with those HCC patients without cirrhosis, in the single setting of HBV infection; (2) some available factors associated with metabolic syndrome (MS), apart from DM, produced no obvious effects in these patients, indicating that the role of DM was independent of other factors of MS; and (3) between the 2 groups, a marginal statistical difference was observed for the courses of past HBV infection whereas no significant difference was found for the courses of DM.

Our positive conclusion that DM is an independent

associated factor for HCC has been put forward by some previous studies[1,4,9,10], however, we answered this question from another viewpoint; based on our available literature and current knowledge, this was the first time that HCC patients, not healthy or non-liver cancer patients, served as controls. Therefore, some possible factors associated with carcinoma could be controlled easily. To address this question, we included a new patient cohort: hospital-based Chinese HCC patients with HBV-related cirrhosis were studied. Although the entire study population came from one single tertiary referral hospital and the overall numbers were not very large, we provided enough numbers of patients based on our sample estimation for statistical analysis. Moreover, one of our big strengths was the strict inclusion and exclusion criteria, allowing some potential influencing factors to be controlled. Maybe it was because of the number of patients and samples that a marginal statistical difference was found for the comparison of DM in the univariable analysis and a very wide 95% CI was observed in the multivariable analysis. We hope our results and conclusion will be validated in greater numbers of patients and more referral hospitals.

Cirrhosis is one of the most important risk factors for HCC; to determine the role of DM in HCC, comparison of the cirrhotic patients with HCC patients was necessary and we have conducted this comparison (data not shown). Twenty-seven patients (12.1%) were diabetic in our study population which was lower than previous reports and the sample estimation. The reasons were deduced to be as follows: (1) when considering the influence of the course of DM for HCC, 12 diabetic patients were excluded because the diagnosis of DM was less than 1 year before the diagnosis of HCC. In fact, the total number was 39 cases and the overall percentage of diabetic patients was 17.4% which was consistent with other studies; (2) it has been confirmed that HCV and heavy alcohol consumption play important roles in the onset and development of DM, however, these patients had been excluded to control any possible influence; and (3) our diagnosis was mainly based on the serum glucose; OGTT was only used when necessary. Although the aforementioned issues perhaps play some role in affecting the results and conclusion, we provide relatively sufficient evidence to answer the study question, especially as no statistical difference was found for the role of DM in the univariate analysis.

Besides DM, three cirrhosis-related factors were shown to be possible associated factors for HCC in these patients, including prolonged prothrombin time/INR, lower platelet count and lower albumin level. As for other factors, it is well known that increasing age and male sex are risk factors for HCC; however, no differences were found in our study. The reason was deduced to be that similar HCC patients, not non-cancer or non-liver cancer patients, were used as controls and the role of other risk factors was limited.

Non-alcoholic fatty liver disease (NAFLD), including its most severe form, NASH, has been considered as a risk factor for HCC[17-19]. Unfortunately, biopsies were obtained from a minority of our patients (32, 14.3%) because it was unnecessary for most of the confirmed HCC patients, and re-evaluation cannot be performed. Another limitation was that most of the cirrhotic patients were diagnosed clinically rather than on a biopsy and the role of cirrhosis could be underestimated. However, we followed strictly the diagnostic criteria which were recommended by the authorized institutes and used widely in clinical practice. We believed that our results are most likely applicable to clinical practice.

The pathogenesis mechanisms remain unclear and some are suggested as follows. The first is regarding obesity, diabetes and NAFLD in HCC. Obesity can lead to insulin resistance (IR) and steatosis, which are associated with the release of inflammatory mediators and production of cytokines. NASH can result in some typical histologic characteristics[10]. Therefore, diabetes and obesity can cause hepatic inflammation, leading to oxidative stress and lipid peroxidation, resulting in hepatocyte injury and necrosis, and subsequently HCC. The second suggested mechanism is that IR leads to a state of hyperinsulinemia which, via interaction with the insulin receptor, promotes increased phosphorylation and activation of downstream pathways[20]. The latter have been shown to play some role in tumorigenesis by decreasing apoptosis and increasing mitogenesis. The third mechanism involves insulin-like growth factor (IGF) which, while acting through separate binding proteins and receptors, has the same downstream intracellular mediators as the insulin receptor pathway[21,22]. Recent studies have reported altered IGF signaling in 90% of HCCs, including the autocrine production of IGFs, IGF binding proteins, and IGF binding protein proteases as well as IGF receptor expression[23-25]. The clarification of these mechanisms would be helpful to address the question of an association of DM with HCC.

In addition, considering that cirrhotic patients with HCV infection are twice as likely to have type-2 DM than patients with HBV infection[26,27], a very obvious difference was presumed between HCV infection and HBV infection regarding the role of DM in HCC and the reason remains unclear. One study has shown that more severe insulin resistance was present in non-cirrhotic patients with HCV infection than in patients with HBV infection[26]. IR was associated with the presence of serum HCV core antigen, the severity of hepatic fibrosis and decreased expression of hepatic insulin receptor substrate (IRS) 1 and IRS2 in patients with HCV infection. HCV core down-regulated the expression of IRS1 and IRS2 in human hepatoma cell lines as well as in whole animals. These observations suggested that HCV causes changes in specific hepatic molecules regulating glucose metabolism and results in severe insulin resistance. A possible mechanism suggested was that HCV core-induced suppressor of cytokine signaling (SOCS) 3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination[26]. However, another study reported an association of exogenous insulin or sulphonylurea treatment with an increased incidence of HCC in

patients with HCV infection, especially in those without cirrhosis[27]. These findings make the DM question more difficult to answer and further studies are required.

In addition to the aforementioned issues, some limitations should be acknowledged. The first limitation was due to the nature of our hospital-based case-control study, in that some data could not be obtained and some possible factors could not be adjusted. For example, NAFLD and NASH have been regarded as risk factors for HCC, but we could not assess these changes. Actually, biopsy was unnecessary for these confirmed HCC patients and was not recommended. Secondly, the diagnosis of cirrhosis and HCC was mostly based on imaging findings, which could lead to some underestimation and diagnostic bias. This concern has been addressed above. The third constraint was limited generalizability, because the entire study population came from a single tertiary referral hospital and extending these results to the overall population may be a concern. In addition, some diagnostic parameters were unavailable from the clinical records for those diseases listed in the exclusion criteria. The setting was designed as the HBV infection; however, we believe that, except for HCV and heavy alcohol intake, co-existence with HBV as the cause or risk factor for HCC was limited.

In conclusion, besides the 3 cirrhosis-related parameters (INR, albumin and platelet count), DM was found to be the sole independent associated factor for HCC in Chinese patients with hepatic cirrhosis, compared with those HCC patients without cirrhosis, in the single setting of HBV infection. These patients should be especially closely monitored. Future research should clarify these issues: (1) the basic oncogenic mechanisms by which diabetes "directly" predisposes to HCC, especially in animal models and experiments; (2) the role of DM in the genesis of HCC and the factors involved in its complications; (3) the impact of DM on the natural history of patients with HCC; (4) the impact of early diagnosis and treatment of DM in HCC; and (5) the benefits of controlling DM in the management of HCC and subsequent complications.

COMMENTS

Background

Diabetes mellitus has been put forward as a potential risk factor for hepatocellular carcinoma (HCC) by some studies; however, no consensus has been reached about the "true" role of diabetes mellitus (DM) in HCC, at least as a "true" independent factor in patients with hepatitis B virus (HBV) infection.

Research frontiers

The authors found that, besides the three cirrhosis-related parameters [international normalized ratio (INR), albumin and platelet count], DM was the sole independent factor for HCC in Chinese patients with cirrhosis, compared with those HCC patients without cirrhosis, in the single setting of HBV infection.

Innovations and breakthroughs

This is the first time that HCC patients, not healthy or non-liver cancer patients, served as controls, and some possible factors associated with carcinoma could thus be controlled easily.

Applications

HCC patients with diabetes should be especially closely monitored and future research should clarify the associated issues.

Peer review

In this paper, Gao *et al* examined the role of diabetes mellitus and other associated factors in Chinese HCC patients with cirrhosis, compared with those HCC patients without cirrhosis, in the single setting of HBV infection. They found that besides the three cirrhosis-related parameters (INR, album in and platelet count), DM was the sole independent factor associated with HCC in patients with HBV-related cirrhosis. The presented data are excellent.

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BRIEF ARTICLE

HOGG1 polymorphism in atrophic gastritis and gastric cancer after *Helicobacter pylori* eradication

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Abstract

AIM: To investigate the association between Ser326Cys human oxoguanine glycosylase 1 (hOGG1) polymorphism and atrophic gastritis and gastric cancer after *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: A total of 488 subjects (73 patients with gastric cancer, 160 with atrophic gastritis after *H. pylori* eradication and 255 controls) were prospectively collected. Polymerase chain reaction-restriction fragment length polymorphism analysis was performed to distinguish hOGG1 Ser326Cys polymorphism. Statistical analysis was conducted by two-sample *t* test for continuous variables and χ^2 test for categorical variables. Logistic regression models were used to find the risk factors for gastric cancer and atrophic gastritis.

RESULTS: Neither the hOGG1 Ser/Cys nor the Cys/Cys genotype was associated with gastric cancer. Compared with the Ser/Ser genotype, odds ratio (OR) for Ser/Cys was 0.96, (95% CI: 0.51-1.84) and OR for Cys/Cys was 1.1 (95% CI: 0.48-2.1). No association was detected between hOGG1 polymorphism and Lauren type of gastric cancer (P = 0.61) either. However, Ser/Cys and Cys/Cys were significantly associated with atrophic gastritis with OR: 1.76 for Ser/Cys (95% CI: 1.03-3.0) and 2.38 for Cys/Cys (95% CI: 1.34-4.23). After controlling for age, gender, smoking and alcohol, there were still significant associations with OR: 2.05 for Ser/Cys (95% CI: 1.14-3.68) and 2.76 for Cys/Cys (95% CI: 1.47-5.18).

CONCLUSION: HOGG1 polymorphisms (Cys/Cys and Ser/Cys) are associated with atrophic gastritis. No significant association is detected between hOGG1 polymorphisms (Cys/Cys or Ser/Cys) and gastric cancer.

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Key words: Human oxoguanine glycosylase 1 polymorphism; Atrophic gastritis; Gastric cancer

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INTRODUCTION

Gastric carcinoma develops in the following sequence: superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and cancer according to Correa's model[1]. *Helicobacter pylori* (*H. pylori*) infection is an important cause of chronic atrophic gastritis. The tissue damage and cell destruction are either caused by direct release of cytotoxins, lipase and phospholipase or indirectly by reactive oxygen species (ROS) released from polymorphonuclear leucocytes[2]. ROS is thought to be one of the pathogeneses for both atrophic gastritis and cancer[3]. However, the mechanism is still unclear for atrophic gastritis developing in *H. pylori* negative patients.

Oxygen-free radicals (OFR) are generated in small amounts in the course of normal metabolic reactions. However, OFR can react with complex cellular molecules such as fats, proteins, or DNA and cause further damage to them. Some oxidative DNA lesions are pro-mutagenic and oxidative damage has been proposed to play a role in the development of certain cancers[4,5]. Hydroxyl radicals are important for DNA damage. This radical is so reactive that it can damage all components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone[6]. One of the most common lesions formed by ROS modifications is 8-Hydroxy-2'-deoxyguanosine (8-OHdG). A specific DNA glycosylase/apurinic (AP) lyase, 8-hydroxy-2'-deoxyguanosine-glycosylase/apruinic lyase (ogg1), which catalyses the release of 8-OHdG and the cleavage of DNA at the AP site was found in *Escherichia coli* and yeast[4,5]. The human homologue of this gene, hOGG1 has been identified[6,7]. The gene product of hOGG1 can exhibit greatest specificity and activity for 8-OHdG:dC and is inactive against 8-oxodeoxyguanosine:dA[8,9]. HOGG1 has also been shown to excise 2,6-dianimo-4-hydroxy-5-formamidopyrimidine residues in a similar manner to its yeast homologue[10]. A C/G polymorphism at position 1245 in the 1α-specific exon 7 of the hOGG1 results in an amino acid substitution from serine to cysteine in codon 326. A number of hOGG1 polymorphisms have been described and a Ser/Cys substitution in exon 7 is highly prevalent[8,9,11,12]. The hOGG1 protein encoded by the wild-type 326Ser allele exhibited substantially higher DNA repair activity than the 326Cys. Some studies have suggested that the Ser326Cys hOGG1polymorphism may be associated with increased risk for lung[8], stomach[9], orolaryngeal[11,12], bladder[13] as well as gallbladder cancers[14] The aim of this study was to investigate the association between hOGG1 genotype and gastric cancer as well as atrophic gastritis.

MATERIALS AND METHODS

Subjects

This is a prospective case-control study in patients with atrophic gastritis and gastric cancer and healthy controls consecutively enrolled at Sir Run Run Shaw Hospital, China from April 2005 to March 2008.

All enrolled gastric cancer patients were histologically confirmed prior to operation and chemotherapy. Healthy controls were patients with normal endoscopic findings during the recruiting period. Gastric cancer patients were classified according to Lauren type. The inclusion criteria for atrophic gastritis were: endoscopically diagnosed atrophic gastritis according to Sydney system[15] and documented *H. Pylori* infection eradication for at least 1 year. *H. pylori* was confirmed to be negative in both histopathology and ^{13}C Urea breath test. The exclusion criteria for atrophic gastritis included newly diagnosed atrophic gastritis with *H. Pylori* infection or the *H. Pylori* which was eradicated within 1 year; the *H. Pylori* status was unknown or negative before diagnosis; patients with any kind of gastric operation history; and either histology or ^{13}C Urea breath test was considered to be positive for *H. pylori* infection.

Five biopsy specimens taken from the antrum, the angulus and the corpus of the stomach were embedded in paraffin wax, stained with haematoxylin-eosin and by Giemsa method. Mononuclear cell infiltration, polymorphonuclear cell infiltration, glandular atrophy, intestinal metaplasia, and the density of *H. pylori* were graded from 0 to 3, according to the updated Sydney system[15] by an experienced pathologist.

Each participant completed a self-structured questionnaire about alcohol and tobacco consumption. Alcohol drinking was defined as severe (a total amount of 20 g/d or more for 10 years), none (less than once a month) and mild (any amount in between). Smoking was defined as none, mild (less than 20 cigarettes per day) and severe (more than 20 cigarettes per day). The study was approved by the Sir Run Run Shaw Hospital Institutional Review Board. Each participant signed an informed consent form.

DNA genotyping assays

DNA was extracted from 10 mL whole blood according to the protocol of QIAamp DNA blood kit handbook. The basic method for detecting polymorphism was based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Generated fragments were separated by a 4% Metaphor agarose gel, stained with ethidium bromide. Puc19 DNA/*Msp* I (*Hpa* II) Marker, 23 (MBI Fermentas) or DNA Molecular Weight Marker Ⅷ (Roche Molecular Biochemicals, USA) were used. DNA was subject to PCR using fluorescent primers directed against the marker of hOGG1[8]. PCR protocols were presented in detail as follows: 20 pmol of primers (5'-ACTAGTCTCACCAGCCGTGAC-3' and 5'-TGGCCTTTGAGGTAGTCACAG-3') reacted with 1 mmol/L $MgCl_2$ and 2.5 U of *Taq* DNA polymerase in 50 μL systems. The PCR reaction began with denaturation at 95℃ for 14 min, and then was taken at 95℃, 1 min; 59.5℃, 1 min; and 72℃, 1 min for 30 cycles.

The hOGG1 PCR product (10 μL) was incubated with 2 U of *Fnu*4H I (New England Biolabs) overnight at 37℃, ended with 65℃ for 20 min. *Fnu*4H I (cuts cysteine alleles

Table 1 Clinical characteristics of subjects

	Total patients (*n* = 488)	Atrophic gastritis (*n* = 160)	Gastric cancer (*n* = 73)	Controls (*n* = 255)
Mean age (SD, yr)	48.6 (11.9)	51.4 (10.6)	59.6 (11.2)	43.6 (10.3)
Gender (%)				
Male	57.2	48.7	65.8	60.0
Female	42.8	51.3	34.2	40.0
Smoking (%)				
None	69.4	75.6	52.1	70.5
Mild/moderate	21.4	19.4	30.1	20.0
Severe	9.2	5.0	17.8	9.5
Alcohol (%)				
None	61.6	68.8	41.1	63.0
Mild/moderate	28.1	25.6	41.1	26.0
Severe	10.3	5.6	17.8	11.0
HOGG1 (%)				
Ser/Ser	24.5	16.7	28.8	28.2
Ser/Cys	47.0	35.3	25.8	46.7
Cys/Cys	28.5	48.0	45.4	25.1

HOGG1: Human oxoguanine glycosylase 1.

Table 2 Clinical characteristics of different grades of atrophic gastritis

	Grade 1 (*n* = 68)	Grade 2 (*n* = 68)	Grade 3 (*n* = 23)
Mean age (SD, yr)	51.3 (10.8)	51.1 (9.8)	52.9 (12.8)
Gender (%)			
Male	47.1	50.0	52.2
Female	53.9	50.0	47.8
Smoking (%)			
None	76.5	75.0	73.9
Mild/moderate	16.2	20.6	26.1
Severe	7.3	4.4	0.0
Alcohol (%)			
None	70.6	64.7	73.9
Mild/moderate	22.1	29.4	26.1
Severe	7.3	5.9	0.0
HOGG1 (%)			
Ser/Ser	18.2	15.1	13.0
Ser/Cys	62.1	36.4	43.5
Cys/Cys	19.7	48.5	43.5

HOGG1: Human oxoguanine glycosylase 1.

generated 123/124 and 169/170 bp fragments from primary 293 bp amplicon.

Quality control

(1) Blind the researchers to case control status; (2) include blanks in each plate in different well positions; (3) include multiple and duplicate control subjects in each plate in different well positions; (4) determine 10% as an acceptable amount of missing data and rerun assays if there is more missing data in either cases or controls; and (5) perform an Hardy-Weinberg Equilibrium test for each SNP before testing any hypothesis.

Statistical analysis

Statistical analysis was performed by SAS software (SAS Institute Inc, Version 9.1, Cary NC.). Discrete variables were analyzed by the Pearson χ^2 test and continuous variables by the Student's *t* test or generalized regression models. Logistic regression models were fitted to find the risk factors for gastric cancer and atrophic gastritis. For all analyses, significance was determined at a level of $P < 0.05$ (two-tailed).

RESULTS

Clinical characteristics in different groups

Totally, 488 subjects were included in this study (160 patients with atrophic gastritis, 73 with gastric cancer and 255 controls). All gastritis patients were *H. pylori* negative, which was confirmed by both histopathology and ^{13}C Urea breath test. The clinical characteristics of the subjects are summarized in Tables 1-3. Patients with gastric cancer were significantly older than those in the control group (59.6 ± 11.2 years *vs* 43.6 ± 10.3 years, $P < 0.0001$) and atrophic gastritis group (59.6 ± 11.2 years *vs* 51.4 ± 10.6 years, $P < 0.0001$). Gastric cancer group had a significantly higher ratio of males than atrophic gastritis group (65.8% *vs* 48.7%, $P = 0.02$).

Table 3 Clinical characteristics of different grades of intestinal metaplasia

	Grade 1 (*n* = 44)	Grade 2 (*n* = 76)	Grade 3 (*n* = 40)
Mean age (SD, yr)	50.7 (9.5)	51.4 (11.2)	51.9 (10.1)
Gender (%)			
Male	47.7	48.7	50.0
Female	52.3	51.3	50.0
Smoking (%)			
None	86.4	71.1	72.5
Mild/moderate	6.8	25.0	22.5
Severe	6.8	3.9	5.0
Alcohol (%)			
None	77.3	60.5	75.0
Mild/moderate	15.9	32.9	22.5
Severe	6.8	6.6	2.5
HOGG1 (%)			
Ser/Ser	13.9	18.6	15.8
Ser/Cys	53.5	42.7	52.6
Cys/Cys	32.6	38.7	31.6

HOGG1: Human oxoguanine glycosylase 1.

HOGG1 genotype in different groups

The hOGG1 genetic polymorphism was determined using PCR and RFLP (Figure 1). As shown in Table 4, neither the hOGG1 Ser/Cys nor the Cys/Cys genotype was associated with gastric cancer, compared with the Ser/Ser genotype (OR: 0.96 for Ser/Cys, 95% CI: 0.51-1.84 and OR: 1.1 for Cys/Cys, 95% CI: 0.48-2.1). No association was detected between hOGG1 polymorphism and Lauren type of gastric cancer ($P = 0.61$) either. Ser/Cys and Cys/Cys were significantly associated with atrophic gastritis (OR: 1.76, 95% CI: 1.03-3.0 and OR: 2.38, 95% CI: 1.34-4.23). After controlling for age, gender, smoking and alcohol, there were still significant associations, with OR: 2.05 for Ser/Cys, 95% CI: 1.14-3.68 and 2.76 for Cys/Cys, 95% CI: 1.47-5.18.

There was no statistically significant association be-

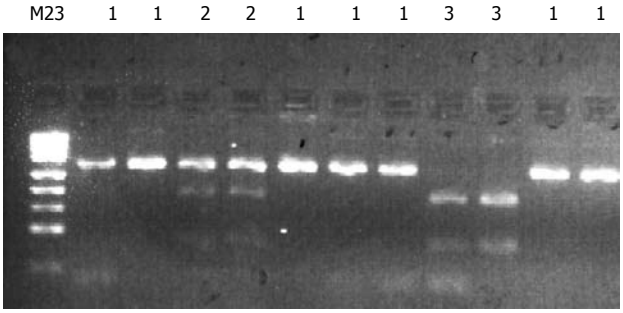

Figure 1 Selected genotyping assays. The human oxoguanine glycosylase 1 genetic polymorphism was determined using polymerase chain reaction and restriction fragment length polymorphism. 1: Genotype Ser/Ser in 293 bp; 2: Genotype Cys/Ser in 293/169/124 bp; 3: Genotype Cys/Cys in 169/124 bp; M23: Marker 23, PUC19DNA/*Msp* Ⅰ (*Hpa* Ⅱ) Marker, 23 (Fermentas life science Co.).

Table 4 Odds ratios for association of genotypes and gastric cancer/atrophic gastritis

HOGG1 subtypes	Gastric cancer *vs* control		Atrophic gastritis *vs* control	
	OR (95% CI)	Adjusted OR (95% CI)[1]	OR (95% CI)	Adjusted OR (95% CI)[1]
Ser/ser	1	1	1	1
Ser/Cys	0.96 (0.51-1.84)	1.29 (0.57-2.93)	1.76 (1.03-3.0)	2.05 (1.14-3.68)
Cys/Cys	1.1 (0.48-2.10)	1.22 (0.48-3.14)	2.38 (1.34-4.23)	2.76 (1.47-5.18)

[1]Adjusted for age, gender, other risk factors such as alcohol and smoking. HOGG1: Human oxoguanine glycosylase 1; OR: Odds ratios.

tween intestinal dysplasia and hOGG1 polymorphism (P = 0.75). HOGG1 Cys/Cys group had statistically significantly higher rate of moderate and severe atrophic gastritis than Ser/Cys and Ser/Ser group (P = 0.03). Smoking was a risk factor for gastric cancer (mild smoking 35% *vs* 20% and moderate smoking 15% *vs* 9.45%, P = 0.02), but not a risk factor for atrophic gastritis. Alcohol was a risk factor for gastric cancer (mild drinking 37.5% *vs* 26% and moderate smoking 17.5% *vs* 11%, P = 0.03), but not a risk factor for atrophic gastritis.

DISCUSSION

The exact mechanism by which oxidative stress contributes to the development of aging and carcinogenesis is still unclear. The importance of oxidative damage in chronic gastritis, either in the presence or absence of *H. pylori*, has been confirmed by various studies[2,16,17]. It is shown in the present study that there are different ratios of oxidative repair enzyme gene polymorphism between atrophic gastritis patients and healthy controls. This difference implies that the accumulation of oxidative DNA damage plays a role in atrophic gastritis. A characteristic pattern of modifications can be described both chemically and structurally for ROS-induced DNA damage as follows: modification of all bases, production of base-free sites, deletions, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal rearrangements[18].

One of the most common lesions formed by ROS

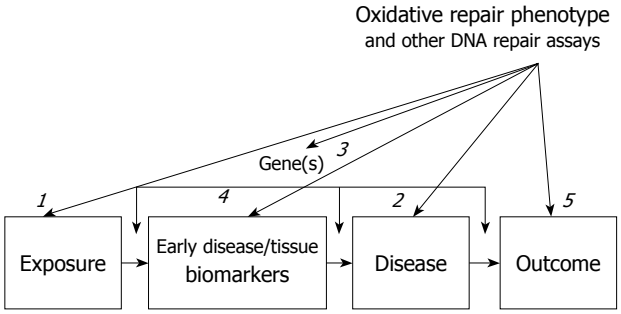

Figure 2 Categories in the molecular epidemiology of oxidative repair[34].

modifications is 8-OHdG. Among the polymorphisms found, the C/G polymorphism at position of 1245 in the 1 a-specific exon 7 of the hOGG1 gene, which results in amino acid substitution from serine (Ser) to cysteine (Cys) in codon 326 is highly prevalent[19,20]. The proportion of homozygous *C* (Ser326) individuals is different from one to another ethnic group, from 12% in Chinese, 25.8% in Micronesians, 27.7% in Japanese, 39.9% in Australian Caucasian, 24%-57.1% in Germans, 63.7% in Hungarians to 74.5% in Melanesians[21]. Although there are some contradictory results about the polymorphisms in hOGG1 affection repair function and carcinogenesis, most of these researches showed that the Cys allele was associated with cancer[22], such as esophageal[23], lung[24], gastric[25], prostate[26], nasopharyngeal cancers[22,27,28]. Only limited researches found that in Caucasian populations, Ser326 confers risk to prostate cancer[20]. Similar to our result, some studies found that Ser326Cys polymorphism has no contribution to gastric cancer and lung cancer[21,29]. Similar results were also found in breast cancer and colorectal cancer[30-32]. We did not evaluate other sequence variants, such as 11657A/G or 7143A/G, which was found to be associated with prostate cancer[14].

Oxidative DNA damage has been proposed to be related to a series of diseases. To understand the role of DNA repair activity, accurate, reproducible and specific phenotype assays should be developed and tested in human populations in molecular epidemiology studies. As presented by Caporaso[33] in 2003 (Figure 2), there are five categories of questions that can be addressed in a molecular epidemiology study.

In the first category, the exposure to alcohol and tobacco are interesting targets for gastric cancers. Both alcohol drinking and smoking can cause oxidative damage, their roles should be further studied. The present study showed that both alcohol and smoking were risk factors for gastric cancer. It was found that nicotine, at 0.8 μmol/L, the very low sub-micromolar level occurring in the tissues of smokers, may increase oxidative stress, induces apoptosis, and enhance the ability of NaDOC to activate the 153 kDa growth arrest and DNA damage promoter[34]. Some studies revealed that a frequent drinking habit elevated the odds ratio (OR) for stomach cancer in Cys/Cys compared with Ser/Ser and Ser/Cys carriers, suggesting that the hOGG1 Ser(326)Cys polymorphism may alter the impact

of some environmental factors on stomach cancer development[35].

Oxidative damage is a crucial step of *H. pylori* pathogenicity, being mechanistically related to the link between *H. pylori* infection and gastric carcinoma[36,37]. Many studies showed *H. pylori*-related oxidative DNA damage using various methodological approaches[16,38-43]. The severity of inflammation and damage associated with *H. pylori* infection is dependent on the ability of mucosal cells to counteract the increased load of reactive oxygen species[44]. *H. pylori* infection with increased oxidative damage to DNA occurred in the early stage of gastritis. The oxidative DNA damage is more apparent in gastric mucosa with severe disease than with chronic gastritis[3]. Both bacterial factors and the host response may be involved in the oxidative damage[44,45]. Patients with hOGG1 Cys/Cys genotype have a lower ability to clear ROS which contributed to the high level of DNA damage and led to epithelial cell death[43]. Farinati *et al*[46] and Konturek *et al*[47] showed that hOGG1 1245C-->G polymorphism was common in both gastric cancer and atrophic gastritis patients, but very rare in controls, and correlated more closely with 8-OHdG levels than did *H. pylori* infection or cagA status. The present study suggested that apoptosis induced by *H. pylori* may be one of the earliest events in the onset and progression of atrophic gastritis. *H. pylori* infection induced an up-regulation of Bax and down-regulation of Bcl-2 apoptosis[46]. Once the apoptosis begins, it does not depend on the existence of *H. pylori* but associated with the host capacity of DNA repair. van der Hulst *et al*[48] followed 155 cases of gastritis after *H. pylori* eradication for 1 year, and also could not find the improvement of atrophy and intestinal metaplasia. Forbes followed 54 cases of gastritis for 7 years, among them 32 cases received *H. pylori* eradication therapy, but the outcome of atrophy and intestinal metaplasia was the same between the two groups[49]. The current study found that some patients had improvement of atrophy and intestinal metaplasia after 1 year of *H. pylori* eradication and some had no improvement. Those patients might have deficiency in oxidative damage repair which was caused by either *H. pylori* infection or other exterior injury, such as alcohol or tobacco due to antioxidative enzyme polymorphism[46]. The present scientific consensus is that the *H. pylori* oncogenic role is mediated by the chronic active inflammation it elicits in the gastric mucosa. Although the ultimate basic mechanism of carcinogenesis is unknown, strongly suggestive evidences showed that oxidative stress played a pivotal role in the process[39]. We found that alcohol and tobacco rather than interior hOGG1 polymorphism were risk factors for gastric cancer.

In conclusion, Ser326Cys hOGG1 polymorphism plays an important role in atrophic gastritis after eradication of *H. pylori* for 1 year. However, no association was found between this polymorphism and gastric cancer, although it could be secondary to a not large enough sample size. Smoking and alcohol are risk factors of gastric cancer regardless of different kinds of Ser326Cys HOGG1 polymorphism. More prospective studies are needed to confirm our findings and further reveal the causal relationship between Ser326Cys hOGG1 polymorphism and atrophic gastritis/gastric cancer.

COMMENTS

Background

Although oxidation injury caused by *Helicobacter pylori* (*H. pylori*) is the mechanism of atrophic gastritis and gastric cancer, a large proportion of atrophic gastritis can not be reversed after *H. pylori* eradication. The defect of anti-oxidation barrier might be related to the occurence of atrophic gastritis and gastric cancer. The polymorphism of human oxoguanine glycosylase 1 (hOGG1) is thought to be closely related with the repairing level of DNA oxidation injury.

Research frontiers

hOGG1 is one of the most important antioxidative enzymes. Among several *hOGG1* gene polymorphisms, the Ser→Cys polymorphism at position 326 is related to decreased repair function. However, the association between hOGG1 polymorphism and gastric cancer or atrophic gastritis in post-*H. pylori* eradication patients remains unclear.

Innovations and breakthroughs

This is the first study to report that hOGG1 polymorphisms (Cys/Cys and Ser/Cys) are associated with atrophic gastritis patients after *H. pylori* eradication. No significant association was detected between hOGG1 polymorphisms (Cys/Cys or Ser/Cys) and gastric cancer.

Applications

The results from this study may hypothesize some different pathways involved in the gastric cancer and atrophic gastritis after *H. pylori* eradication with different hOGG1 polymorphisms. Biological approaches will be adopted to disclose the detailed mechanism in molecular pathway and gene sets level.

Peer review

The authors examined the association between HOGG1 polymorphism and atrophic gastritis and gastric cancer in post-*H. pylori* eradication patients. It was shown that hOGG1 polymorphisms (Cys/Cys and Ser/Cys) were associated with atrophic gastritis in a Chinese population with post-*H. pylori* eradication. No significant association was detected between hOGG1 polymorphisms (Cys/Cys or Ser/Cys) and gastric cancer. The results are interesting and may hypothesize the different underlying pathways leading to the outcomes in this population.

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BRIEF ARTICLE

Paeonol inhibits tumor growth in gastric cancer *in vitro* and *in vivo*

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Abstract

AIM: To investigate the anti-tumor effects of paeonol in gastric cancer cell proliferation and apoptosis *in vitro* and *in vivo*.

METHODS: Murine gastric cancer cell line mouse forestomach carcinoma (MFC) or human gastric cancer cell line SGC-7901 was cultured in the presence or absence of paeonol. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and cell cycle and apoptosis by flow cytometry and TUNEL staining. Tumor growth after subcutaneous implantation of MFC cells in mice was monitored, and the effects of treatment with paeonol were determined.

RESULTS: *In vitro*, paeonol caused dose-dependent inhibition on cell proliferation and induced apoptosis. Cell cycle analysis revealed a decreased proportion of cells in G0/G1 phase, with arrest at S. Paeonol treatment in gastric cancer cell line MFC and SGC-790 cells significantly reduced the expression of Bcl-2 and increased the expression of Bax in a concentration-related manner. Administration of paeonol to MFC tumor-bearing mice significantly lowered the tumor growth and caused tumor regression.

CONCLUSION: Paeonol has significantly growth-inhibitory and apoptosis-inducing effects in gastric cancer cells both *in vitro* and *in vivo*.

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Key words: Paeonol; Gastric cancer; Cell proliferation; Apoptosis

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world. Gastric cancer represents roughly 2% (25 500) of all new cancer cases yearly in the United States[1,2]. However, gastric cancer is much more common in many parts of the world including China. With delayed symptoms, most of gastric

cancer patients are often diagnosed at an advanced stage, and the 5-year survival remains less than 20%[1]. Surgical resection remains the only curative treatment. But with high rates of local recurrence and systemic spread (including peritoneal metastases), attempts to improve the outcomes of this disease have incorporated the use of adjuvant chemotherapy. However, the high incidence rate of severe side effects of the drugs used in the chemotherapy limits its therapeutic results. Thus, there has been growing interest in developing more effective chemotherapeutic agents for gastric cancer.

Natural products are potential sources of novel anticancer drugs that may be applicable to cancer chemotherapy. The anti-tumor potential of components from Chinese herbal medicines has been of great interest. With thousands of years of experience, Chinese herbal medicines are considered as a rich source of new therapeutic agents. Paeonol is the main component of a Chinese herbal medicine prepared from the root bark of *Paeonia moutan*. It has various pharmacological and physiological effects such as sedation, hypnosis, antipyresis, analgesia, anti-oxidation, anti-inflammation, anti-bacteria, immuno-regulation and anti-tumor effects[3-6]. Our previous studies showed that paeonol inhibited the proliferation of different tumor cell lines *in vitro* and suppressed tumor growth in xenograft tumor models *in vivo*[7-9].

In this study, we found for the first time that paeonol exerted the impressive biological effects in blocking the growth of gastric cancer cells *in vitro*, and in an allograft mouse model as well. We also observed that the growth inhibitory effects of paeonol were mediated by induction of apoptotic cell death *via* increasing the Bax/Bcl-2 ratio. The results of this investigation may provide a scientific explanation for the traditional application of this herbal medicine in gastric cancer therapy.

MATERIALS AND METHODS

Drugs and chemicals

The compound paeonol with 98% purity was purchased from Tianshi Pharmaceutical Factory of Tongling (Cat. No. 010521) (Tonglin, Anhui, China). For *in vivo* experiments, paeonol was suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) to the desired concentrations. *In vitro,* the cells were seeded and treated with the control vehicle (0.1% DMSO), or different concentrations of paeonol for the indicated time. 5-fluorouracil (5-FU) injection was obtained from Shanghai Haipu Pharmaceutical Factory, China (Cat. No. 031109) (Shanghai, China). RPMI-1640 medium was from GIBCO BRL, Life Technologies Inc. (Grand Island, New York, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and acridine orange (AO) were from Sigma Co. (St. Louis, MO, USA). DNA-Prep-Reagents Kit was bought from Beckman Coulter Co. (Miami, FL, USA. Cat. No. 760279K). Rabbit polyclonal antibodies against human Bcl-2 and Bax were all purchased from Lab Vision Corporation (Fremont, California, USA) and streptavidin-biotin-peroxidase (S-P) reagents kit was obtained from Fuzhou Maxim Biotech, Ltd. (Fuzhou, Fujian, China).

Animals and cell lines

Murine gastric cancer cell line mouse forestomach carcinoma (MFC) and human gastric cancer cell line SGC-7901 were purchased from Shanghai cell bank, Chinese Academy of Sciences. Six-week-old male healthy Kunming mice were obtained from the Animal Department of Anhui Medical University, and were maintained on a 12 h light/12 h dark cycle from 6:00 AM to 18:00 PM under a regulated environment (20 ± 1℃). Animals were housed in plastic cages with free access to food and water. All procedures followed the guidelines for use of animals in research set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Cell culture

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37℃ in humid atmosphere with 5% CO_2.

Cell proliferation assay: Cells were cultured in 96-well plates at a density of 1×10^4 cells with 100 μL medium overnight, and then treated with various concentrations of drugs. After 44 h of drug exposure, MTT solution (5 mg/mL) was added to the plates. The cells were incubated at 37℃ for another 4 h. Formazine was solved in 150 μL/well DMSO and absorbance was detected at 490 nm using ELx 800 Strip reader (Bio-Tek, USA). Each drug concentration was tested in five replicates from which the mean, standard deviation and coefficient of variation were calculated. The drug concentration capable of inhibiting cell growth by 50% relative to untreated controls was calculated as follows: Inhibition percentage (%) = (1-$A_{490\ nm}$ of experimental well)/$A_{490\ nm}$ of control well. The median inhibitory concentration (IC_{50}) (defined as the drug concentration inhibiting cell growth by 50%) was calculated from the dose-response curves.

Cell cycle analysis: Cells were seeded (2×10^5 cells/well) in 6-well plates, treated with or without paeonol for 48 h and then trypsinized, washed in phosphate-buffered saline and fixed in ice-cold 70% ethanol-phosphate-buffered saline. DNA was labeled with propidium iodide. Cells were sorted by flow cytometry, and cell cycle profiles were determined using Mcycle software (Beckman Coulter, Fullerton, CA).

Apoptosis assay: Apoptosis was analyzed by two methods. First, sub-G1 DNA analysis was conducted by flow cytometry. Cells were cultured in 6-well plates. Non-adherent cells were removed by gentle washing, and the medium was removed and replaced with fresh medium containing paeonol at the desired concentrations. After 24 h of drug exposure, cells were collected and centrifuged at 2000 r/min in a 15 mL tube for 5 min, fixed with 70% ethanol for 4 h, washed twice with phosphate buffered sa-

line (PBS), and then 500 μL propidium iodide (PI) staining buffer was added in the dark at room temperature for 30 min according to the procedure of DNA-Prep Coulter reagents kit. A minimum of 1×10^5 cells treated for each group were analyzed using an EPICS XL-MCL Coulter counter. Second, acridine orange fluorescence staining was performed as follows. Cells were seeded overnight in 6-well plates containing cover slips. After incubation with the drug for 48 h, the cover slips were washed twice with PBS, fixed with 95% ethanol for 15 min, acidified with 1% acetic acid for 30 s, dyed with 0.1 mg/mL acridine orange for 10 min, differentiated with 0.1 mol/L $CaCl_2$ for 2 min, and then washed with PBS for 3 times. The cover slips were sealed and observed under fluorescence microscope.

Apoptotic cells in sections of mouse tumor tissues were detected using an *in situ* apoptosis detection kit (Promega) according to the instructions of the manufacturer. Cells were visualized under light microscope.

Animal tumor allograft model and treatment with drugs

Kunming mice were injected with MFC cells (ip), and the second generation of the cells from the mice with ascites was collected under aseptic condition. The collected cells were diluted in normal saline to a concentration of 1×10^{10} cells/L and injected into the right upper flank regions of each mouse. After 7 d, they were weighed and randomized into 5 groups (n = 12-20): A negative control group was given an equal amount of normal saline (intragastric administration), a positive control group was treated with intraperitoneal injection of 5-FU (2.5 mg/kg per day) and three paeonol groups were intragastrically administrated with paeonol (100, 200 and 400 mg/kg per day). Drugs were administrated daily by gavage for 11 d. In addition, we used the mice without tumor injection as normal groups. On day 12, all mice were weighed and killed, and the tumor was removed and weighed.

Immunohistochemical analysis for Bcl-2 and Bax

Cells were cultured in 6-well plates containing cover slips overnight. After exposure to drug for 24 h, the cover slips were washed twice with PBS, fixed with 4% paraformaldehyde solution for 25 min. The standard streptavidinbiotin-peroxidase method was used for immunohistochemical stain. Briefly, endogenous peroxidase activity was blocked by incubation in endogenous peroxidase blocking solution for 10 min followed by incubation with normal non-immune serum for 10 min at room temperature. Samples were incubated overnight at 4℃ with primary antibodies against Bcl-2 and Bax, washed with PBS and incubated with biotinylated secondary anti-rabbit antibody for 30 min at 37℃. After washing, the samples were incubated with streptavidin-peroxidase at 37℃ for 20 min. Diaminobenzidine/hydrogen peroxidase was used as a chromogen and samples were counterstained with hematoxylin. As negative control, PBS was used instead of primary antibody and other steps were followed in the same way.

The immunohistochemical results were quantitatively analyzed by a biological image analysis system which consists of Nikon ECLIPSE 80i biology microscope, Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Japan), and JEOA 801D morphologic biological image analysis software version 6.0 (Jie Da Technologies, Inc, China). Samples were observed in three randomly selected optical fields under microscopy (× 400) and the average optical density was measured.

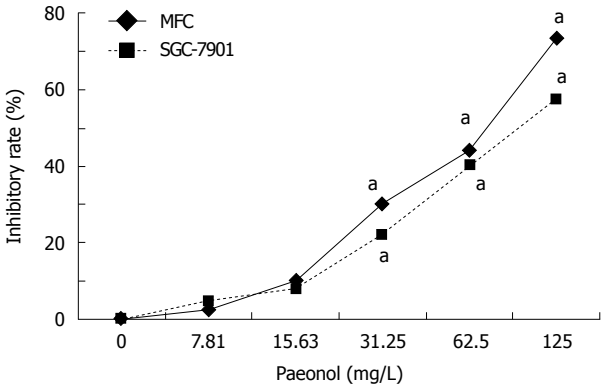


Figure 1 Paeonol inhibits the proliferation of mouse forestomach carcinoma and SGC-7901 cells *in vitro*. Mouse forestomach carcinoma (MFC) or SGC-7901 cells (1×10^4 cells) were incubated either alone (control) or in the presence of different concentrations of paeonol for 48 h and cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Treatment with paeonol caused dose-dependent inhibition of cell proliferation. $^aP < 0.05$, paeonol treated *vs* untreated control. Data are mean ± SD (n = 6).

Statistical analysis

Data were expressed as mean ± SD. Analysis of variance test was used for determining differences between groups, and $P < 0.05$ was regarded as statistically significant.

RESULTS

Anti-proliferative effects of paeonol in cultured gastric cancer cells

To evaluate the effects of paeonol on cell viability, gastric cancer cell line MFC or SGC-7901 cells were cultured with varying concentrations of paeonol. Exposure to paeonol for 48 h produced a dose-dependent suppression of cell proliferation in both MFC and SGC-790 cells (Figure 1). The IC_{50} of paeonol on MFC and SGC-790 cells were 60.10 and 82.60 mg/L, respectively.

Paeonol induces S phase cell cycle arrest in cultured gastric cancer cells

To determine whether paeonol decreased viability of cultured gastric cancer cells by inhibiting cell growth, we investigated the effect of paeonol on cell cycle distribution. After propidium iodide staining and flow cytometric analysis, paeonol treated gastric cancer cells revealed a dose-dependent increase accumulation in the number of cells in S phase at 24 h. Concomitant with this blocking, there was a dose-dependent decrease in the number of cells accumulating in the G0/G1 phase, and also a decrease in G2/M phase but only in MFC cell line, which

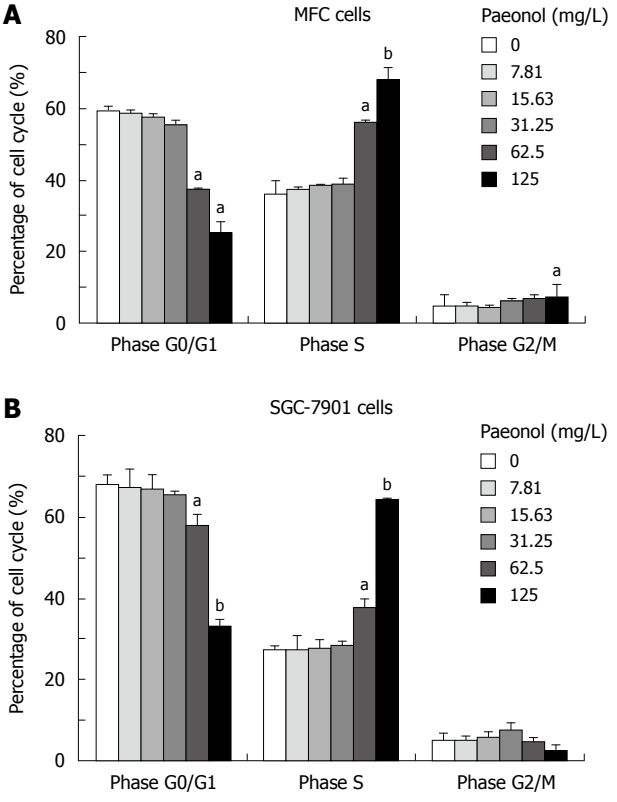


Figure 2 Effects of paeonol on cell cycle of mouse forestomach carcinoma and SGC-7901 cells *in vitro*. A: Mouse forestomach carcinoma (MFC) cells; B: SGC-7901 cells. The number of cells in G0/G1, S, G2/M phases was determined by flow cytometry. Values are expressed as the mean ± SD from three replicate experiments ($n = 6$). $^aP < 0.05$, $^bP < 0.01$, paeonol treated *vs* untreated control.

is consistent with the finding that paeonol blocks the cell cycle at the S phase (Figure 2).

Induction of apoptosis by paeonol in cultured gastric cancer cells

As arrest of cell cycle progression in tumor cells is usually associated with concomitant activation of cell apoptosis pathways, we checked the induction of apoptosis after paeonol-treated gastric cell cultures. By flow cytometric analysis, the number of cells with sub-G1 DNA content, which represents apoptotic cell population, was significantly increased in a concentration-dependent manner in both MFC and SGC-790 cells (Figure 3A-C) after 24 h paeonol treatment. Consistent with this finding, there was an increased number of apoptotic cells stained with acridine orange after paeonol treatment compared with untreated gastric cancer cells. The cells treated with paeonol for 48 h showed typically apoptotic changes, including reduction in cell volume, chromatin condensation and deformed and fragmented nuclei (Figure 3D).

Induction of apoptosis by paeonol is associated with increase of Bax/Bcl-2 ratio in vitro

The streptavidin-peroxidase method was used to examine the expression of Bcl-2 and Bax. Positive Bcl-2 and Bax staining was identified by brown and yellow staining mainly in cytoplasm or membrane (Figure 4). Treatment of paeonol in gastric cancer cell line MFC and SGC-790 cells significantly reduced the number of Bcl-2 positive cells, with weak to moderate staining in a concentration-related manner while the number of Bax positive cells was increased in the paeonol-treated group in a concentration-related manner.

Paeonol inhibits gastric tumor growth in vivo associated with induction of apoptosis and inhibiting proliferation

Based on the observed anti-proliferative and proapoptotic effects of paeonol in gastric cancer cell lines *in vitro*, we next tested whether paeonol treatment could affect growth of gastric cancer cells *in vivo*. For these studies, we established the experimental model of MFC tumor-bearing mice and then treated these animals with paeonol (100, 200 and 400 mg/kg per day, ip). We also used 5-FU (2.5 mg/kg per day, ip) as positive control. Most of the mice developed visible subcutaneous MFC tumors 7 d after MFC cell injection. The mice with visible subcutaneous MFC tumors were divided randomly into 5 groups. Paeonol or 5-FU treatment was started and lasted 11 d. The volume of tumor was calculated every 2 d (Figure 5A). When the experiment was terminated 11 d later, mice treated with paeonol (100, 200, and 400 mg/kg per day) had reduction in tumor growth by 37.55%, 51.62% and 54.15%, respectively compared with the model controls. The inhibitory rate of positive control group treated with 5-FU injection was 69.68%. Interestingly, 3/20 mice died in the model control group while 0/12 mice died in all paeonol groups, and 2/12 mice died in 5-FU group during the experiment, indicating no severe drug toxicity of paeonol under these doses.

Histological analysis of MFC tumors from untreated mice revealed poorly differentiated, infiltrating gastric cancer with high mitosis rates. In contrast, tumors from mice receiving paeonol showed few mitotic cells (Figure 5B). Compared with the control tumors, mitotic rates were significantly lower in tumors from mice receiving paeonol.

We found that paeonol not only decreased the proliferative rate of subcutaneously implanted gastric cancer, but also significantly increased the rate of apoptosis. Thus, while MFC tumors from untreated mice showed almost no TUNEL staining (Figure 5C), tumor cells from mice receiving paeonol showed frequent condensed nuclei and cytoplasmic shrinkage.

DISCUSSION

Paeonol is a natural product extracted from the root bark of *Paeonia Suffruticosa Andrew*[10]. It is a white needle crystal with a relatively low-melting point of 51-52℃. The molecular formula of paeonol is $C_9H_{10}O_3$ and the molecular weight is 166.18[11]. Our previous study demonstrated the anti-neoplastic activity of paeonol in various cell lines, such as human erythromyeloid cell line K562, breast cancer gene cell line T6-17, human hepatoma cell line Bel-7404, HepG2 and SMMC-7721, cervical cancer cell line Hela, and human colorectal cancer cell line HT-29[7-9,12,13]. In this study, we found that paeonol inhib-

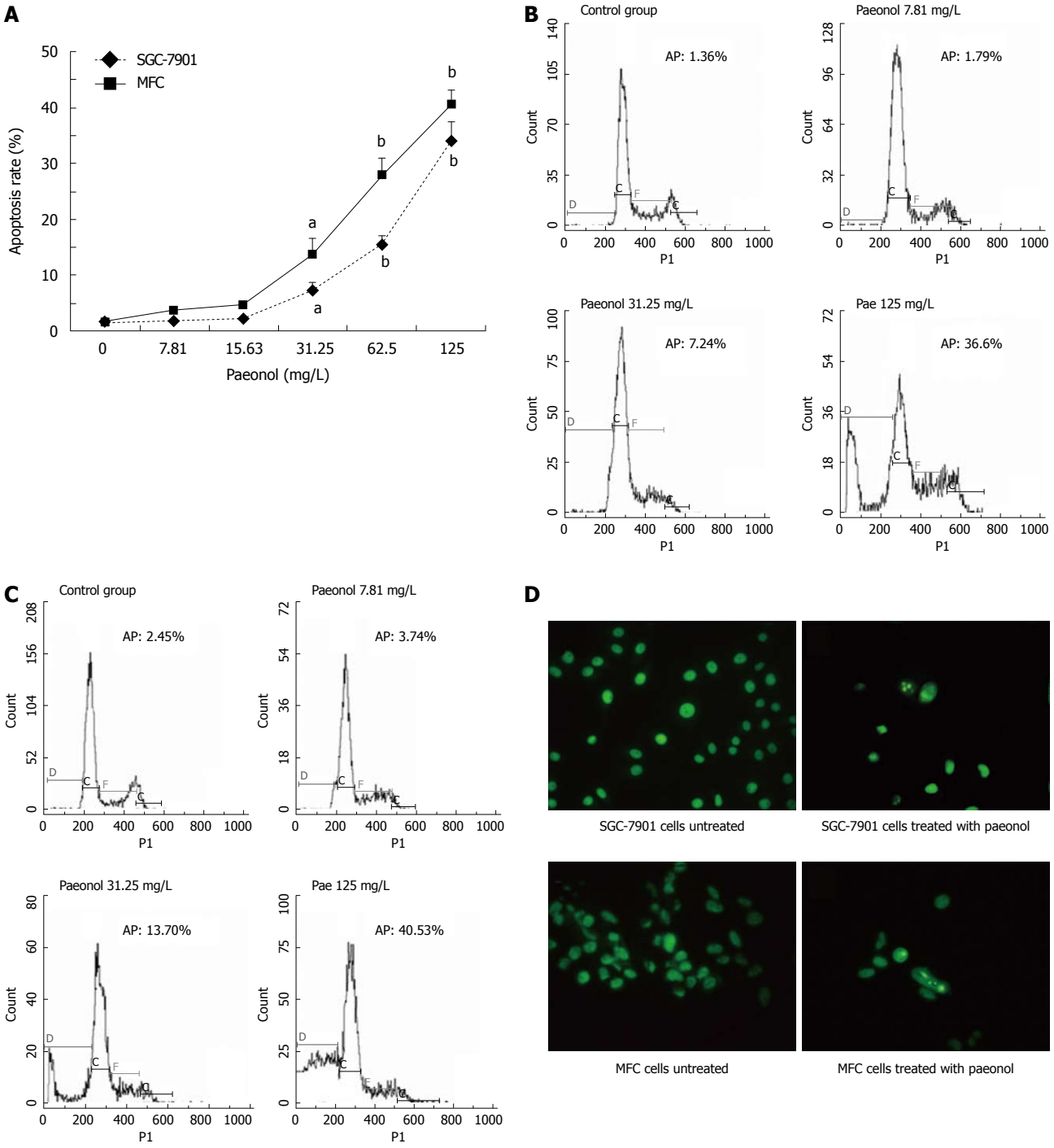


Figure 3 Induction of apoptosis by paeonol in cultured mouse forestomach carcinoma and SGC-7901 cells. A: Paeonol increased the rate of apoptosis, as determined by the number of cells with sub-G1 DNA content by flow cytometry. Values are expressed as the mean ± SD from three replicate experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, paeonol treated *vs* untreated control; B, C: Effects of paeonol on apoptosis of gastric cancer cell line SGC-7901 (B) and mouse forestomach carcinoma (MFC) cells (C) by flow cytometry. One representative flow cytometry tracing was shown from three replicate experiments. AP: Apoptotic percentage; D: Fluorescent staining of nuclei (× 320) in paeonol-treated and untreated cells by acridine orange. SGC-7901 and MFC cells treated with 62.5 mg/L paeonol for 48 h. Condensed and fragmented nuclei and apoptotic bodies were seen in the paeonol-treated cells, but not in the control.

ited the proliferation of two kinds of gastric cancer cell lines in a dose-dependent manner *in vitro*. Moreover, treatment of MFC tumor bearing mice with various doses of paeonol (100, 200 and 400 mg/kg per day) significantly suppressed the tumor growth. Our results indicated that the anti-tumor effect of paeonol was related to induction of apoptosis.

We analyzed the effects of paeonol in two gastric cancer cell lines *in vitro*. In both MFC and SGC-7901 cells, paeonol caused dose-dependent growth inhibition and arrest, and similar results have been observed in other cancer cell lines in our previous studies[8,9]. Flow cytometric analysis of the effects of paeonol on the cell cycle in treated gastric cancer cells revealed a dose-dependent

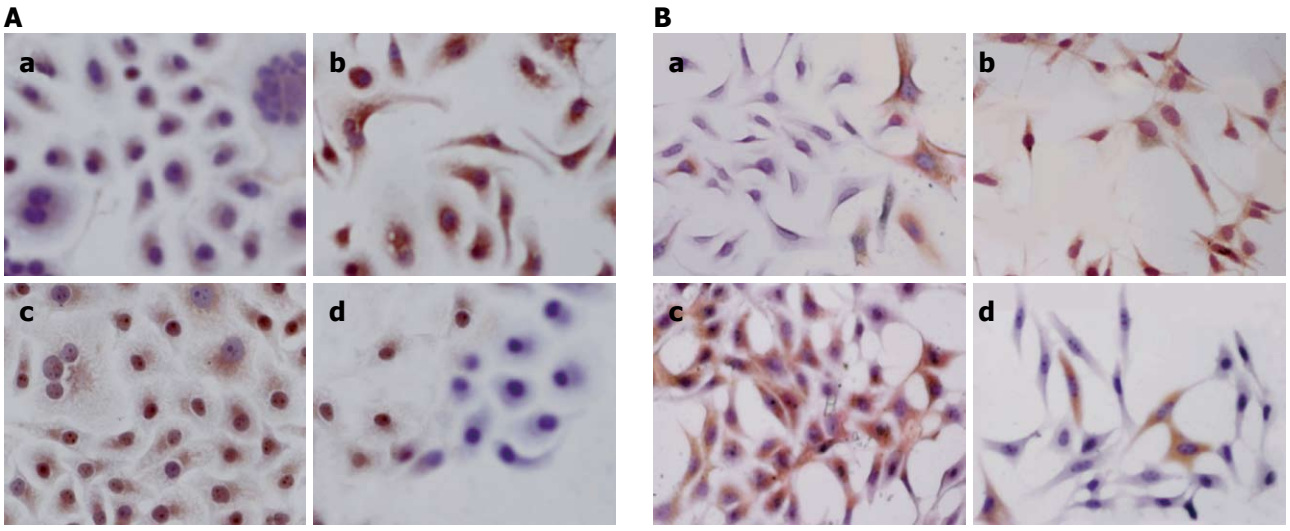
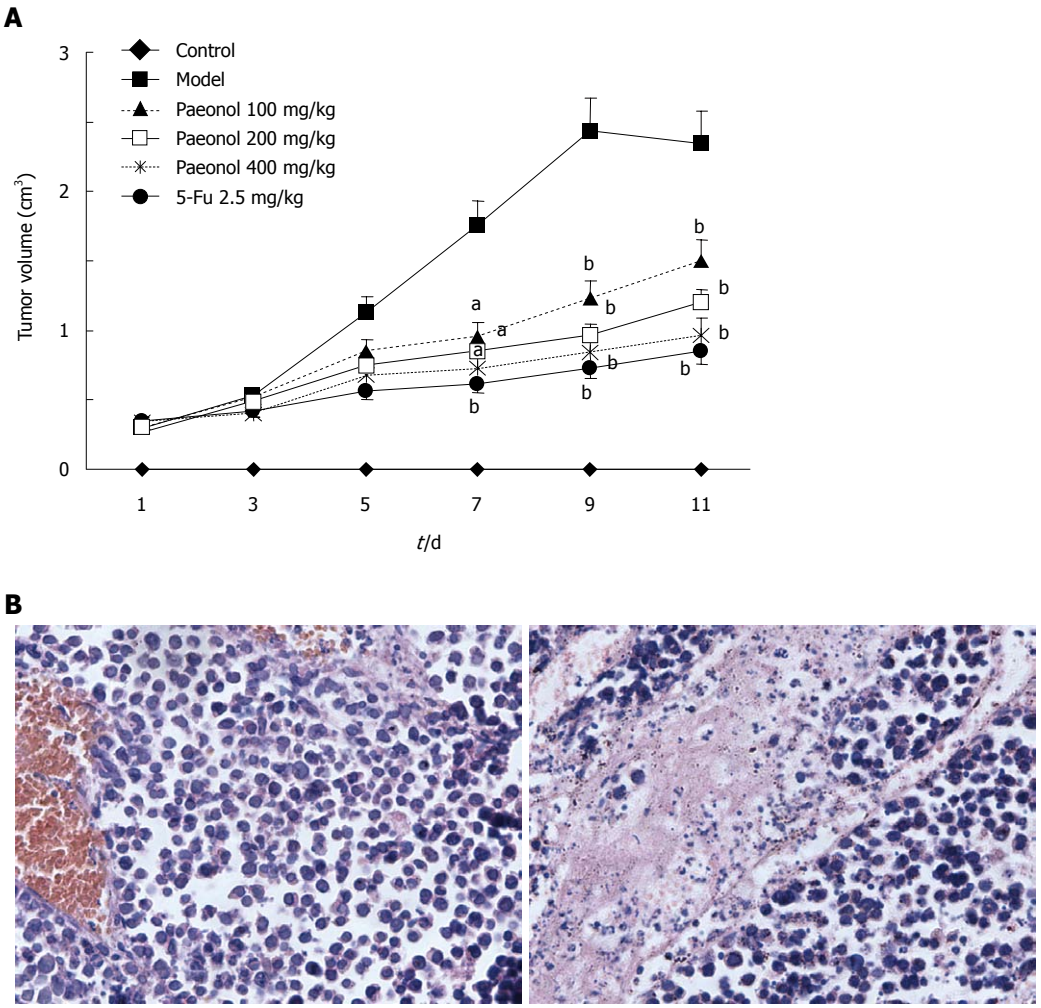


Figure 4 Expression of Bcl-2 and Bax in mouse forestomach carcinoma and SGC-7901 cells with or without paeonol treatment (× 200). A: SGC-7901, cells were treated with 62.5 mg/L paeonol or with vehicle control for 48 h. a: Untreated control; b: Paeonol treatment. Bax expression was detected by immunohistochemical analysis. Cells treated with paeonol had a significant increase in the expression of Bax. c: Untreated control; d: Paeonol treatment. Bcl-2 expression was detected by immunohistochemical analysis. Cells treated with paeonol had a significant decrease in the expression of Bcl-2; B: Mouse forestomach carcinoma, cells were treated with 62.5 mg/L paeonol or with vehicle control for 48 h. a: Untreated control; b: Paeonol treatment. Bax expression was detected by immunohistochemical analysis. Cells treated with paeonol had a significant increase in the expression of Bax. c: Untreated control; d: Paeonol treatment. Bcl-2 expression was detected by immunohistochemical analysis. Cells treated with paeonol had a significant decrease in the expression of Bcl-2.



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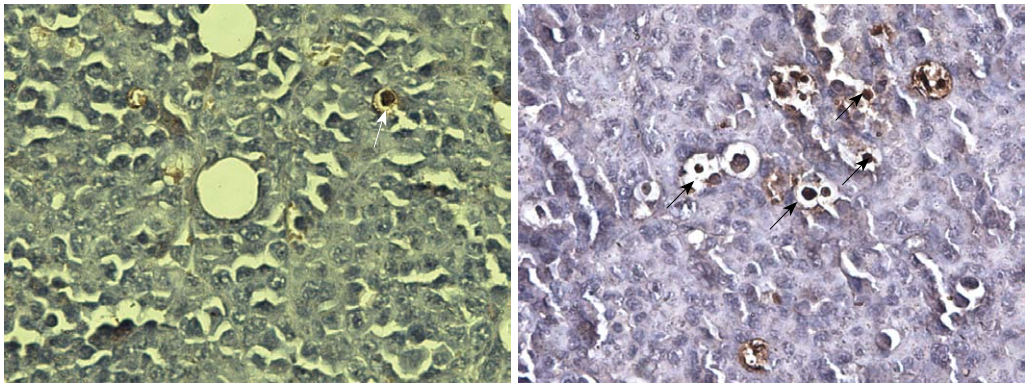

Figure 5 Paeonol inhibits gastric tumor growth *in vivo* associated with induction of apoptosis and inhibiting proliferation. A: Effects of paeonol or 5-fluorouracil (5-FU) in tumor volume. Values are expressed as the mean ± SD (n = 12-14). $^{a}P < 0.05$, $^{b}P < 0.01$, paeonol treated *vs* model group; B: Hematoxylin-eosin-stained sections of mouse forestomach carcinoma (MFC) tumors from untreated controls showed numerous cells in mitosis (left), paeonol (200 mg/kg) treatment suppressed cell proliferation, as indicated by the small number of mitotic cells (right) (× 400); C: TUNEL stained sections of MFC tumors from untreated control displayed occasionally apoptotic cells (left). Tumors from mice treated with paeonol (200 mg/kg) had a prominent population of cells with condensed or fragmented nuclei and cytoplasm shrinkage, indicating apoptosis (right) (× 400). The arrows indicate apoptotic cells.

decrease in cell proliferation, a concomitant and proportionate accumulation of cells in phase S, and an increase of cells in the sub-G1 fraction. The latter finding indicates increased apoptosis, a finding confirmed by acridine orange fluorescence staining. These results *in vitro* suggested that paeonol might have anti-proliferative effects and arrest the cell cycle, leading to cancer cell apoptosis.

We also investigated the underlying mechanisms for the apoptosis induction of gastric cancer cells. Apoptosis is a physiological process in controlling cell number and proliferation that helps maintain the homeostasis of multi-cellular organisms[14,15]. The hypothesis that failure to undergo apoptosis contributes to the development of resistance to anticancer agents has been the subject of extensive research[16]. Therefore, agents that facilitate apoptosis should improve therapeutic efficacy. In the present study, the cells treated with paeonol showed morphological alterations typical of the apoptotic process, including reduction in cell volume, chromatin condensation, deformed and fragmented nuclei, and so on. Similarly, apoptotic peak appeared before G_1 phase after paeonol treatment, which resulted from the internucleosomal degradation of DNA. Although understanding of the detailed signaling pathways that trigger apoptosis is incomplete, this process is controlled by a number of complex proteins. The Bcl-2 family proteins are key regulators of apoptosis and are over-expressed in many malignancies[17]. To examine the mechanism of apoptosis, we examined the expression of Bcl-2 protein family, which is an important regulator of apoptosis. We found that paeonol decreased the expression of Bcl-2 and increased the expression of Bax, the ratio of Bcl-2/Bax decreased correspondingly, which could be an important mechanism contributing to the induction of tumor cell apoptosis by paeonol.

Having observed substantial suppression of gastric cancer cell growth by paeonol treatment i*n vitro*, we conducted experiments designed to test the potential for paeonol to exert protective effects against gastric cancer *in vivo*. It was recognized that the model used in this study, i.e. mice injected with gastric cancer cells subcutaneously, is an artificial one and is not necessarily equivalent to the stepwise development of gastric cancer in human. Nonetheless, the results here showed that treatment with different doses of paeonol inhibited tumor growth in the tumor-bearing mice.

In conclusion, the results obtained in the present study indicate that paeonol has significantly growth-inhibitory and apoptosis-inducing effects in gastric cancer cells both *in vitro* and *in vivo*. These promising data provide a rationale for further exploration of paeonol as a therapeutic agent for gastric cancer.

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COMMENTS

Background

Gastric cancer is a common tumor which seriously threatens the human health worldwide. Currently, a variety of cytotoxic and anti-proliferative agents have been tested in gastric cancer. However, the high rate of severe side effects of these drugs limits their therapeutic results. Thus, there is a critical need to develop more strategies for chemotherapy of gastric cancer.

Research frontiers

Chinese herbal medicines are now attracting great attention in the world, which also show promising effects in gastric cancer treatment. Paeonol, a natural product extracted from the root of *Paeonia Suffruticosa Andrew*, has shown anti-neoplastic activities in both cell lines and animal models.

Innovations and breakthroughs

This is the first report on the anti-proliferation, induction of apoptosis by paeonol in gastric cancer *in vitro* and *in vivo*.

Applications

Paeonol might be useful as a new agent in gastric cancer.

Terminology

Paeonol is isolated from the herb *Pycnostelma paniculatum* (Bunge) K.S., and the root of the plant *Paeonia Suffruticosa Andrew*. It is a white needle crystal with a relatively low melting point of 51-52℃. The molecular formula of paeonol is $C_9H_{10}O_3$ and the molecular weight is 166.18.

Peer review

The paper describes the effects of paenol on cell lines and a mouse model of gastric tumor. The data have a potential interest in the field of cancer therapy. It would be interesting to have some pieces of information on the chemical structure of paeonol and to discuss the possible molecular mechanism of the action of this substance.

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CASE REPORT

Febrile cholestatic disease as an initial presentation of nodular lymphocyte-predominant Hodgkin lymphoma

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Abstract

Febrile cholestatic liver disease is an extremely unusual presentation of Hodgkin lymphoma (HL). The liver biopsy of a 40-year-old man with febrile episodes and cholestatic laboratory pattern disclosed an uncommon subtype of HL, a nodular lymphocyte-predominant HL (NLPHL). Liver involvement in the early stage of the usually indolent NLPHL's clinical course suggests an aggressiveness and unfavorable outcome. Emphasizing a liver biopsy early in the diagnostic algorithm enables accurate diagnosis and appropriate treatment. Although rare, HL should be considered in the differential diagnosis of cholestasis.

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Key words: Cholestatic disease; Hodgkin lymphoma; Nodular lymphocyte-predominant Hodgkin lymphoma

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INTRODUCTION

Febrile cholestatic disease is a rare initial presentation of Hodgkin lymphoma (HL)[1]. Initial liver involvement is extremely rare in indolent types of HL and more frequent in aggressive types of HL such as mixed cellularity and lymphocytic depletion[2]. Liver involvement increases up to 60% postmortem in advanced stages of the disease, as a result of intra and extrahepatic infiltration by the tumor or due to paraneoplastic phenomenon[3]. In this report, we present the case of a 40-year-old man with febrile cholestatic disease as the initial presentation of an uncommon subtype of HL - a nodular lymphocyte-predominant HL (NLPHL). We also discuss the challenges of the diagnostic workup and treatment, and review the literature.

CASE REPORT

In November 2008, a 40-year-old man was referred to our hospital due to febrile episodes and abnormal liver tests that developed during the previous month. Initial liver function tests indicated cholestasis without hyperbilirubinemia [alkaline phosphatase (AP) 1030 IU/L, γ-glutamyl transferase (GGT) 419 U/L and total bilirubin 14 μmol/L]

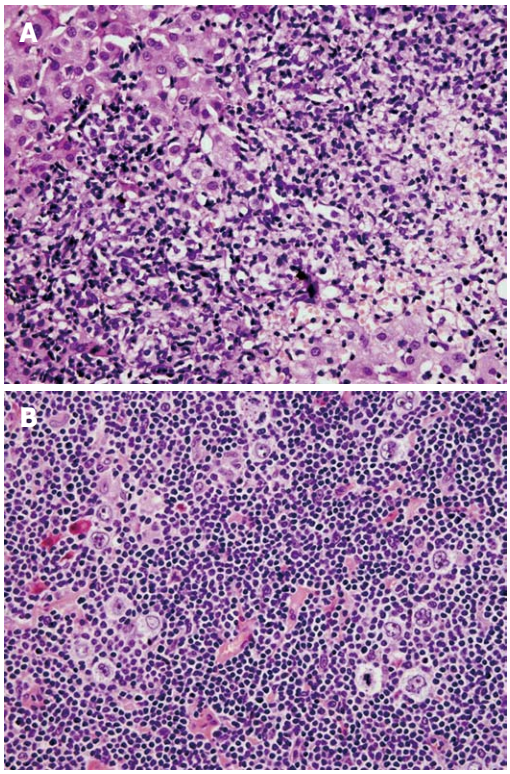

Figure 1 Nodular lymphocyte-predominant Hodgkin lymphoma of the liver and axillary lymph node. A: A portal tract showing a dense lymphoid infiltrate. The multiple sections revealed just a few scattered, CD20 positive neoplastic cells (HE stain, × 40); B: The popcorn (Hodgkin lymphoma) cells with the typically lobulated nuclei in a background of small lymphoid cells (HE stain, × 40).

with an increased level of transaminase activity [aspartate aminotransferase (AST) 170 IU/L and alanine aminotransferase (ALT) 156 IU/L]. A 5-d treatment of amoxicillin-clavulanate for *Klebsiella pneumonia* urinary infection, preceding the symptoms, was promptly discontinued with no improvement in liver tests. The initial physical examination was unremarkable. Abdominal ultrasound (US) revealed a slightly hyperechogenic liver without focal lesions and normal biliary system. Serology (hepatitis A, B, C, human immunodeficiency virus, *Epstein-Barr* virus and cytomegalovirus), autoimmune studies (antinuclear, antimitochondrial, anti-smooth muscle and anti-liver-kidney microsomal antibodies) and tumor markers (α-fetoprotein, carbohydrate antigen 19-9 and prostate-specific antigen) tested negative. There was no history of intravenous drugs or hepatotoxic substances abuse.

On admission, functional liver tests showed slight progression of cholestatic pattern: AP 1511 IU/L, GGT 516 U/L, total bilirubin 17 μmol/L, AST 200 IU/L and ALT 197 IU/L. Repeat physical examination revealed moderate hepatosplenomegaly with no peripheral lymphadenopathy. Abdominal US showed an enlarged, hyperechogenic liver without focal lesions and an enlarged spleen. Abdominal computed tomography (CT) scan confirmed the aforementioned finding with additional slightly enlarged retroperitoneal lymph nodes. Chest CT scan was normal.

Three months after initial symptoms, liver biopsy showed portal areas filled with mixed inflammatory infiltrate, containing lymphocytes, histiocytes, and rare atypical lymphatic cells with large nuclei (CD20+, CD45+). In addition to mild interface hepatitis, there were also signs of mild canalicular and cytoplasmic cholestasis (Figure 1A).

US of the neck, inguinal and axillary region showed a single enlarged (2 cm) left axillary lymph node. Fine needle aspiration (FNA) of the lymph node was suggestive for HL. An extirpated axillary lymph node showed a pseudonodular pattern with expanded follicular dendritic network (highlighted with CD23 antibody), and scattered atypical "popcorn" cells (LP cells) that stained positively for CD20, CD45, PAX5 and EMA, and negatively for CD30, CD15, and EBV-LMP (Figure 1B) reconfirming the diagnosis of nodular lymphocytic predominant HL. No bone marrow infiltration was found.

The final diagnosis was advanced NLPHL (clinical stage ⅣB) and the initial treatment consisted of four cycles of the ABVD protocol (doxorubicin, bleomycin, vinblastine and dacarbazine). Evaluation after treatment showed no B-symptoms or signs of peripheral adenopathy, but hepatosplenomegaly and abdominal lymphadenopathy persisted. Disease activity was confirmed by liver FNA and treatment was continued with the bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone (BEACOPP) protocol. On the ninth day of the new protocol the patient died due to sepsis. Postmortem liver specimen revealed scanty lymphatic portal infiltrate without LP cells.

DISCUSSION

In this case report, the diagnosis of HL was established by a liver biopsy identifying a rare subtype of HL, NLPHL. The clinical and laboratory characteristics were consistent with febrile cholestatic disease with the exclusion of other possible causes such as biliary tract obstruction, bacterial and viral infections, autoimmune disease or hepatotoxic substances.

NLPHL is a rare subtype of HL (representing 5% of HL) with unique clinicopathologic features and an indolent clinical course[4]. The major pathohistological feature is the presence of malignant LP cells, variants of Reed-Sternberg cells ($CD20^+CD15^-CD30^-$) within a nodular pattern of infiltrating lymphocytes. Although initial presentation of NLPHL is peripheral lymphadenopathy in some rare cases, other sites may also be involved (spleen, liver, lung and bone marrow)[5-10].

Febrile cholestatic liver disease as a first symptom is an extremely unusual presentation of HL. In the only published series of 421 HL patients, 1.4% of patients initially had cholestasis and fever with no peripheral adenopathies[1]. Liver involvement of any kind at the time of presentation of HL is infrequent and ranges from 5-14%, but increases with advanced stages of the disease up to 60% postmortem[3].

Cholestasis in HL can be the result of parenchymal infiltration by the tumor, biliary tract obstruction second-

ary to extrahepatic lymphoma or paraneoplastic phenomenon[11]. Parenchymal involvement is the most common mechanism of cholestasis and may consist of diffuse or focal infiltrates, portal zone cellularity, an epithelioid cell reaction or as lymphoid aggregates[12]. Cholestasis secondary to biliary tract obstruction is rare with a frequency of 0.5%[13]. The most rarely seen mechanism of cholestasis is the paraneoplastic phenomenon in the form of HL-related idiopathic cholestasis (IC) and vanishing bile duct syndrome (VBDS). It remains controversial as to whether HL-related IC and VBDS represent different pathohistological entities or a spectrum of the same disease. The exact mechanism of ductal destruction and cholestasis is unclear, but it is hypothesized that cytotoxic T lymphocytes or toxic cytokines from lymphoma cells are responsible for ductopenia[14].

In the presented case, the mechanism of liver injury was parenchymal infiltration by tumor, manifested as cholestasis without hyperbilirubinemia and increased transaminase activity. Liver involvement is a rare initial presentation of NLPHL (2%-3%) but may lead to fulminant liver failure as recently described[15].

Moreover, liver involvement is more frequent in aggressive types of HL such as mixed cellularity and lymphocytic depletion[2]. The prognosis for patients with liver disease as the initial manifestation of HL is generally poor[1,15-17], but not the rule, since some case reports show favorable response to chemotherapy[18-22]. Unfortunately, this was not the case with our patient where 3 mo after the appearance of symptoms, ABVD chemotherapy resulted in a poor therapeutic response. After the first cycle of the more aggressive BEACOPP therapy, the anti-lymphoma effect was sufficient, but toxicity led to a fatal outcome.

HL should be considered in the differential diagnosis of cholestasis. Due to limitations in laboratory and morphological investigations, liver biopsy should be considered in the early phase of the diagnostic algorithm to confirm the diagnosis and enable appropriate treatment.

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CASE REPORT

Delayed internal pancreatic fistula with pancreatic pleural effusion postsplenectomy

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Abstract

The occurrence of pancreatic pleural effusion, secondary to an internal pancreatic fistula, is a rare clinical syndrome and diagnosis is often missed. The key to the diagnosis is a dramatically elevated pleural fluid amylase. This pancreatic pleural effusion is also called a pancreatic pleural fistula. It is characterized by profuse pleural fluid and has a tendency to recur. Here we report a case of delayed internal pancreatic fistula with pancreatic pleural effusion emerging after splenectomy. From the treatment of this case, we conclude that the symptoms and signs of a subphrenic effusion are often obscure; abdominal computed tomography may be required to look for occult, intra-abdominal infection; and active conservative treatment should be carried out in the early period of this complication to reduce the need for endoscopy or surgery.

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Key words: Pancreatic fistula; Pleural effusion; Splenectomy; Subphrenic effusion; Postoperative complications

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Jin SG, Chen ZY, Yan LN, Zeng Y. Delayed internal pancreatic fistula with pancreatic pleural effusion postsplenectomy. *World J Gastroenterol* 2010; 16(35): 4494-4496 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i35/4494.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i35.4494

INTRODUCTION

A pancreatic fistula is a common complication after pancreatic surgery, trauma, and inflammation, *etc*. However, the emergence of a delayed postoperative internal pancreatic fistula with pancreatic pleural effusion is still relatively rare. Here we report such a case after splenectomy.

CASE REPORT

A 52-year-old man was admitted to our department for hepatic cirrhosis with splenomegaly and hypersplenism. Physical examination showed a smooth, hard spleen palpated under the left rib margin. Laboratory examinations showed no obvious abnormality except on routine blood examination (white blood count, hemoglobin, and platelet count were 1.90×10^9/L, 65 g/L, and 24×10^9/L, respectively). Abdominal computed tomography (CT) showed hepatic cirrhosis and splenomegaly. Endoscopic examination showed mild esophageal varicose veins without signs of bleeding. Thus splenectomy was conducted and the splenic bed was sewn up with a 4-0 Prolene suture to cover the rough surface of the splenic bed tissue and to prevent subphrenic infection. In the operation, we examined the diaphragm and tail of the pancreas carefully and found no obvious injury. A drainage tube was placed at the left subphrenic fossa. The operation was successful.

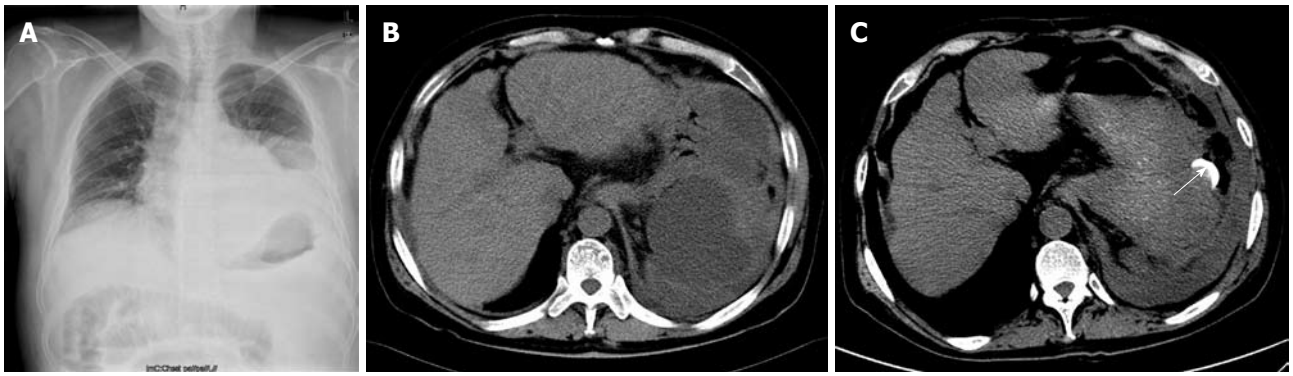

Figure 1 Imaging of the patient. A: Chest X-ray film showing a left pleural effusion; B: Computed tomography reveals encapsulated fluid about 16 cm × 9 cm in the left subphrenic fossa; C: Abdominal paracentesis (arrow) was conducted and subphrenic effusion decreased significantly.

During the first 4 d after surgery, the patient recovered smoothly. Amylase in the drainage fluid was normal on the 4th d postoperatively and the drainage tube was removed the following day. Subsequently, however, a fever of unknown origin occurred and fluctuated between 37.5℃ and 40℃ in the following days, without other abnormal symptoms and signs. Then ultrasound examination of the abdomen, including the portal venous system, a chest X-ray, and blood culture were performed to determine the cause, but no obvious positive results were found initially. We had initially considered the cause was spleen fever.

The patient's condition gradually worsened. Dyspnea and acute heart failure occurred but it was not until the 17th d postoperatively that a left pleural effusion was found in the chest X-ray film (Figure 1A), and a left subphrenic effusion encapsulating about 16 cm × 9 cm was revealed by abdominal CT (Figure 1B). Immediately, abdominal paracentesis and thoracocentesis under ultrasound guidance were conducted, and slightly turbid alutaceous liquid was drained out. Amylase values of the protein-rich fluid from the peritoneal cavity and thoracic cavity were significantly elevated at 19 202 IU/L and 17 531 IU/L, respectively. In the following 20 d, more than 2000 mL sterile fluid were drained from the peritoneal cavity and thoracic cavity (Figure 1C). The patient gradually recovered.

DISCUSSION

The occurrence of pancreatic pleural effusion, secondary to internal pancreatic fistula, is a rare clinical syndrome and diagnosis is, therefore, often missed. The fluid accumulation is attributed to disruption of the pancreatic duct or to rupture of a pseudocyst. The key to the diagnosis is a dramatically elevated pleural fluid amylase. Effusions in association with acute pancreatitis, esophageal perforation, and thoracic malignancy are important to consider in the differential diagnosis of an elevated pleural fluid amylase but are usually easy to exclude.

The pancreatic duct disruption can also develop posteriorly. Extravasated fluid travels in a cephalad direction through the retroperitoneum to reach the thoracic cavity, or by the lymphatic system and stomata[1-3] of the diaphragm flow into the pleural cavity. Stomata in the peritoneum covering the inferior surface of the diaphragm were first described by von Recklinghausen in 1863. These stomata communicate with lymphatic vessels within the diaphragm. This pancreatic pleural effusion is also called a pancreatic pleural fistula, according to Michael[4]. Pancreatic pleural effusions are typically large and have a tendency to recur. This is in contrast to sympathetic effusions without significant elevated amylase that occur in the setting of acute pancreatitis or secondary to subphrenic abscesses, and tend to be small and self-limiting.

In this case, we supposed that the pathogenesis of the internal pancreatic fistula arose as a result of posterior pancreatic duct rupture. As a minor leakage encapsulated by surrounding tissues, pancreatic leakage was not obvious initially, but it became significant when the inflammation regressed, so a delayed internal pancreatic fistula presented. Because of the short duration of the complication and rapid recovery, CT failed to show the pancreatic pleural fistula, and endoscopic retrograde cholangiopancreatography[5-7] examination was also not considered necessary. An internal pancreatic fistula with pleural effusion can usually be managed nonoperatively by percutaneous drainage and reoperation is rarely required[8,9].

From the treatment of this case, we have come to some important conclusions: (1) a delayed internal pancreatic fistula can occur postsplenectomy; (2) patients who continue to have a fever and slow clinical progress may require CT of the abdomen to look for occult, intra-abdominal infection accounting for the fever[10]; and (3) active conservative treatment should be carried out in the early period of this complication to reduce the need for endoscopy or surgery.

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Walled-off pancreatic necrosis: Wishing our pancreatitis nomenclature was correct

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Abstract

The ultimate reason why pancreatologists have strived to establish definitions for inflammatory pathologies of the pancreas is to improve patient care. Although the Atlanta Classification has been used for around for 17 years, considerable misunderstanding of the key elements of the nomenclature still persists. While a recent article by Stamatakos *et al* aimed to deal with an entity not clearly defined in the 1993 document, it is replete with factual and conceptual errors as well as contradictory statements.

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Key words: Severe pancreatitis; Acute necrotizing pancreatitis; Walled-off pancreatic necrosis

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TO THE EDITOR

I am puzzled as to why the editorial process did not identify the multitude of major factual and conceptual errors in the article "Walled-off pancreatic necrosis" by Stamatakos *et al*[1]. Without consistency in terminology among experts, our patients will not benefit from high-quality studies such as the Dutch PANTER trial which showed a reduction in morbidity of infected pancreatic necrosis managed with a step-up percutaneous approach compared with the Berger technique of open necrosectomy[2]. Walled-off pancreatic necrosis (WOPN) refers to (peri)pancreatic necrosis in a patient who has been nursed through 10-12 wk of illness and presents with pressure symptoms or rarely with infection[3]. This time-dependent maturation results in separation and demarcation of the solid component and sometimes liquefaction[3].

The authors have been permitted to perpetuate the misconception that a pancreatic pseudocyst contains necrosis when the Atlanta Classification clearly states that it does not[4].

Statements such as the following indicate that the authors do not understand the term of wall off pancreatic necrosis: "WOPN was formerly known as pancreatic abscess", "WOPN occurs … in complicated cases of pseudocysts", "little or no necrotic material is present", "superinfection of pseudocysts is one way that WOPN may develop" and "bacterial flora … is the sine qua non of WOPN". The authors stated that WOPN was defined in 2005 but have referenced articles from 2001 and 1993 when outlining the epidemiology and pathophysiology of this condition.

The authors seem confused about patient outcome from severe pancreatitis: the greatest predictor of mortality is deteriorating organ dysfunction rather than the extent of

necrosis or infection[5]. On the one hand, they stated that "mortality rate approaches 100% if surgical intervention … (is) not undertaken for WOPN". On the other hand, they stated that "if bacteremia can be restrained, necrosectomy can be avoided".

With disregard for current evidence, the authors recommended total parenteral nutrition[6] and prophylactic antibiotics[7] for patients with severe pancreatitis.

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Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
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April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

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New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

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Orlando, FL, United States
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10th OESO World Congress on Diseases of the Oesophagus 2010

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International Liver Association's Fourth Annual Conference

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La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

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Boston, MA, United States
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Maximization of personal benefits

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Seon Hahn Kim, *Seoul*
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Martin D Zielinski, *Rochester*
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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 36 September 28, 2010

EDITORIAL

4499 Clinical implications of accessory pancreatic duct
Kamisawa T, Takuma K, Tabata T, Egawa N

4504 A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease
Rahimi R, Shams-Ardekani MR, Abdollahi M

TOPIC HIGHLIGHT

4515 Diagnosis and management of pancreatic neuroendocrine tumor in von Hippel-Lindau disease
Tamura K, Nishimori I, Ito T, Yamasaki I, Igarashi H, Shuin T

4519 Recent standardization of treatment strategy for pancreatic neuroendocrine tumors
Imamura M

REVIEW

4526 Liver disease and erythropoietic protoporphyria: A concise review
Casanova-González MJ, Trapero-Marugán M, Jones EA, Moreno-Otero R

ORIGINAL ARTICLE

4532 Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome
Malinen E, Krogius-Kurikka L, Lyra A, Nikkilä J, Jääskeläinen A, Rinttilä T, Vilpponen-Salmela T, von Wright AJ, Palva A

4541 Lowered HGK expression inhibits cell invasion and adhesion in hepatocellular carcinoma cell line HepG2
Han SX, Zhu Q, Ma JL, Zhao J, Huang C, Jia X, Zhang D

4549 Infantile hepatic hemangioendothelioma: A clinicopathologic study in a Chinese population
Zhang Z, Chen HJ, Yang WJ, Bu H, Wei B, Long XY, Fu J, Zhang R, Ni YB, Zhang HY

BRIEF ARTICLE

4558 High prevalence of viable *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's disease
Mendoza JL, San-Pedro A, Culebras E, Cíes R, Taxonera C, Lana R, Urcelay E, de la Torre F, Picazo JJ, Díaz-Rubio M

4564 Risk factors associated with the development of ischemic colitis
Cubiella Fernández J, Núñez Calvo L, González Vázquez E, García García MJ, Alves Pérez MT, Martínez Silva I, Fernández Seara J

4570 Recurrent abscess after primary successful endo-sponge treatment of anastomotic leakage following rectal surgery
Riss S, Stift A, Kienbacher C, Dauser B, Haunold I, Kriwanek S, Radlsboek W, Bergmann M

4575 A predictive factor for the response to S-1 plus cisplatin in gastric cancer
Miyazaki I, Kawai T, Harada Y, Moriyasu F

4583 Clinicopathological evaluation of duodenal well-differentiated endocrine tumors
Ishido K, Tanabe S, Higuchi K, Sasaki T, Katada C, Azuma M, Naruke A, Koizumi W, Mikami T

4589 Endoscopic removal of gastric ectopic pancreas: An initial experience with endoscopic submucosal dissection
Ryu DY, Kim GH, Park DY, Lee BE, Cheong JH, Kim DU, Woo HY, Heo J, Song GA

4594 Double balloon endoscopy increases the ERCP success rate in patients with a history of Billroth Ⅱ gastrectomy
Lin CH, Tang JH, Cheng CL, Tsou YK, Cheng HT, Lee MH, Sung KF, Lee CS, Liu NJ

4599 Plasma miR-216a as a potential marker of pancreatic injury in a rat model of acute pancreatitis
Kong XY, Du YQ, Li L, Liu JQ, Wang GK, Zhu JQ, Man XH, Gong YF, Xiao LN, Zheng YZ, Deng SX, Gu JJ, Li ZS

4605 Laparoscopic low anterior resection for rectal carcinoma: Complications and management in 132 consecutive patients
Zhu QL, Feng B, Lu AG, Wang ML, Hu WG, Li JW, Mao ZH, Zheng MH

4611 High expression level of EDIL3 in HCC predicts poor prognosis of HCC patients
Sun JC, Liang XT, Pan K, Wang H, Zhao JJ, Li JJ, Ma HQ, Chen YB, Xia JC

4616 Clinicopathological features of gastric glomus tumor
Fang HQ, Yang J, Zhang FF, Cui Y, Han AJ

CASE REPORT

4621 Complete resection of isolated pancreatic metastatic melanoma: A case report and review of the literature
He MX, Song B, Jiang H, Hu XG, Zhang YJ, Zheng JM

LETTERS TO THE EDITOR

4625 Abnormal colonic cholinergic and nitrergic activities in relation to elastosis in uncomplicated diverticular disease
Golder M

World Journal of Gastroenterology
Volume 16 Number 36 September 28, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
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EDITORIAL

Clinical implications of accessory pancreatic duct

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Abstract

The accessory pancreatic duct (APD) is the main drainage duct of the dorsal pancreatic bud in the embryo, entering the duodenum at the minor duodenal papilla (MIP). With the growth, the duct of the dorsal bud undergoes varying degrees of atrophy at the duodenal end. Patency of the APD in 291 control cases was 43% as determined by dye-injection endoscopic retrograde pancreatography. Patency of the APD in 46 patients with acute pancreatitis was only 17%, which was significantly lower than in control cases ($P < 0.01$). The terminal shape of the APD was correlated with APD patency. Based on the data about correlation between the terminal shape of the APD and its patency, the estimated APD patency in 167 patients with acute pancreatitis was 21%, which was significantly lower than in control cases ($P < 0.01$). A patent APD may function as a second drainage system for the main pancreatic duct to reduce the pressure in the main pancreatic duct and prevent acute pancreatitis. Pancreatographic findings of 91 patients with pancreaticobiliary maljunction (PBM) were divided into a normal duct group (80 patients) and a dorsal pancreatic duct (DPD) dominant group (11 patients). While 48 patients (60%) with biliary carcinoma (gallbladder carcinoma, $n = 42$; bile duct carcinoma, $n = 6$) were identified in PBM with a normal pancreatic duct system, only two cases of gallbladder carcinoma (18%) occurred in DPD-dominant patients ($P < 0.05$). Concentration of amylase in the bile of DPD dominance was significantly lower than that of normal pancreatic duct system (75403.5 ± 82015.4 IU/L *vs* 278157.0 ± 207395.0 IU/L, $P < 0.05$). In PBM with DPD dominance, most pancreatic juice in the upper DPD is drained into the duodenum *via* the MIP, and reflux of pancreatic juice to the biliary tract might be reduced, resulting in less frequency of associated biliary carcinoma.

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Key words: Accessory pancreatic duct; Minor duodenal papilla; Pancreas divisum; Main pancreatic duct; Acute pancreatitis; Pancreaticobiliary maljunction

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INTRODUCTION

The human pancreas develops embryologically from fusion of the dorsal and ventral pancreatic buds. The dorsal pancreatic bud gives rise to the anterior part of the head of the pancreas, in addition to the body and tail, while the ventral pancreatic bud develops into the posterior part of the head of the gland. Fusion of the pancreatic buds is accompanied by anastomosis of the

ducts. The main drainage duct of the ventral pancreatic bud communicates with the main duct of the dorsal pancreatic bud, with the point of union lying between the isthmus and head of the pancreas. This becomes the dominant and more constant pancreatic duct [main pancreatic duct (MPD)]. The proximal part of the main dorsal pancreatic duct (DPD) partially regresses to form the accessory pancreatic duct (APD, Santorini's duct), which opens into the minor duodenal papilla (MIP)[1-5].

Pancreas divisum is a common anatomical variation, in which the dorsal and ventral pancreatic ducts do not unite embryologically. In pancreas divisum, the DPD becomes the main duct and drains most of the pancreas through the MIP. As the MIP is substantially smaller than the major duodenal papilla, a larger secretory capacity might presumably place a significant load on the MIP[6,7]. Although there are controversies regarding the clinical significance of pancreas divisum, correlation between pancreas divisum and pancreatitis has been reported based on the findings such as increased incidence of pancreas divisum in acute idiopathic pancreatitis in endoscopic retrograde cholangiopancreatography (ERCP)[8,9], isolated dorsal pancreatitis as shown by irregular dilatation apparent on dorsal pancreatography alone[10], and improvement after endoscopic or surgical procedures that open the MIP[11-13]. As less than 5% of the population with pancreas divisum develop pancreatic symptoms[14], interrelationships between poor function of the MIP and increased flow of pancreatic juice caused by alcohol or diet might increase dorsal duct pressure and lead to development of complications[6]. On the other hand, as cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations are more frequently found in patients with pancreas divisum associated with idiopathic pancreatitis than in those with pancreas divisum without pancreatitis, it is suggested that predisposing factors such as CFTR gene mutation, are necessary to develop pancreatitis in patients with pancreas divisum[15-17].

Patency of the APD has been assessed by direct injection of material into the pancreatic duct in resected or autopsy specimens[18-20]. A great deal of difficulty is encountered in determining the percentage of cases in which the MIP is patent. Accordingly, the reported patency of the APD has ranged widely from 12%[18] to 82%[19]. Clinical significance of the APD in the typical pancreatic duct system with fusion between the ventral and DPD remains unclear. We have performed endoscopic studies on patency of the APD using dye-injection endoscopic retrograde pancreatography (ERP)[6,21-23]. In this editorial, we elucidate clinical implications of the APD.

PATENCY OF APD BY DYE-INJECTION ERP

From 1989 to 2002, during routine ERP, 2-3 mL of contrast medium containing a small amount of indigocarmine was injected into the MPD *via* a selectively cannulated endoscopic catheter at the usual pressure. Egress of dye from the MIP observed endoscopically indicates the APD patency. This method can be used endoscopically to determine the patency of the APD (Figure 1)[6,21-23].

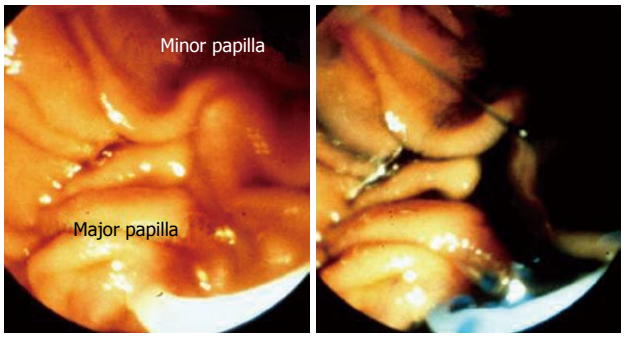

Figure 1 Endoscopic appearance of the major and minor duodenal papilla. In a patient with a patent accessory pancreatic duct, the dye injected into the main pancreatic duct was excreted from the minor duodenal papilla.

Table 1 Relationship between the patency of the accessory pancreatic duct and its terminal shape *n* (%)

Terminal shape of the APD	No. of cases	Patency
Stick	149	71 (48)
Spindle	29	27 (93)[a]
Cudgel	24	21 (88)[a]
Saccular	21	3 (14)[b]
Branch	42	3 (7)[b]

[a]Significantly higher compared with patency of stick-type accessory pancreatic duct (APD); [b]Significantly lower compared with patency of stick-type APD.

Of the 291 controls with normal pancreatogram in the head of the pancreas who underwent ERCP for suspicion of pancreatobiliary diseases other than acute pancreatitis, 43% had a patent APD. The terminal shape of the APD exhibited several consistent radiological features, which were classified into six types. Stick-type APD showed gradual narrowing of the duct (Figure 2A). In branch-type APD, the duct gradually narrowed and gave off several fine terminal branches (Figure 2B). In spindle-type APD, ampullary termination was seen (Figure 2C), while saccular-type APD displayed saccular termination (Figure 2D). Cudgel-type APD is associated with a duct diameter exceeding 2 mm (Figure 2E). In some cases, APD is halfway or none. The most common is stick type (n = 149, 51%), followed by branch type (n = 42, 14%), spindle type (n = 29, 10%), halfway type or none (n = 26, 9%), cudgel type (n = 24, 8%), and saccular type (n = 21, 7%). The terminal shape of the APD was correlated with APD patency. Stick-type APD was patent in 48% of cases. Patency of the spindle-type APD (93%) and cudgel-type APD (88%) was significantly higher than that of the stick-type APD ($P < 0.01$). Patency of the branch-type APD (7%) and saccular-type APD (14%) was significantly lower than that of the stick-type APD ($P < 0.01$) (Table 1).

In 46 patients with acute pancreatitis, 8 (17%) had a patent APD. Patency of the APD of patients with acute pancreatitis was significantly lower than APD patency

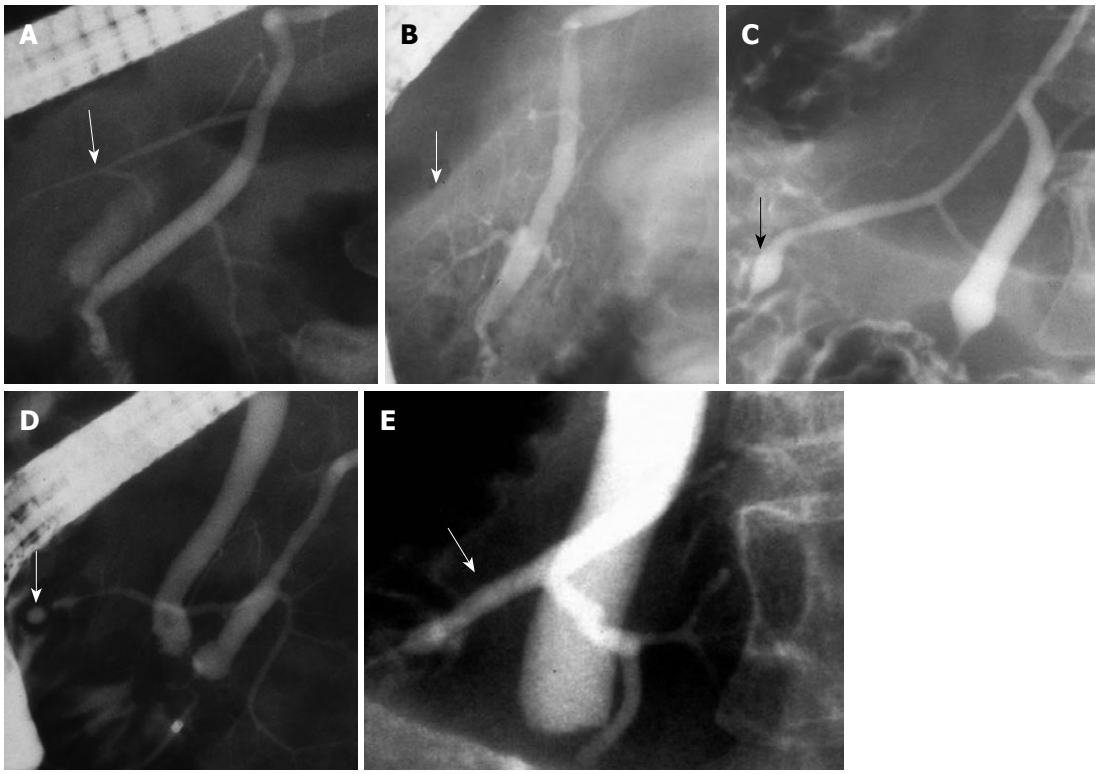

Figure 2 Terminal shape (arrows) of the accessory pancreatic duct. A: Stick type; B: Branch type; C: Spindle type; D: Saccular type; E: Cudgel type.

(43%) of controls ($P < 0.01$). Of the 43 patients with choledocholithiasis, 13 had acute pancreatitis. Patency of the APD in patients with acute biliary pancreatitis was 8%, which was significantly less frequent than in those without acute pancreatitis (43%) ($P < 0.01$).

ANALYSIS OF APD OF PATIENTS WITH ACUTE PANCREATITIS

Pancreatograms of 167 patients with acute pancreatitis, in whom the first side branches in the head were filled, were reviewed. Major etiologies of acute pancreatitis were biliary ($n = 61$), idiopathic ($n = 40$), post-ERCP ($n = 30$), and alcoholic ($n = 24$). The severity of the acute pancreatitis was severe ($n = 13$), moderate ($n = 26$), and mild ($n = 128$).

In respect to the terminal shape of the APD in these 167 patients with acute pancreatitis, stick-type APD ($P < 0.01$), spindle-type APD ($P < 0.01$), and cudgel-type APD ($P < 0.05$) were less frequent, and branch-type APD ($P < 0.01$) and halfway-type or no APD ($P < 0.01$) were more frequent compared with the controls. Based on the underlying data about correlation between the terminal shape of the APD and its patency, the estimated APD patency in 167 patients with acute pancreatitis was 21%, which was significantly lower than that of controls (43%) ($P < 0.01$). APD patency was estimated to be 21% in patients with severe acute pancreatitis, 28% in those with moderate acute pancreatitis, and 20% in those with mild acute pancreatitis.

BILIARY CARCINOMA ASSOCIATED WITH PANCREATICOBILIARY MALJUNCTION SHOWING DORSAL DUCT DOMINANCE

Pancreaticobiliary maljunction (PBM) is a congenital anomaly defined as a union of the pancreatic and biliary ducts that is located outside the duodenal wall. As the action of the sphincter muscle does not functionally affect the union, pancreatic juice frequently refluxes into the bile duct *via* the anomalous junction, resulting in a high incidence of carcinogenesis in the biliary tract[24-26]. Pancreatographic findings of 91 PBM patients were divided into a normal duct group (80 patients) and a DPD dominant group (11 patients). DPD dominance is defined as cases in which ventral pancreatic duct anastomosis with DPD is narrower than DPD. While 48 patients (60%) with biliary carcinoma (gallbladder carcinoma, $n = 42$; bile duct carcinoma, $n = 6$) were identified in PBM with a normal pancreatic duct system, only two cases of gallbladder carcinoma (18%) occurred in DPD-dominant patients ($P < 0.05$) (Table 2).

Although there was no difference in the diameter of ventral pancreatic duct, the maximum diameter of the Santorini's duct in DPD dominance was significantly larger than that of normal pancreatic duct system (2.5 ± 0.6 mm *vs* 0.9 ± 0.3 mm, $P < 0.01$). The Santorini's duct flew straight from the upstream DPD in DPD dominance. Concentration of amylase in the bile of DPD dominance

Table 2 Relationship between pancreatographic finding and biliary carcinoma in patients with pancreaticobiliary maljunction *n* (%)

Pancreatography	Biliary carcinoma +
Normal (n = 80)	48 (60)
Dorsal pancreatic duct dominant (n = 11)	2 (18)

$P < 0.05$.

was significantly lower than that of normal pancreatic duct system (75 403.5 ± 82 015.4 IU/L *vs* 278 157.0 ± 207 395.0 IU/L, $P < 0.05$).

CLINICAL SIGNIFICANCE OF THE APD

Dye-injection ERP is a simple and definitive method for examining APD patency[6,21-23]. In the study using dye-injection ERP, the patency of the APD in 46 patients with acute pancreatitis was 17%, which was significantly lower than in controls (43%). Since acute pancreatitis is a reversible disease and ERP was performed after resolution of pancreatitis, it seems less likely that pancreatic inflammation impedes flow of contrast medium through the pancreatic duct. Estimated patency of the APD based on the data by dye-injection ERP was 21% in 167 patients with acute pancreatitis, which was also significantly lower than in controls.

Though the mechanisms that induce acute pancreatitis are different and are not fully clarified, many authors appear to agree that obstruction of the flow of pancreatic juice is of fundamental importance in the occurrence of biliary acute pancreatitis. During impaction of a stone in the major duodenal papilla, pressures of both bile and pancreatic juice flow increase, but if an efficient mechanism for decompressing the pancreatic duct system is available in individuals with a patent APD, it may prevent acute pancreatitis by reducing pressure in the MPD. Nowak *et al*[27] reported, in a prospective ERCP study, that APD patency was only found in 17% of 47 patients with acute biliary pancreatitis compared with 69% in a control group. A patent APD may function as a second drainage system for the MPD to reduce the pressure in the MPD and prevent acute pancreatitis. However, as there was no relationship between the severity of acute pancreatitis and patency of the APD, APD patency appears to relate to the trigger of acute pancreatitis, but not to the progression of pancreatitis. During ERCP, in cases with non-patent APD, endoscopists should be more cautious to post-ERCP pancreatitis than usual, and alternatively consider prophylactic pancreatic stenting.

Although DPD dominance was observed in about 4% of pancreases[19,28], it was detected in 12% of PBM patients. In PBM patients, the incidence of associated biliary carcinoma in DPD-dominant patients with PBM was significantly less frequent than that of a normal pancreatic duct system. Furthermore, a lower amylase level in the bile was more frequently seen in DPD-dominant patients. From these facts, it is suggested that, in PBM patients with DPD dominance, most pancreatic juice in the upper DPD is drained into the duodenum *via* the MIP, and reflux of pancreatic juice to the biliary tract might be reduced, resulting in less frequency of associated biliary carcinoma.

CONCLUSION

Patency of the APD in patients with acute pancreatitis was significantly lower than in controls. A patent APD may function as a second drainage system to reduce the pressure in the MPD and prevent acute pancreatitis.

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EDITORIAL

A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease

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Abstract

The etiology of inflammatory bowel disease (IBD) is not yet known, but many factors such as defects in the immune system, oxidative stress, microbial content in the gastrointestinal tract, nuclear factor (NF)-κB, nitric oxide (NO), cyclooxygenase-2 (Cox-2), and leukotriene B4 (LB4) are thought to play a role in its pathogenesis. In traditional Iranian medicine (TIM), several medicinal plants are thought to be effective for the treatment of IBD. In this study, information on all of these remedies were derived from all available old sources such as documents or notes and books and were added to the information derived from modern medical databases covering all *in vitro*, *in vivo* and clinical trials. For some of these plants, only one or two mechanisms of action have been found such as in *Cassia fistula*, *Lepidium sativum*, and *Bunium persicum*. However, for some plants various mechanisms of action are known. For example, *Commiphora mukul* is effective in IBD due to its immunomodulatory, antioxidant, and antibacterial properties and it decreases NF-κB, NO and Cox-2. Another herb, *Plantago ovata*, has immunomodulatory, antioxidant, anti-inflammatory and wound healing activities and decreases NO and LB4. Considering the mechanisms of action of these plants, the combination of some of them may be useful because of their many mechanisms of action such as *Pistacia lentiscus*, *Bunium persicum*, *Solanum nigrum*, *Plantago ovata*, *Boswellia*, *Solanum nigrum*, *Plantago ovata* and *Commiphora mukul*. For some of the herbal products used in TIM such as oleogum resin from *Commiphora myrrha*, seeds of *Ocimum basilicum*, seeds of *Linum usitatissimum*, gum resin of *Dracaena cinnabari*, seeds of *Plantago major*, seeds of *Lallementia royleana*, and seeds of *Allium porrum*, there is no or not enough studies to confirm their benefits in IBD. It is suggested that an evaluation of the effects of these plants on different aspects of IBD should be performed.

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Key words: Herbal medicine; Inflammatory bowel disease; Medicinal plants; Traditional Iranian medicine

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to two chronic diseases that cause inflammation of the intestines: ulcer-

ative colitis (UC) and Crohn's disease (CD). The etiology of IBD is unclear. The most accepted hypothesis currently implicates a combination of one or more of the following factors: immune dysregulation (caused by genetic or environmental factors), abnormal gastrointestinal (GI) tract luminal factors (such as microorganisms constituting the GI tract flora), oxidative stress, and defects in the GI mucosal barrier that allow luminal factors to penetrate into the mucosa[1,2].

In our previous paper, we reviewed all medicinal plants used worldwide for the treatment of IBD[3] by including all *in vitro*, *in vivo*, and clinical studies that examined medicinal plants for the treatment of IBD. Added to that information, there is information and data that are only found in documents, notes, or books by traditional Iranian medicine (TIM) scientists. In TIM, there is a GI disease known as "Zahir" which seems to be identical to IBD regarding the explained symptoms of the disease. Zahir is defined as tenesmus of the rectum during defecation followed by secretion of mucosa and bloody diarrhea[4]. Various natural remedies have been used in TIM for IBD. These remedies have been used for many years by Iranian physicians such as Rhazes and Avicenna for the treatment of IBD in humans. Different mechanisms of action have been described in traditional Iranian publications accounting for the usefulness of these plants in IBD, which include anti-inflammatory, antiulcer, wound healing, and antidiarrheal activities[5,6]. In the present work, these remedies are revised individually and possible evidence of their efficacy in modern medicine is reviewed. For this purpose, electronic databases including Pubmed, Scopus, Embase, and Google Scholar were searched for each of the plants in TIM and all retrieved articles were examined to obtain studies giving any *in vitro*, *in vivo*, or clinical evidence of the efficacy of these herbs in the treatment of IBD. The retrieved studies directly evaluated these herbs on IBD animal models or humans, or indirectly surveyed their efficacy on the mechanisms involved in the pathogenesis of IBD.

FACTORS INVOLVED IN THE PATHOGENESIS OF IBD

Immune system

There is evidence of defective responses in both the innate and the adaptive immune systems in IBD[7]. The behavior of the cells mediating innate immunity such as neutrophils, macrophages, dendritic cells, and natural killer cells are altered, and defective mucosal T helper (Th) cell responses and greater expression of cytokines such as interleukin (IL)-1-β, IL-6, IL-12, tumor necrosis factor α (TNF-α) and interferon (IFN)-γ were demonstrated in patients with IBD[8,9]. Recent meta-analyses confirmed the efficacy of anti-TNF-α drugs for induction of remission in UC[10] but did not confirm them for induction of response and remission in CD[11].

Oxidative stress

Oxidative stress is a potential etiological and/or triggering factor for IBD, because the damaging effects of reactive oxygen molecules have been well established in the inflammation process[12-14]. Although some conflicting results exist, it seems that patients with IBD demonstrate excessive oxidized molecules compared with healthy control subjects in a variety of organic systems (e.g. GI tract, blood, and respiratory system)[12]. Recent studies have shown decreased total antioxidant capacity and increased reactive oxygen molecules in patients with IBD[13-15].

Microbes

Some studies have suggested a role for the microbial content of the GI tract in the pathogenesis of IBD[16]. The disease occurs in areas of the GI tract with the highest concentrations of luminal bacteria. Normal, nonpathogenic enteric bacteria can induce chronic intestinal inflammation in genetically susceptible hosts with defective immunoregulation, bacterial clearance, or mucosal barrier function. It has been shown that the concentration of intestinal bacteria in IBD is higher than normal and increases progressively with severity of the disease[17-19]. Probiotics have been found to be useful in the management of irritable bowel syndrome[20] and pouchitis[21]. Antibacterials and probiotics have been demonstrated to be effective in UC *via* modification of the gut bacterial flora[22,23]. However, current meta-analyses have only confirmed the efficacy of antibiotics for CD[24] and failed to demonstrate the efficacy of probiotics in maintaining remission and preventing clinical and endoscopic recurrence in CD[25].

Nuclear factor-κB

These proteins are a family of structurally related eukaryotic transcription factors that promote the expression of over 150 genes, many of which play important roles in the regulation of inflammation and apoptosis[26]. Excess or inappropriate activation of nuclear factor (NF)-κB has been observed in human IBD[27,28]. Thus, inhibitors of NF-κB can be used as a treatment strategy for the management of IBD.

Nitric oxide

Nitric oxide (NO) is a short-life molecule produced by the enzyme known as NO synthase (NOS), in a reaction that converts arginine and oxygen into citrulline and NO. There are three isoforms of the enzyme: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Interestingly, NO has both beneficial and detrimental roles in the body. It seems that constitutive forms of NO synthase (cNOS) including nNOS and eNOS are critical to normal physiology, while inhibition of these enzymes may cause cellular damage. On the other hand, induction of iNOS causes injury; therefore, specific inhibition of this enzyme can be beneficial. Three key observations confirm the detrimental role of iNOS in inflammation. Firstly, since large quantities of NO are produced by iNOS relative to the two cNOS isoforms, excess NO may contribute to inflammation through nitrosation,

Table 1 ***In vitro*** **studies on plants used in traditional Iranian medicine for the treatment of inflammatory bowel disease**

Study	Plant	Part	Results
Watt *et al*[39]	*Althaea officinalis*	Whole plant ethanol extract	Antibacterial activity against *E. coli*
Yoshikawa *et al*[40]	*Boswellia carterii*	Mono- and triterpenes isolated from this oleogum resin	↓NO production in lipopolysaccharide-activated mouse peritoneal macrophages
Chevrier *et al*[41]	*Boswellia carterii*	Ethanol extract of oleogum resin	Immunomodulatory properties
Camarda *et al*[42]	*Boswellia carterii*	Essential oil isolated from oleogum resin	Antimicrobial activities against various microorganisms including fungi, Gram-positive and Gram-negative bacterial strains
Moghtader *et al*[43]	*Bunium persicum*	Essential oil of seed	Strong anti-bacterial effects
Shahsavari *et al*[44]	*Bunium persicum*	Essential oil of seed	Antioxidant properties
Kumar *et al*[45]	*Cassia fistula*	Crude extract of fruit	Significant antimicrobial activity
Francis *et al*[46]	*Commiphora mukul*	Terpenoids and guggulusteroids	↓Lipid peroxidation and Cox inhibitory activities
Manjula *et al*[47]	*Commiphora mukul*	Crude extract of gum resin	↓Proliferative response of peripheral blood mononuclear cells,
Matsuda *et al*[48]	*Commiphora mukul*	Methanolic extract of gum resin	↓inflammatory mediators such as IFN-δ, IL-12, TNF-α, IL-1β and NO, ↓NO production in lipopolysaccharide-activated mouse peritoneal macrophages
Saeed *et al*[49]	*Commiphora mukul*	The essential oil, chloroform extract and seven sesquiterpenoids compounds of oleogum resin	Wide range of inhibitory activity against both Gram positive and Gram negative bacteria
Fattouch *et al*[50]	*Cydonia oblonga*	Pulp and peel polyphenolic extract	Radical scavenging and antimicrobial activities
Silva *et al*[51]	*Cydonia oblonga*	Pulp and peel methanolic extract	Antioxidant activity
Hamauzu *et al*[52]	*Cydonia oblonga*	Pulp and peel phenolic extract	Superior antioxidant functions to that of chlorogenic acid and ascorbic acid as standard antioxidants
Kaur *et al*[53]	*Foeniculum vulgare*	Aqueous and organic seed extracts	Antibacterial activity comparable to standard antibiotics
De Marino *et al*[54]	*Foeniculum vulgare*	n-butanol and aqueous extract of fruit	Moderate antioxidant activity
Baliga *et al*[55]	*Foeniculum vulgare*	Aqueous extract	↓NO
Fukuda *et al*[56]	*Juglans regia*	Polyphenols	↓Lipid peroxidation, ↑antioxidant activity
Zhou *et al*[57]	*Pistacia lentiscus*	Oleogum resin	↓Pro-inflammatory substances such as NO and prostaglandin E2, ↓expression of iNOS and Cox-2 at both protein and mRNA levels, potent hydroxyl radical scavenging activity
Westerhof *et al*[58]	*Plantago ovata*	Mucopolysaccharides of seed	Wound cleansing and healing properties, limits scarring
Al-Fatimi *et al*[59]	*Solanum nigrum*	Methanolic extract of fruit	Free radical scavenging activities in the DPPH assay
Heo *et al*[60]	*Solanum nigrum*	A glycoprotein (SNL glycoprotein) isolated from fruit	Scavenging effects on both superoxide anion and hydroxyl radical
Aquil *et al*[61]	*Terminalia chebula*	Ethanolic extract of fruit	Broad-spectrum antibacterial activity, synergistic interaction with tetracycline, chloramphenicol and ciprofloxacin against *S. aureus* and/or *E. coli*
Kim *et al*[62]	*Terminalia chebula*	Butanol fraction of fruit	Profound growth-inhibitory activity against six intestinal bacteria especially Clostridium perfringens and *E. coli*
Moeslinger *et al*[63]	*Terminalia chebula*	Aqueous extract of fruit	↓Inducible nitric oxide synthesis

E. coli: *Escherichia coli*; NO: Nitric oxide; Cox: Cyclooxygenase; IFN: Interferon; IL: Interleukin; TNF-α: Tumor necrosis factor α; iNOS: Inducible nitric oxide synthase; mRNA: Messenger ribonucleic acid; DPPH: 1,1-diphenyl-2-picrylhydrazyl; SNL: *Solanum nigrum* L.; *S. aureus*: *Staphylococcus aureus*.

oxidative damage, and enhanced inflammatory cytokines. Secondly, expression patterns of iNOS correlate with prolonged inflammation. Thirdly, inhibition of iNOS results in reduced inflammation[29,30]. There is evidence that IBD is associated with an overproduction of NO by iNOS[31]. Increased luminal and salivary NO has also been detected in IBD patients[32,33]. It was shown that inhibition of iNOS blunted dextran sulfate sodium (DSS) colitis in mice[34].

Cyclooxygenase-2

Cyclooxygenase-2 (Cox-2) is another involved factor in IBD acting through synthesis of prostaglandins. Thus, selective Cox-2 inhibitors, such as celecoxib, are another class of drugs that have been claimed to be effective in IBD[35,36].

Leukotriene B4

Leukotriene B4 is a pro-inflammatory mediator with a role in several inflammatory diseases such as IBD. Inhibition of this mediator can reduce inflammation and ameliorate IBD[37].

MODERN EVIDENCE FOR THE EFFICACY OF MEDICINAL PLANTS IN TIM USED FOR THE TREATMENT OF IBD

Pistacia lentiscus

Oleogum resin from *Pistacia lentiscus* (*P. lentiscus*) known as "Mastaki" is an efficacious remedy for the treatment of IBD in TIM[38]. Supplementation with oleogum resin from *P. lentiscus* delayed the onset and progression of the disease and helped prevent weight loss in the DSS model of colitis (Tables 1[39-63] and 2[64-81]). In addition, oleogum resin inhibited the production of pro-inflammatory substances such as NO and prostaglandin E2. Western blotting and reverse transcription polymerase chain reaction (RT-PCR) analyses have shown that oleogum resin from *P. lentiscus*

Table 2 *In vivo* studies on plants used in traditional Iranian medicine for the treatment of inflammatory bowel disease

Study	Model	Species	Plant	Part	Results
Wang *et al*[64]	Carrageenan- or dextran- induced paw edema	Rat	*Althaea rosea*	Ethanolic extract of flower	Anti-inflammatory and analgesic effect
Fan *et al*[65]	Complete Freund's adjuvant-induced inflammation	Rat	*Boswellia carterii*	Gum resin extract	Lengthened paw withdrawal latency, ↓paw edema, ↓spinal Fos protein expression, no noticeable adverse effects observed
Banno *et al*[66]	TPA-induced inflammation	Mouse	*Boswellia carteri*	Compounds isolated from methanol extract of the resin	Marked anti-inflammatory activity
Kiela *et al*[67]	DSS- and TNBS-induced colitis	Mouse	*Boswellia serrata*	Gum resin extract	Ineffective in ameliorating colitis, ↑the basal and IL-1β-stimulated NF-κB activity in intestinal epithelial cells in vitro as well as reverse proliferative effects of IL-1β, hepatotoxicity effect with pronounced hepatomegaly and steatosis was observed
Mencarelli *et al*[68]	TNBS-induced colitis	Mouse	*Commiphora mukul*	Guggulsterone	↓Severity of disease and the fecal score and colon inflammation, ↓IL-2, IL-4 and IFN-γ as well as T cell proliferation
Cheon *et al*[69]	DSS-induced colitis	Mouse	*Commiphora mukul*	Guggulsterone	↓NF-κB signaling pathway, attenuates acute colitis
Birdane *et al*[70]	Ethanol-induced gastric lesions	Rat	*Foeniculum vulgare*	Aqueous extract	↓Gastric mucosal lesion, ↓lipid peroxidation, ↑antioxidant activity
Choi *et al*[71]	Carrageenan-induced paw edema, arachidonic acid-induced ear edema, formaldehyde-induced arthritis, DNFB-induced contact hypersensitivity reaction	Mouse	*Foeniculum vulgare*	Fruit methanolic extract	Anti-inflammatory and central analgesic effect, ↓lipid peroxidation, ↑antioxidant activity
Al-Yahya *et al*[72]	Carrageenan-induced paw edema	Rat	*Lepidium sativum*	Ethanolic extract of seed	Anti-inflammatory and analgesic activities, potentiate gastric ulcer induced by indomethacin
Kim *et al*[73]	DSS-induced colitis	Mouse	*Pistacia lentiscus*	Oleogum resin	Delayed the onset and progression of the disease, prevent weight loss
Al-Said *et al*[74]	Gastric mucosal damage induced by pyloric ligation, aspirin, phenylbutazone, and reserpine	Rat	*Pistacia lentiscus*	Oleogum resin	↓Intensity of gastric mucosal damage
Rodríguez-Cabezas *et al*[75]	TNBS-induced colitis	Rat	*Plantago ovata*	Seed	Ameliorated the development of colonic inflammation, ↓some of the pro-inflammatory mediators such as NO, leukotriene B4, and TNF-α; ↑production of short chain fatty acids, butyrate and propionate
Rodríguez-Cabezas *et al*[76]	TNBS-induced colitis	Rat	*Plantago ovata*	Seed	↓Colonic myeloperoxidase activity, restoration of colonic glutathione levels
Joo *et al*[77]	DSS-induced colitis	Mouse	*Solanum nigrum*	A glycoprotein (SNL glycoprotein) isolated from fruit	↓NO production, ↓free radical formation, suppressive effect on activities of NF-κB, regulates the expression of iNOS and Cox-2
Jainu *et al*[78]	Acetic acid-induced gastric ulcers	Rat	*Solanum nigrum*	Fruit extract	↓Gastric lesions induced by cold restraint stress (76.6%), indomethacin (73.8%), pyloric ligation (80.1%) and ethanol (70.6%) with equal or higher potency than omeprazole, ↓gastric secretory volume and acidity and pepsin secretion, ↑rate of healing of ulcers, ↓H(+)K(+)ATPase activity, ↓gastrin secretion
Akhtar *et al*[79]	Aspirin-induced gastric ulcers	Rat	*Solanum nigrum*	Powder from aerial parts and its methanolic extract	↓Ulcer index, ↓acid and pepsin secretions
Mahesh *et al*[80]	-	Rat	*Terminalia chebula*	Aqueous extract of fruit	Modulate oxidative stress and enhance antioxidant status in the liver and kidney
Bhattacharya *et al*[81]	-	Rat	*Terminalia chebula*	Ethanolic extract of fruit	↑Rate of healing of gastric lesion induced by indomethacin, ↓lipid peroxidation

TNBS: 2,4,6-Trinitrobenzene sulfonic acid; DNBS: 2,4-dinitrobenzene sulfonic acid; DSS: Dextran-sulfate sodium, TPA: 12-O-tetradecanoylphorbol-13-acetate; IL: Interleukin; NF-κB: Nuclear factor κB; IFN: Interferon; DNFB: 2,4-dinitrofluoro benzene; NO: Nitric oxide; TNF-α: Tumor necrosis factor α; iNOS: Inducible nitric oxide synthase; SNL: *Solanum nigrum* L.; Cox: Cyclooxygenase.

inhibited the expression of iNOS and Cox-2 at both the protein and mRNA levels. It has shown potent hydroxyl radical scavenging activity; however, it has scavenged NO and superoxide radicals very poorly (Table 1)[57]. Oleogum resin from *P. lentiscus* at an oral dose of 500 mg/kg produced a significant reduction in the intensity of gastric mucosal damage induced by pyloric ligation, aspirin, phenylbutazone, and reserpine in rats (Table 2)[74]. Treating CD

patients with oleogum resin from *P. lentiscus* resulted in the reduction of TNF-α secretion ($P = 0.028$). Macrophage migration inhibitory factor (MIF) release was significantly increased ($P = 0.026$) meaning that random migration and chemotaxis of monocytes/macrophages was inhibited. No significant changes were observed in IL-6, monocyte chemotactic protein-1 (MCP-1), and intracellular antioxidant glutathione (GSH) concentrations showing that oleogum resin from *P. lentiscus* acts as an immunomodulator on peripheral blood mononuclear cells (PBMCs) by a TNF-α inhibitory and a MIF stimulatory activity[82]. Another study performed on CD patients demonstrated a significant reduction in the CD activity index (CDAI) ($P = 0.05$) due to oleogum resin from *P. lentiscus* as compared to pretreatment values. Plasma IL-6 and C-reactive protein (CRP) were significantly decreased. Total antioxidant potential (TAP) was significantly increased ($P = 0.036$). No patient or control exhibited any side effects[83]. A double-blind clinical trial was carried out on patients with symptomatic and endoscopically proven duodenal ulcers, to compare therapeutic responses to oleogum resin from *P. lentiscus* and placebo over a period of 2 wk. The results from this study demonstrated symptomatic relief in 80% of patients treated with oleogum resin from *P. lentiscus* and in 50% patients treated with placebo. Endoscopically proved healing occurred in 70% of patients treated with oleogum resin from *P. lentiscus* and in 22% patients treated with placebo. The differences between the treatments were highly significant ($P < 0.01$). Oleogum resin from *P. lentiscus* was well tolerated and did not produce side effects. This study showed that oleogum resin from *P. lentiscus* has an ulcer healing effect (Table 3[82-88]).

Commiphora mukul

Gum resin from *Commiphora mukul* (*C. mukul*) known as "Moghl" is another natural product used in TIM for IBD[4,89]. Guggulsterone (GS), a steroid isolated from the gum resin of *C. mukul*, has been investigated in two models of intestinal inflammation induced in mice by trinitrobenzene sulfonic acid (TNBS) and oxazolone. The results showed that GS protects mice against the development of signs and symptoms of colon inflammation. GS effectively attenuated the severity of disease, the fecal score and colon inflammation as assessed by measuring the macroscopic and microscopic damage scores. *In vitro*, mechanistic studies carried out using CD4+ cells isolated from the intestinal lamina propria demonstrated that GS effectively regulates the function of effector T cells. The net biological effects resulting from exposure to GS includes attenuation of the generation of IL-2, IL-4 and IFN-γ as well as T cell proliferation (Table 1)[68]. GS blocked the NF-κB signaling pathway and attenuated DSS-induced acute murine colitis (Table 2)[69]. Several compounds in the gum resin from *C. mukul* have shown lipid peroxidation and Cox inhibitory activities[46]. The anti-inflammatory effect of *C. mukul* gum has been studied in PBMCs and showed an inhibitory effect on the proliferative response of PBMC. Further studies on inflammatory mediators such as IFN-γ, IL-12, TNF-α, IL-1β and NO showed down-regulation, whereas no inhibition was observed in the case of the anti-inflammatory cytokine IL-10[47]. The methanolic extract of the gum resin from *C. mukul* was found to inhibit NO production in lipopolysaccharide-activated mouse peritoneal macrophages[48]. The essential oil, chloroform extract, and seven sesquiterpenoid compounds isolated from the oleogum resin of *C. mukul* demonstrated a wide range of inhibitory activity against both gram positive and gram negative bacteria (Table 1)[49].

Foeniculum vulgare

The fruit of *Foeniculum vulgare* (*F. vulgare*) known as "Razianeh" in TIM has been used for the treatment of IBD[87]. The aqueous and organic seed extracts have shown significant antibacterial activity comparable to standard antibiotics[53]. n-Butanol and aqueous fruit extracts of *F. vulgare* showed moderate radical scavenging properties *in vitro* (Table 1)[54]. Pretreatment with aqueous extracts of *F. vulgare* significantly reduced ethanol-induced gastric lesions in rats. In addition, this extract significantly reduced lipid peroxidation and increased antioxidant activity[70]. Oral administration of *F. vulgare* fruit methanolic extract to mice exhibited inhibitory effects against acute and subacute inflammatory diseases and type Ⅳ allergic reactions and showed a central analgesic effect. Moreover, it significantly increased the plasma antioxidant activity and decreased lipid peroxidation (Table 2)[71]. The aqueous extract of *F. vulgare* showed a significant NO scavenging effect *in vitro* (Table 1)[55].

Terminalia chebula

The black fruit of *Terminalia chebula* (*T. chebula*) known as "Halile siah" in TIM has been used for the treatment of IBD[4]. The aqueous extract of *T. chebula* has been shown to effectively modulate oxidative stress and enhance antioxidant status in the liver and kidney of aged rats[80]. The ethanolic extract of *T. chebula* accelerated the rate of healing of gastric lesions induced by indomethacin and inhibited lipid peroxidation in the gastric tissue of rats (Table 2)[87]. In addition, the ethanolic extract has been tested against specific multidrug-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (*S. aureus*) and extended spectrum β-lactamase-producing enteric bacteria and has shown broad-spectrum activity. This extract has also shown synergistic interaction with tetracycline, chloramphenicol and ciprofloxacin against *S. aureus* and/or *Escherichia coli* (*E. coli*)[61]. In addition, the butanol fraction of *T. chebula* fruit had profound growth-inhibitory activity against six intestinal bacteria, especially *Clostridium perfringens* and *E. coli*[62]. An aqueous extract from *T. chebula* was found to inhibit inducible nitric oxide synthesis by decreasing iNOS protein and iNOS mRNA levels (Table 1)[63].

Lepidium sativum

The seed of *Lipidium sativum* known as "Tokhm taretizak" is another famous drug used in TIM for IBD[90]. The ethanolic extract from the seed of this plant has

Table 3 Clinical studies on plants used in traditional Iranian medicine for the treatment of inflammatory bowel disease

Study	Study design	No. of patients	Disease	Plant	Part of plant	Control group	Duration of treatment	Result
Kaliora *et al*[82]	Open, comparing CD patients with healthy volunteers	10 patients and 8 controls	CD	*Pistacia lentiscus*	Oleogum resin	-	4 wk	↓TNF-α secretion, ↑macrophage migration inhibitory factor release meaning that random migration and chemotaxis of monocytes/ macrophages was inhibited
Kaliora *et al*[83]	Open, comparing CD patients with healthy volunteers	10 patients and 8 controls	CD	*Pistacia lentiscus*	Oleogum resin	-	4 wk	Significant reduction of CD Activity Index, ↓Plasma IL-6 and C-reactive protein, ↑total antioxidant potential, no side effects observed
Madisch *et al*[84]	Double-blind, randomized, placebo-controlled, multicenter	31	Collagenous colitis	*Boswellia serrata*	Gum resin extract	Placebo	6 wk	The proportion of patients in clinical remission was higher in the *Boswellia serrata* extract group than in the placebo group; Compared to placebo, *Boswellia serrata* extract treatment had no effect on histology and quality of life
Gupta *et al*[85]	Randomized	30	Chronic colitis	*Boswellia serrata*	Gum resin	Sulfasalazine	6 wk	Eighteen out of 20 patients treated with *Boswellia* gum resin showed an improvement in one or more of the parameters including stool properties, histopathology as well as scanning electron microscopy, hemoglobin, serum iron, calcium, phosphorus, proteins, total leukocytes and eosinophils; In the sulfasalazine group, 6 out of 10 patients showed similar results in the same parameters, 14 out of 20 patients treated with *Boswellia* gum resin achieved remission, while in the case of sulfasalazine the remission rate was 4 out of 10
Gupta *et al*[86]	Randomized	30	UC	*Boswellia serrata*	Gum resin	Sulfasalazine	6 wk	All tested parameters including stool properties, histopathology, scanning microscopy of rectal biopsies, and blood parameters including hemoglobin, serum iron, calcium, phosphorus, proteins, total leukocytes and eosinophils improved after treatment with *Boswellia serrata* gum resin. The rate of remission was similar in the two studies group (82% in the *Boswellia serrata* group *vs* 75% in the sulfasalazine group)
Al-Habbal *et al*[87]	Double-blind controlled	38	Duodenal ulcer	*Pistacia lentiscus*	Oleogumresin	Placebo	2 wk	Symptomatic relief in 80% of patients on oleogum resin from *P. lentiscus* and 50% in patients on placebo, endoscopically proven healing occurred in 70% of patients on oleogum resin from *P. lentiscus* and 22% of patients on placebo, no side effects observed
Fernández-Bañares *et al*[88]	Open label, multicenter, randomized	92	UC	*Plantago ovata*	Seed	Mesalamine	12 mo	40% relapse rate in the *P. ovata* seed group and 35% in the mesalamine group and 30% in the *Plantago ovata* plus mesalamine group

UC: Ulcerative colitis; CD: Crohn's disease; iNOS: Inducible nitric oxide synthase; Cox: Cyclooxygenase.

shown significant anti-inflammatory and analgesic activities in rats. However, it has been shown to potentiate gastric ulcer induced by indomethacin in these animals (Table 2). The mechanism of action of this seed seems to be inhibition of prostaglandin synthesis[72].

Plantago ovata and *P. psyllium*

The seed isolated from *Plantago ovata* (*P. psyllium*) and *P. psyllium* called "Esfarzah" is also used as an effective drug in the treatment of IBD[56,90]. Dietary fiber supplementation with 5% *P. ovata* seeds ameliorated the development of colonic inflammation in transgenic rats as evidenced by an improvement in intestinal cytoarchitecture. This effect was associated with a decrease in some of the pro-inflammatory mediators involved in the inflammatory process such as NO, leukotriene B4, and TNF-α. The

intestinal contents from fiber-treated colitis rats showed a significantly higher production of short chain fatty acids, butyrate and propionate, than non-treated colitis animals. *In vitro* studies revealed a synergistic inhibitory effect of butyrate and propionate on TNF-α production[75]. A significant reduction in colonic myeloperoxidase activity and restoration of colonic glutathione levels were also shown by this supplementation in a similar study (Table 2)[76]. Mucopolysaccharides derived from the husk of *P. ovata* have properties beneficial for wound cleansing and wound healing. It also limits scarring (Table 1)[58]. An open label, multicenter, randomized clinical trial was conducted to compare the efficacy and safety of *P. ovata* seeds (10 g *bid*) with mesalamine (500 mg *tid*) in maintaining remission in UC. After 12 mo, the relapse rate was 40% (14 of 35 patients) in the *P. ovata* seed group, 35% (13 of 37) in the mesalamine group, and 30% (9 of 30) in the *P. ovata* plus mesalamine group. The results of this study showed that *P. ovata* seeds might be as effective as mesalamine in maintaining remission in UC (Table 3)[88].

Bunium persicum

The fruit of *Bunium persicum* (*B. persicum*) known as "zireh kermani" is another natural product used for the treatment of IBD in TIM[6]. It is an economically important medicinal plant growing wild in the dry regions of Iran. The essential oil of *B. persicum* has strong anti-bacterial effects. This property could be the result of relatively high amounts of terpinenes and cumin aldehyde in the essential oil[43]. In addition, this essential oil has shown antioxidant properties. It was able to reduce the oxidation rate of soybean oil in the accelerated condition at 60℃ (Table 1)[44].

Cassia fistula

Fruit from *Cassia fistula* (*C. fistula*) known as "Flous" is another drug for the treatment of IBD in TIM[87]. The only known mechanism related to the beneficial effect of this plant is its antimicrobial properties. Crude extract of *C. fistula* exhibited significant antimicrobial activity (Table 1)[45].

Cydonia oblonga

Fruit from *Cydonia oblonga* known as "Beh" is also used for the treatment of IBD[91]. This fruit has shown radical scavenging and antimicrobial activities[50]. The phenolic extract exhibited the strongest antioxidant activity among the other extracts[51]. The antioxidant functions of its phenolic extracts were superior to that of chlorogenic acid and ascorbic acid as standard antioxidants (Table 1)[52].

Solanum nigrum

The fruit of *Solanum nigrum* (*S. nigrum*) known as "Tajrizi" is another natural product for the treatment of IBD in TIM[4,5]. A glycoprotein isolated from this fruit [*Solanum nigrum* L. (SNL) glycoprotein] has demonstrated a dose-dependent inhibitory effect on NO production and free radical formation in DSS-induced colitis in mice. It exhibited a suppressive effect on the activities of NF-κB and regulated the expression of iNOS and Cox-2 in the downstream signaling pathway (Table 2)[77]. *S. nigrum* fruits showed effective free radical scavenging activities in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay which seemed to be related to the SNL glycoprotein[59]. The SNL glycoprotein has remarkable scavenging effects on both the superoxide anion and hydroxyl radical, but exhibited slightly higher scavenging effects on the superoxide anion generated by the enzymatic hypoxanthine/xanthine oxidase system than on hydroxyl radicals generated by the Fenton reaction (Table 1)[60]. Treatment with *S. nigrum* extract significantly inhibited the gastric lesions induced by cold restraint stress (76.6%), indomethacin (73.8%), pyloric ligation (80.1%) and ethanol (70.6%) with equal or higher potency than omeprazole in experimental ulcer models. It also showed concomitant attenuation of gastric secretory volume, acidity and pepsin secretion in ulcerated rats. In addition, it accelerated the healing of acetic acid-induced ulcers after 7 d of treatment. Furthermore, it significantly inhibited H^+K^+ATPase activity and decreased gastrin secretion in the ethanol-induced ulcer model. The severity of the reaction of the ulcerogen and the reduction in ulcer size by *S. nigrum* extract was evident from histological findings (Table 2)[78,79].

Juglans regia

The kernel of *Juglans regia* (*J. regia*) known as "gerdou" has been used for the treatment of IBD in TIM[62]. Polyphenol compounds isolated from n-butanol extract of *J. regia* demonstrated a significant decrease in lipid peroxidation and a remarkable increase in antioxidant potential (Table 1)[56].

Boswellia carterii

Oleogum resin from *Boswellia carterii* (*B. carterii*) and *Boswellia serrata* (*B. serrata*) known as "Kondor" in TIM is another efficacious remedy for IBD[6,90]. Various studies have shown the anti-inflammatory effect of this oleogum resin (Table 2)[65,66]. Some new mono- and triterpenes isolated from this oleogum resin have exhibited NO production inhibitory activity in lipopolysaccharide-activated mouse peritoneal macrophages[40]. The ethanol extract of this oleogum resin has immunomodulatory properties *in vitro*[41]. The antimicrobial activities of the essential oil isolated from the oleogum resin of *B. carterii* have been demonstrated against various microorganisms including fungi, and gram-positive and gram-negative bacterial strains (Table 1)[42]. The results of a study evaluating the effectiveness of *Boswellia* extracts in controlled settings of DDS- or TNBS-induced colitis in mice suggested that *Boswellia* is ineffective in ameliorating colitis in these models. Moreover, individual boswellic acids were demonstrated to increase the basal and IL-1β-stimulated NF-κB activity in intestinal epithelial cells *in vitro* as well as reverse the proliferative effects of IL-1β. In addition, a hepatotoxic effect of *Boswellia* with pronounced hepatomegaly and steatosis was observed (Table 2)[67]. Patients with chronic diarrhea and histologically proven collagenous colitis were randomized to receive either oral *B. serrata* extract 400 mg three times daily for 6 wk or placebo. After 6 wk, the proportion of patients in clinical remission was higher in the

Table 4 Mechanisms of action of the plants used for the treatment of inflammatory bowel disease in traditional Iranian medicine

Plant	Activities								
	Immunomodulatory[1]	Antioxidant[2]	Antibacterial	↓NF-κB	↓NO	↓Cox-2	↓LB4	Anti-inflammatory	Wound healing
Althaea spp.			*					*	
Boswellia carterii and *Boswellia serrata*	*		*		*			*	
Bunium persicum		*	*						
Cassia fistula			*						
Commiphora mukul	*	*	*	*	*	*			
Cydonia oblonga		*	*						
Foeniculm vulgare		*	*		*			*	*
Juglans regia		*							
Lepidium sativum								*	
Pistacia lentiscus	*				*	*			*
Plantago ovata	*	*			*		*	*	*
Solanum nigrum		*		*	*	*			*
Terminalia chebula			*		*				*

[1]Modulating immune system by effects on factors of innate immunity including neutrophils, macrophages, dendritic cells, and natural killer cells are altered and defective mucosal helper T (Th) cell responses and greater expression of cytokines such as interleukin (IL)-1-β, IL-6, IL-12, tumor necrosis factor α (TNF-α) and interferon (IFN)-γ; [2]Decreasing factors involved in oxidative stress and lipid peroxidation and/or increased factors enhanced antioxidant capacity. Asterisks indicate having that kind of effect under the column heading. LB4: Leukotriene B4; NO: Nitric oxide; Cox-2: Cyclooxygenase-2; NF-κB: Nuclear factor κB.

B. serrata extract group than in the placebo group ($P = 0.04$). Compared to placebo, *B. serrata* extract treatment had no effect on histology and quality of life[84]. Thirty patients with chronic colitis were randomized to receive either a preparation of the gum resin from *B. serrata* (900 mg daily divided in three doses for 6 wk) or sulfasalazine (3 g daily divided in three doses for 6 wk). Of 20 patients treated with *Boswellia* gum resin, 18 patients showed an improvement in one or more of the parameters including stool properties, histopathology as well as scanning electron microscopy, in addition to hemoglobin, serum iron, calcium, phosphorus, proteins, total leukocytes, and eosinophils. In the sulfasalazine group, 6 of 10 patients showed similar results in the same parameters. Of 20 patients treated with *Boswellia* gum resin, 14 achieved remission, while in the case of sulfasalazine, the remission rate was 4 of 10[85]. In a similar study, patients with UC received either *B. serrata* gum resin preparation (350 mg three times daily for 6 wk) or sulfasalazine (1 g three times daily) and all tested parameters including stool properties, histopathology, scanning microscopy of rectal biopsies, blood parameters including hemoglobin, serum iron, calcium, phosphorus, proteins, total leukocytes and eosinophils improved after treatment with *B. serrata* gum resin. The rate of remission was similar in the two studies group (82% in the *B. serrata* group *vs* 75% in the sulfasalazine group) (Table 3)[86].

Althaea spp.

The flower and seed of various species of *Althaea* known as "Khatmi" in TIM have been claimed to be efficacious in IBD[87]. The ethanol extract of *Althaea officinalis* demonstrated significant antibacterial activity against *E. coli* (Table 1)[39]. The ethanol extract of the flower of *Althaea rosea* showed anti-inflammatory and analgesic effects in carrageenan- or dextran-induced rat paw edema (Table 2)[41].

CONCLUSION

Various herbal preparations have been used in TIM for the treatment of IBD. For many of the plants used in these formulations, there are various studies demonstrating their efficacy in IBD. These studies included *in vitro*, *in vivo*, and clinical trials which are summarized in detail in Tables 1-3, respectively. Table 4 briefly shows the modes of action of these plants in IBD. These medicines have shown their usefulness in IBD by different mechanisms of action including inhibiting the production of NO, Cox-2 and leukotriene B4, immunomodulatory properties, antimicrobial activities, antioxidant activities, and antiulcer and wound healing properties. As shown in Table 4, for some of these plants, only one or two mechanisms of action have been found such as in *Juglans regia*, *Cassia fistula*, *Lepidium sativum*, and *Bunium persicum*. However, in some of the plants various mechanisms of action are known. For example *Commiphora mukul* is effective in IBD due to its immunomodulatory, antioxidant, and antibacterial properties and it decreases NF-κB, NO and Cox-2. Another herb, *Plantago ovata*, has immunomodulatory, antioxidant, anti-inflammatory and wound healing activities and decreases NO and leukotriene B4. Considering the mechanisms of action of these plants, the combination of some of them may be useful due the numerous mechanisms involved in IBD, such as *Pistacia lentiscus*, *Bunium persicum*, *Solanum nigrum*, *Plantago ovata*, *Boswellia*, *Solanum nigrum*, *Plantago ovata* and *Commiphora mukul*.

Based on the published studies, some plants are likely to be more effective in the management of current IBD cases such as *Pistacia lentiscus*, *Plantago ovata* and *Commiphora mukul*. No exact relationship was found between the class of plants investigated and their efficacy which supports

the hypothesis of a complicated pathogenesis of IBD.

No potential adverse events have been reported for these remedies. There is only one study showing the ineffectiveness of the gum resin from *B. serrata* in ameliorating colitis in mouse DSS- and TNBS-induced colitis. Moreover, this study demonstrated its hepatotoxic effect[67]. However, other studies on gum resin from this plant have demonstrated its benefit in IBD such as inhibiting NO production[40], immunomodulatory properties[41], antimicrobial[40], anti-inflammatory activities[65,66], and inducing clinical remission[84,85].

For some of the herbal products used in TIM such as oleogum resin from *Commiphora myrrha*, seeds of *Ocimum basilicum*, seeds of *Linum usitatissimum*, gum resin from *Dracaena cinnabari*, seeds of *Plantago major*, seeds of *Lallementia royleana*, and seeds of *Allium porrum*, there are no or not enough studies to confirm their benefits in IBD. It is suggested that an evaluation of the effects of these plants on different aspects of IBD should be performed.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Diagnosis and management of pancreatic neuroendocrine tumor in von Hippel-Lindau disease

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Abstract

The pancreatic manifestations seen in patients with von Hippel-Lindau (VHL) disease are subdivided into 2 categories: pancreatic neuroendocrine tumors (NET), and cystic lesions, including simple cyst and serous cystadenoma. The VHL-associated cystic lesions are generally asymptomatic and do not require any treatment, unless they are indistinguishable from other cystic tumor types with malignant potential. Because pancreatic NET in VHL disease are non-functioning and have malignant potential, it is of clinical importance to find and diagnose these as early as possible. It will be recommended that comprehensive surveillance using dynamic computed tomography for abdominal manifestations, including pancreatic NET, should start from the age of 15 years in VHL patients. Unlike sporadic non-functioning NET without VHL disease, in which surgical resection is generally recommended, VHL patients at lower metastatic risk of pancreatic NET should be spared the risks of operative resection.

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Key words: Von Hippel-Lindau disease; Pancreas; Neuroendocrine tumor, Diagnosis; Clinical protocols

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INTRODUCTION

Von Hippel-Lindau (VHL) disease is an autosomal dominant disorder that develops a variety of tumors and cysts in the central nervous system (CNS) and visceral organs[1]. The prevalence of patients with VHL disease was reported to be 1 in 100000 of the population and 1 family in 1 million of the population[2]. Tumor types seen in VHL disease include hemangioblastomas in the CNS and retina, renal cell carcinoma, pheochromocytomas and pancreatic neuroendocrine tumors (NET)[1]. During their growth, these tumors impair the function of the primary organs and sometimes metastasize to distant organs, and thus are thought to have malignant potential. A number of studies in the United States and Europe have reported the clinical characteristics of these tumors, including pancreatic NET[3-9].

PANCREATIC MANIFESTATIONS IN VHL DISEASE

The pancreatic manifestations seen in patients with VHL disease are subdivided into 2 categories: NET as solid tumors, and cystic lesions, including a simple cyst and serous cystadenoma[1,5,10]. Fortunately, cystic lesions complicated with VHL disease are generally asymptomatic and do not require any treatment (Figure 1)[1]. It is necessary to differentially diagnose them from other cystic tumor types, such as intraductal papillary mucin-producing tumors or mucinous cystic tumors, because these mucinous cystic tumors have malignant potential. When cystic lesions seen in patients with VHL disease are indistinguishable from these tumor types or are causative of compression symptom onto adjacent organs, operative resection of the cystic lesion in the pancreas would be considered.

Unlike cystic lesions seen in the pancreas of patients with VHL disease, NET can be locally invasive and can metastasize, resulting in much higher clinical significance[1,6]. NET occur in 8%-17% of patients with VHL disease[1]. The malignant potential of sporadic pancreatic NET, which is not associated with VHL disease, varies depending on the functional properties of the tumors. None of the patients with pancreatic NET associated with VHL disease has been reported to present with hormonal syndrome[3,8]. Sporadic non-functioning NET behave in a malignant fashion with a metastatic spread in 60% to 90%, in marked contrast to the findings in cases with pancreatic NET associated with VHL disease, as previously described (metastatic disease in 11%-20%)[11]. The reason is thought to be as follows. In the case of sporadic non-functioning pancreatic NET, there are no hormonal symptoms, hence the tumors are first identified when they grow larger than 5 cm. In contrast, in the case of pancreatic NET in patients with VHL disease, the tumors can be diagnosed at a relatively early stage by screening examination for abdominal manifestations of the disease[11].

In general, pancreatic NET with or without VHL disease show a slow growth phenotype and thus the patients have a good prognosis. Blansfield *et al*[12] reported that the death rate as a result of metastatic pancreatic NET was 0.3% in patients (n = 633) with VHL disease. Pancreatic NET tend to have a high frequency in patients with pheochromocytoma (VHL type 2) as previously described[3,11]. However, Hammel *et al*[5] reported that patients with pancreatic lesions had significantly fewer pheochromocytomas than those without pancreatic lesions (14/122 *vs* 16/36, $P < 0.0001$). Taken together, there is no consensus to date regarding coexistence of pancreatic NET and pheochromocytoma.

DIAGNOSIS OF PANCREATIC NET IN VHL DISEASE

Ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI) can be used to detect primary NET and their metastases. Octreotide scintigraphy has a sensitivity that exceeds the combination of the others. However, smaller tumors can be difficult to visualize with octreotide scintigraphy. Positron emission tomography with 5-hydroxytryptophan or L-dopa can be an option for detection of small tumors[13], although only a limited number of institutes have employed these methodologies. In almost all hospitals over the world, dynamic CT is the most sensitive method for detection at present, since pancreatic NET are strongly enhanced on dynamic CT (Figure 2)[14]. MRI is also an effective method for metastatic liver lesions[15].

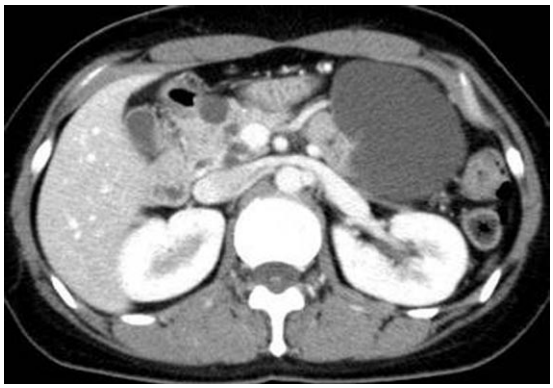

Figure 1 Abdominal computed tomography shows several cystic lesions in the pancreas (32-year-old female).

Figure 2 Contrast-enhanced abdominal computed tomography reveals several pancreatic mass lesions that are strongly enhanced (33-year-old female)[14].

MANAGEMENT OF PANCREATIC NET IN VHL DISEASE

In past reports, the youngest age at diagnosis of pancreatic NET in patients with VHL disease is 12 years old[16], and the second youngest age is 16 years old[12]. The surveillance of renal cell carcinoma (RCC) in VHL disease has been begun from the age of 15, therefore it will be recommended that comprehensive surveillance of abdominal organs including pancreas starts from the age of 15 by abdominal dynamic CT in view of the risk from

Table 1 Treatment recommendations for pancreatic neuroendocrine tumors with von Hippel-Lindau disease[12]

	Treatment recommendation
Prognostic criteria	
Tumor size ≥ 3 cm	
Mutation in exon 3	
Tumor doubling time ≤ 500 d	
None of the criteria	Followed by CT/MRI every 2-3 yr
1 criterion	Followed by CT/MRI every 6-12 mo
2 or 3 criteria	Consider surgical intervention

CT: Computed tomography; MRI: Magnetic resonance imaging.

radiation exposure and renal dysfunction caused by contrast media. In addition, patients with VHL disease require particular attention to distinguish pancreatic NET from metastatic RCC, because pancreatic metastasis from RCC is visualized as a hypervascular tumor as well as pancreatic NET. If pancreatic NET are not found by dynamic CT in the first abdominal surveillance (at the age of 15 years), the patient can be followed with comprehensive surveillance including that for RCC and pheochromocytoma every 2-3 years[12].

Sporadic non-functioning NET without VHL disease behave in a malignant fashion, therefore surgery is recommended to avoid later development of malignancy in all cases with tumor size greater than 2 cm[17,18]. In contrast, in the case of pancreatic NET with VHL disease, the indication for surgery should be carefully decided, because the patients commonly have multiple or recurrent tumors. The problem of surveillance is how to manage pancreatic NET without metastasis.

Blansfield *et al*[12] proposed 3 criteria to predict metastatic disease of pancreatic NET in patients with VHL disease: (1) tumor size greater than or equal to 3 cm; (2) presence of a mutation in exon 3; and (3) tumor doubling time less than 500 d (Table 1). If the patient has none of these criteria, they suggested that the likelihood of the patient's lesion resulting in metastatic disease is very low and that the patient can be followed with a medical history and physical examination and radiologic surveillance on 2-3 years cycles. If the patient has 1 criterion, the patient should be followed more closely every 6 mo to 1 year to detect the emergence of a second criterion. If the patient has 2 or 3 criteria, the patient should be considered for surgical management because of the greater likelihood of future malignancy from pancreatic NET[12]. The treatment strategy in patients with the metastatic disease is still controversial, depending on histological tumor types.

CONCLUSION

It is of clinical importance to find and diagnose pancreatic NET in patients with VHL as early as possible. It is recommended that comprehensive surveillance for abdominal manifestations in VHL patients including pancreatic NET should start from the age of 15. In general, pancreatic NET with or without VHL disease show a slow growth phenotype and patients have a good prognosis. VHL patients at lower metastatic risk from pancreatic NET should be spared the risks of surgical resection.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Recent standardization of treatment strategy for pancreatic neuroendocrine tumors

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Abstract

Recent advances in localization techniques, such as the selective arterial secretagogue injection test (SASI test) and somatostatin receptor scintigraphy have promoted curative resection surgery for patients with pancreatic neuroendocrine tumors (PNET). For patients with sporadic functioning PNET, curative resection surgery has been established by localization with the SASI test using secretin or calcium. For curative resection of functioning PNET associated with multiple endocrine neoplasia type 1 (MEN 1) which are usually multiple and sometimes numerous, resection surgery of the pancreas and/or the duodenum has to be performed based on localization by the SASI test. As resection surgery of PNET has increased, several important pathological features of PNET have been revealed. For example, in patients with Zollinger-Ellison syndrome (ZES), duodenal gastrinoma has been detected more frequently than pancreatic gastrinoma, and in patients with MEN 1 and ZES, gastrinomas have been located mostly in the duodenum, and pancreatic gastrinoma has been found to co-exist in 13% of patients. Nonfunctioning PNET in patients with MEN 1 becomes metastatic to the liver when it is more than 1 cm in diameter and should be resected after careful observation. The most important prognostic factor in patients with PNET is the development of hepatic metastases. The treatment strategy for hepatic metastases of PNET has not been established and aggressive resection with chemotherapy and trans-arterial chemoembolization have been performed with significant benefit. The usefulness of octreotide treatment and other molecular targeting agents are currently being assessed.

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Key words: Gastrinoma; Glucagonoma; Insulinoma; Multiple endocrine neoplasia type 1; Octreotide; Pancreas preserving total duodenectomy; Pancreatic neuroendocrine tumors; Selective arterial secretagogue injection test; Somatostatin receptor scintigraphy

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INTRODUCTION

As pancreatic neuroendocrine tumors (PNET) are rarely encountered in hospitals, standardization of diagnosis and/or the treatment strategy have not progressed until recently. However, recent advances in localization techniques, such as the selective arterial secretagogue injection test (SASI test) and somatostatin receptor scintigraphy (SRS) have promoted curative resection surgery of PNET[1,2]. As the number of resections has rapidly

increased, a few important characteristic pathological features of PNET have been revealed year by year. The World Health Organization pathological classification of PNET was evolutionally simplified in 2003 at the Lion Meeting, and the term carcinoid was declared a misnomer[3] (Table 1). Recently, a study group in the EU published a few guidelines on gastroenteropancreatic neuroendocrine tumors (GEPNET)[5,6]. In this work I will review important progress in the standardization of both surgical and medical treatment strategies for PNET.

EPIDEMIOLOGY OF PNET

In Western countries, PNET is found in about 1 per 100 000 population and represents 1%-2% of all pancreatic neoplasms[5-7]. In the USA, it is suggested that the incidence and prevalence of PNET has substantially increased over the last 30 years probably due to the rapid progress of innovative diagnostic techniques[8]. On the other hand, there have been a few epidemiological studies on NET in Japan[9,10]. In 2006, the Japanese NET study group (NET Work Japan) performed a nationwide survey to examine the epidemiology of GEPNET in Japan, using a stratified random sampling method to select departments of medical facilities where GEPNET were treated in 2005[9,10]. The first survey revealed that the overall prevalence was 2.23 patients per 100 000 population [95% confidence interval (CI): 1.93-2.76] per year. The total number of patients treated for functioning PNET was estimated to be 1627 (95% CI: 1.10-1.57), and the overall prevalence of insulinoma and gastrinoma was 0.84 and 0.23 per 100 000 population per year, respectively. Furthermore, the results in the second survey showed that the incidence of PNET in 2005 was estimated to be 1.01 per 100 000 population per year (95% CI: 0.88-1.25). Accordingly, the incidence of functioning PNET and non-functioning PNET was 0.50 and 0.51 per 100 000 population per year, respectively[9,10]. As the incidence of PNET in the USA has been reported to be about 0.32 per year per 100 000 population by Yao *et al*[11] PNET seems to develop about three times more frequently in Japan compared to that in the USA.

RECENT STANDARD OF DIFFERENTIAL DIAGNOSIS OF FUNCTIONING PNET

Characteristic clinical symptoms of functioning PNET

Recurrent peptic ulcers in gastrinoma, necrolytic migratory erythema in glucagonoma, and watery diarrhea in VIPoma are characteristic symptoms due to an excessive increase of the responsible hormone in blood. These symptoms easily lead to the correct diagnosis when the measurement of blood hormone levels is promptly followed. However, the symptoms due to hypoglycemia do not easily lead to the diagnosis of insulinoma[12]. This may sound strange, but it is true. The diagnosis of insulinoma is the most difficult among the functioning PNET. Patients with insulinoma are often misdiagnosed for long periods. The patient eats much food and looks healthy but somewhat strange without any organic illness. We should be very careful in diagnosing insulinoma as there are a number of diseases that cause hypoglycemia, and a variety of special tests are required for insulinoma diagnosis, which will be described below.

Recently, the differential diagnosis of gastrinoma has also become difficult. This is due to both the easy and long-term use of proton pump inhibitors for recurrent peptic ulcers or regurgitation esophagitis without a precise assessment of both serum gastrin levels and gastric hyperacidity status[13,14].

Measurement of serum hormone levels

The measurement of serum hormone levels is very useful for the differential diagnosis of PNET other than insulinoma. The normal range of serum gastrin levels in patients with gastrinoma is quite different in patients with and without a history of gastrectomy[1]. When a patient undergoes a distal gastrectomy, normal serum gastrin levels are usually lower than 90 pg/mL[1]. Jensen's group in NIH performed an aggressive study on both the fasting serum gastrin levels and the gastrin provocative testing of both patients with gastrinoma and normal volunteers[14,15]. They revealed that various physiological conditions were correlated with basal serum gastrin levels, and have recommended that an increase of 120 pg/mL or more as the positive range for the intravenous secretin test[14,15].

Inhibition test and stimulation test for diagnosis of symptomatic GEPNET

C-peptide inhibition test with hog insulin: This test is not 100% reliable for the diagnosis of insulinoma[12], but it can be completed in only 2 h and can serve as a valuable screening tool. Although this test might not be popular currently, we have favored this test for a long time similar to the group at the Mayo Clinic[15].

Intravenous secretin test for insulinoma: When 2 U/kg • body weight of secretin is intravenously administered, plasma insulin level rises more than 200% within 4 min in normal volunteers, but does not rise more than 100% in patients with insulinoma[16,17]. We have developed this test and used it for patients in whom other tests were non-diagnostic in the differential diagnosis of insulinoma.

Intravenous secretin test for gastrinoma: A bolus injection of 2 U/kg • body weight of secretin into the peripheral vein increases the serum level of gastrin by more than 100 pg/mL in patients with gastrinoma, but does not increase the serum level of gastrin in those without gastrinoma. This well known test has been successfully used for the differential diagnosis of gastrinoma since 1972[18]. Although this test has been proved to be useful for years, we have to be careful as this test is also positive in patients with hypergastrinemia due to atrophic gastritis. It has been shown that antral G-cells also have secretin receptors and release gastrin when stimulated with pharmacological doses of secretin[19].

Table 1 World Health Organization classification of pancreatic neuroendocrine tumors

WHO classification	Well-differentiated neuroendocrine tumor	Well-differentiated neuroendocrine carcinoma	Poorly-differentiated neuroendocrine carcinoma
Biological behavior	Benign/uncertain behavior	Low malignancy	High malignancy
Metastases	-	+	+
Ki-67/MIB-1 index (%)	< 2	2-20	> 20
Pathological differentiation	Well-differentiated	Well-differentiated	Poorly-differentiated
Vascular invasion	-/+	+	+
Size (diameter)	≤ 2 cm	> 2 cm	Any size

Alteration of the original Table by Klöppel[4]. WHO: World Health Organization.

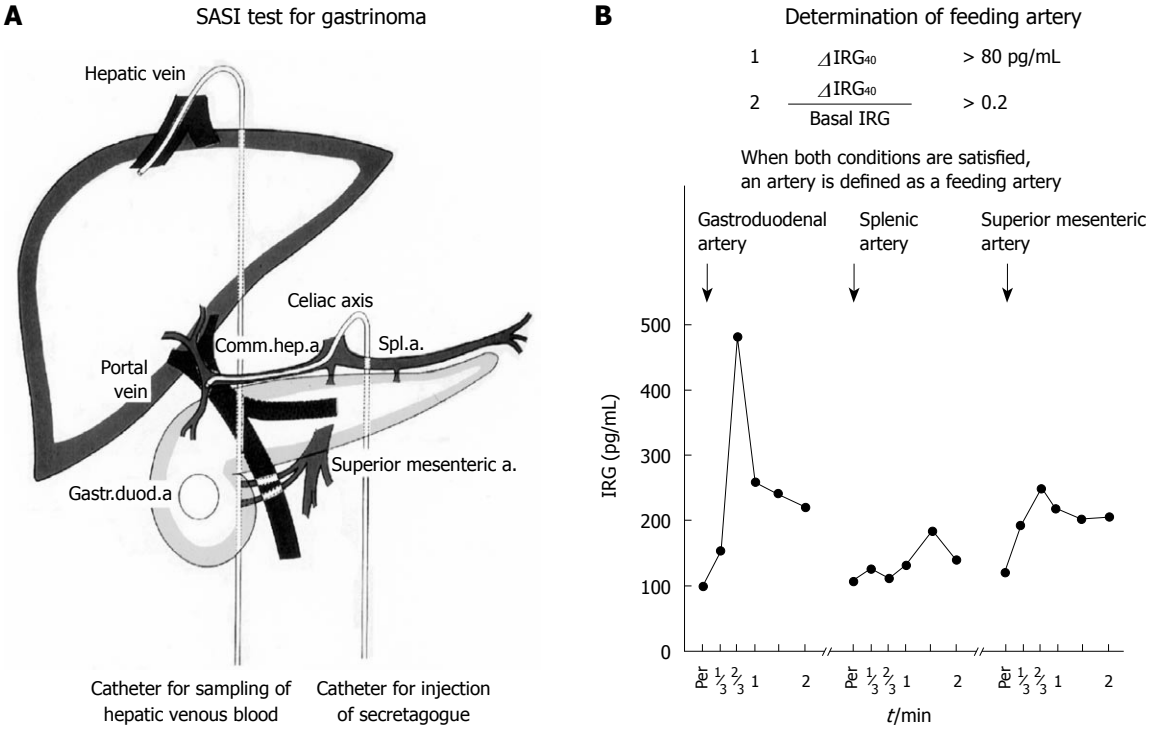

Figure 1 Schema of the selective arterial secretagogue injection test. Results of the selective arterial secretagogue injection (SASI) test in a patient with Zollinger-Ellison syndrome. In this patient, serum immunoreactive gastrin (IRG) at 40 s after the injection of 30 units of secretin rose only after injection into the gastroduodenal artery. Thus, it was diagnosed that the gastrinoma(s) was located in the upper part of the pancreas and/or the duodenum. Gastr.duod.a: Gastroduodenal artery; Spl.a.: Splenic artery; Superior mesenteric a.: Superior mesenteric artery.

RECENT PROGRESS IN LOCALIZATION OF BOTH FUNCTIONING AND NONFUNCTIONING PNET

Imaging techniques such as computed tomography, ultrasonography (US), endoscopic US (EUS), or intraoperative US (IOUS) have been useful for the localization of most PNET greater than 2 cm in diameter[20-23]. However, imaging techniques have difficulty in visualizing PNET less than 5 mm, and cannot identify a functioning PNET among various types of PNET including nonfunctioning PNET[20-23]. As the functioning PNET shows characteristic symptoms even when less than 5 mm, the SASI test is useful for preoperative localization of functioning PNET leading to curative resection surgery[1,20-22].

SRS is indispensable for localization of ectopic NET and the distribution of NET throughout the body[24].

SASI test with secretin or calcium

The SASI test was first described for localization of gastrinoma, and has gradually proved useful for the localization of other symptomatic PNET[1,20-22,25]. At the time of abdominal arteriography, secretagogue (30 U of secretin for gastrinoma and 1 mL of 8.5% calcium gluconate for insulinoma and glucagonoma) is injected into the splenic artery, the gastroduodenal artery and the superior mesenteric artery. Then, 2 mL blood samples are drawn from the hepatic vein through a catheter inserted *via* the femoral vein, before and 20, 40 and 60 s after the injection of secretagogue to detect the change in hormone levels in hepatic venous blood. When the rise in hormone levels 40 s after injection is significantly higher than measurement errors, the artery is diagnosed as a feeding artery of PNET. Functioning PNET is then located in the feeding area of the identified feeding artery. More precise localization is possible by injecting secretagogue into a branch of

the identified artery. When the splenic artery is identified as a feeding artery of insulinoma, more precise localization is possible by injecting calcium solution into the distal, middle and proximal portion of the splenic artery[20]. Both the sensitivity and specificity of the SASI test for both gastrinoma and insulinoma has been shown to be more than 90%, respectively[20,21,25] (Figure 1).

SRS

SRS is clearly able to visualize PNET more than 2 cm in diameter in the body, at a glance, and has contributed to the staging of PNET[26-28]. SRS can visualize 100% of gastrinomas larger than 3 cm in diameter, but only 20% of gastrinomas less than 5 mm, and 30% of gastrinomas less than 1 cm[27]. Thus, SRS visualized 73% of gastrinomas, 100% of glucagonomas, 88% of VIPomas, 73% of non-symptomatic GEPET, and only 46% of insulinomas, depending on both the extent of the presence and the differences in subtypes of somatostatin receptors, and the size of the tumor[27,28]. For the localization of ectopic PNET, SRS is an indispensable test[24].

IOUS

IOUS is useful in estimating the character of a tumor and to measure the distance between a PNET and the main pancreatic duct. In addition, the form and size of a PNET can be measured more correctly with IOUS than any other preoperative imaging technique[29]

Intraoperative rapid assay of blood hormone levels

Rapid immunoassay of insulin (IRI) and radioimmunoassay of gastrin (IRG) are useful for estimating the extent of the curability of surgery. Intraoperative measurement of both blood glucose levels and insulin using the same rate of drip infusion of glucose solution is helpful for estimating the curability of insulinoma resection[12]. The intraoperative secretin test with rapid radioimmunoassay of serum gastrin are useful for confirming the curability of gastrinoma resection surgery[30].

RECENT STANDARD OF SURGICAL TREATMENT OF PNET

The best treatment for PNET is curative surgical resection[5-8,31,32]. This needs to be performed before liver metastasis develops. Most PNET grow without invading the adjacent pancreatic parenchyma, and can reach a size of 1 cm[1,31].

Sporadic PNET

For a benign small PNET such as a benign sporadic insulinoma, enucleation is indicated wherever it is located in the pancreas, as long as it is 5 mm from the main pancreatic duct (MPD)[12,31]. Other sporadic functioning PNET such as gastrinomas, glucagonomas and VIPomas are thought to be potentially malignant and often multiple. Therefore, for these tumors pancreatic resection with lymph node dissection is indicated[31-33]. When the tumors are less than 5 mm in diameter, enucleation might also be indicated. R0 resection surgery for sporadic PNET has brought about complete relief of the characteristic difficult symptoms without recurrence[20,30-32].

PNET associated with MEN 1

In the case of multiple PNET, we must consider whether or not the patient has MEN 1. Serum calcium level and parathyroid hormone level require to be measured first, because the penetration rate of hyperparathyroidism is more than 90% in MEN 1. Genetic analysis is then performed. In patients with MEN 1, both PNET and duodenal NET are often multiple and microscopically numerous, and most are nonfunctioning[34-38].

There has been controversy regarding resection surgery for nonfunctioning PNET in MEN 1[31-33]. Recently, Goudet *et al*[36] revealed in a cohort study of 758 patients with MEN 1, that gastrinoma, nonfunctioning PNET and glucagonomas-vipomas-somatostatinomas had a high risk of death after adjustment for age, gender and diagnosis period. These PNET should be resected as early as possible before the development of hepatic metastases[31,35,36].

So far, extended distal pancreatectomy and enucleation of PNET more than 1 cm in diameter in the pancreatic head has been recommended for the prevention of liver metastases[35]. Total pancreatectomy is, so far, not indicated, because of a significant decrease in the quality of life of patients[37,38]. However, we know that some patients with PNET in MEN 1 rapidly develop liver metastases and die within a few years, therefore we will, in future, perform total pancreatectomy for selected patients based on advanced genetic analysis[38].

Pancreatic hypoglycemia in MEN 1 is often caused by multiple insulinomas which are located mostly in the body or tail of the pancreas[39]. Distal pancreatectomy is indicated for these types of insulinomas guided by the SASI test with calcium[12,39].

Recently, increased resection surgery for gastrinoma in patients with MEN 1 revealed that gastrinomas in MEN 1 were located mostly in the duodenum and rarely in the pancreas[40-43]. We have performed curative resection of gastrinomas in 16 patients with MEN 1 using pancreaticoduodenectomy (PD) or partial duodenal resection or pancreas preserving total duodenectomy (PPTD)[44]. In all patients, duodenal gastrinomas existed; as a single tumor in 42%, multiple tumors in 50% and numerous tumors in 13% (Figure 2). In addition, it was revealed that in two of 16 patients, pancreatic gastrinomas co-existed with multiple duodenal gastrinomas. These were resected guided by localization with the SASI test. When the patient with MEN 1 has more than five duodenal gastrinomas, we would recommend PPTD instead of PD for curative surgery[20,43,44]. The purpose of PPTD is to prevent recurrence of duodenal gastrinoma by total resection of the entire duodenum and to preserve full pancreatic function without resection of the pancreatic head. PPTD can be performed without any complications and seems less invasive than PD.

We have indicated PPTD for multiple duodenal gas-

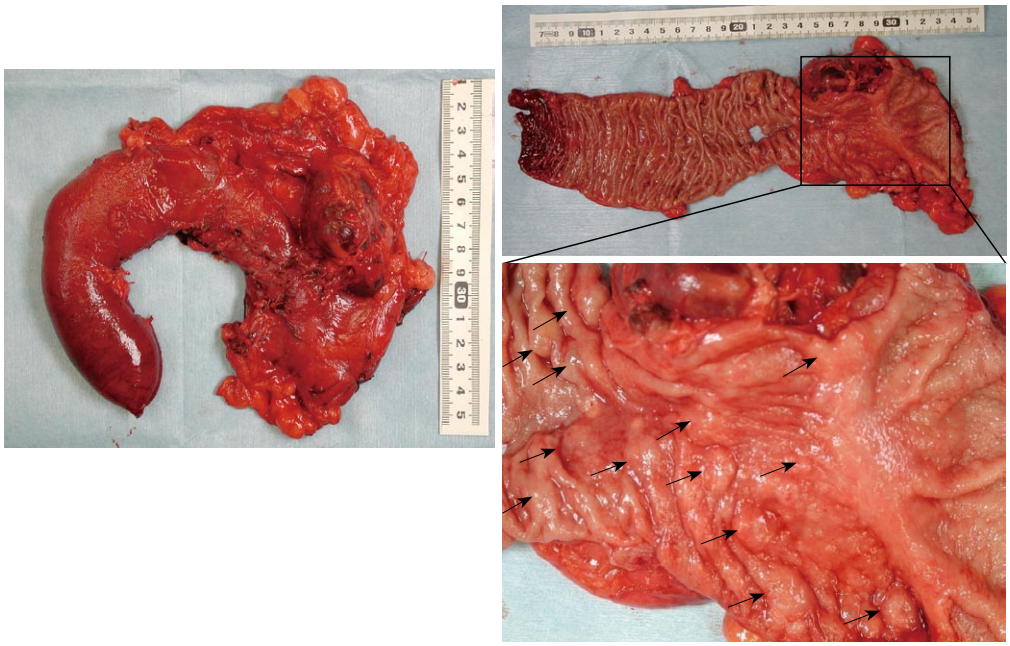

Figure 2 Numerous mucosal gastrinomas in the duodenum. Mucosal tumors with depression (arrows).

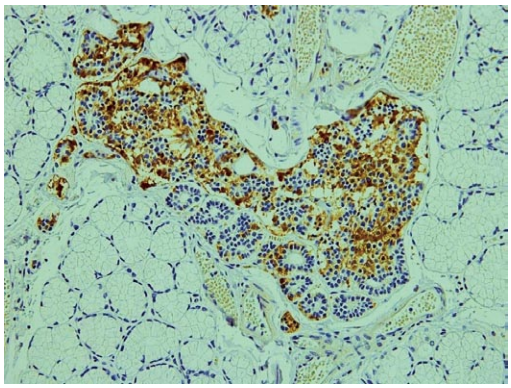

Figure 3 A cluster of G cells in hyperplasia of duodenal Brunner's glands in a patient with multiple endocrine neoplasia type 1 and Zollinger-Ellison syndrome.

trinomas (more than 5 or numerous gastrinomas)[43]. In 7 patients with MEN 1, more than 5 multiple duodenal gastrinomas were suspected during surgery and PPTD was performed. However, postoperative pathological diagnosis revealed that in 3 patients, only one or two duodenal gastrinomas existed, and other submucosal tumors which were thought to be gastrinomas during surgery were diagnosed as hyperplasia of Brunner's glands in the postoperatively fixed paraffin specimen.

We performed immunohistochemical staining of the duodenal Brunner's glands with anti-gastrin serum, and found that there were clusters of gastrin-producing cells in the hyperplasia of duodenal Brunner's glands in all duodenal specimens after PPTD (Figure 3). Recently, Klöppel *et al*[45] reported that in patients with MEN 1, mutations in the menin gene can cause the development of clusters of gastrin-producing cells in the duodenal Brunner's glands, which are thought to be precursor lesions of gastrinoma in patients with MEN 1. This may explain the high rate of postoperative recurrence of duodenal gastrinomas in patients with MEN 1, and may theoretically support the usefulness of PPTD as a curative surgery for these patients[43,44].

TREATMENT OF HEPATIC METASTASES OF PNET

A few guidelines on the treatment of GEPNET have been published, such as the NCCN (National Comprehensive Cancer Network) guideline and Consensus guidelines by the European NET Study Group (ENETS)[5,6,8,44]. In both of these guidelines, resection surgery is first recommended for resectable hepatic metastases of GEPNET when the metastases are limited to the liver[5,6,46-49]. Now, the use of various types of cytotoxic chemotherapy for rapidly growing GEPNET and octreotide for slow growing well-differentiated GEPNET have been standardized[5,6]. These guidelines are also available for PNET.

Hepatectomy for hepatic metastases

It has been proved that resection of hepatic metastases improves the outcome of patients with PNET. Various types of resection surgery have been performed to achieve a macroscopic curative resection of hepatic metastases. Bettini *et al*[49] in Verona have reported on the usefulness of resection surgery combined with cytotoxic chemotherapy for prolongation of survival. They performed hepatic resection surgery whenever more than 90% of the hepatic metastases could be dissected, and used cytotoxic chemotherapy with CDDP, etoposide and 5-fluorouracil (5-FU) with streptozotocin as well as octreotide[49].

Radiofrequency ablation

Radiofrequency ablation (RF) has been performed in ad-

dition to surgical resection of the liver for multiple hepatic metastases, for example, for metastases located deep in the hepatic parenchyma[50]. However, a number of complications after RF have been reported, especially following percutaneous RF. Therefore RF should be performed very carefully[51].

Chemotherapy, octreotide and new molecular targeting drugs

As the few guidelines on GEPNET describe, cytotoxic chemotherapy with CDDP and etoposide, streptozotocin with or without 5-FU, *etc.*, has been recommended for rapidly growing or poorly differentiated GEPNET[5,6]. For slow growing NET, octreotide with or without interferon α has been recommended[5,6].

In addition, prospective studies of mTor inhibitors with or without octreotide and tyrosine kinase inhibitors are currently underway[52,53]. New cytotoxic chemotherapy with temozolomide and capecitabine have also been reported to be effective in a small series of patients with malignant NET[54]. These drugs are also expected to be one of the new agents for PNET.

CONCLUSION

Curative resection surgery for sporadic PNET has almost been standardized using the SASI test for localization of PNET. The treatment strategy for PNET with MEN 1 has not been established, but resection surgery has been proved to contribute to the prolongation of survival in patients with MEN 1. Advances both in new chemotherapy including molecular targeting therapy and genetic analysis of PNET in patients with MEN 1 will lead us to a new treatment strategy for hereditary PNET.

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REVIEW

Liver disease and erythropoietic protoporphyria: A concise review

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Abstract

The porphyries are a group of metabolic disorders characterized by deficiencies in the activity of enzymes involved in the biosynthesis of heme. In erythropoietic protoporphyria (EPP), in the majority of cases an autosomal dominant disease, there is a mutation of the gene that encodes ferrochelatase (FECH). FECH deficiency is associated with increased concentrations of protoporphyrin in erythrocytes, plasma, skin and liver. The prevalence of this inherited disorder oscillates between 1:75000 and 1:200000. Clinical manifestations of EPP appear in early infancy upon first exposure to the sun. Nevertheless, approximately 5%-20% of patients with EPP develop liver manifestations. Retention of protoporphyrin in the liver is associated with cholestatic phenomena and oxidative stress that predisposes to hepatobiliary disease of varying degrees of severity, such as cholelithiasis, mild parenchymal liver disease, progressive hepatocellular disease with end-stage liver disease and acute liver failure. Liver damage is the major risk in EPP patients, so surveillance and frequent clinical and biochemical liver follow-up is mandatory. The diagnostic approach consists in detecting increased levels of protoporphyrin, decreased activity of FECH and genetic analysis of the *FECH* gene. A variety of non-surgical therapeutic approaches have been adopted for the management of EPP associated with liver disease, but none of these has been shown to be unequivocally efficacious. Nevertheless, some may have a place in preparing patients for liver transplantation. Liver transplantation does not correct the constitutional deficiency of FECH. Consequently, there is a risk of recurrence of liver disease after liver transplantation as a result of continuing overproduction of protoporphyrin. Some authors recommend that bone marrow transplantation should be considered in liver allograft recipients to prevent recurrence of hepatic disease.

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Key words: Erythropoietic protoporphyria; Protoporphyrin; Liver; Ferrochelatase

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INTRODUCTION

The porphyrias are a group of metabolic disorders charac-

terized by deficiencies in the activity of enzymes involved in the biosynthesis of heme, first described by Magnus *et al*[1,2]. Most are a result of inborn errors of metabolism, but the metabolic defect in some patient may be acquired[3]. Clinical manifestations of porphyrias can be divided in cutaneous or visceral.

In erythropoietic protoporphyria (EPP) there is a mutation of the gene that encodes ferrochelatase (FECH) in the long arm of chromosome 18. This enzyme catalyzes the insertion of ferrous iron into the protoporphyrin Ⅸ ring to form heme (Figure 1)[4,5]. FECH expression has a heme-dependent negative feedback regulation at a post-transcriptional level in such a way that FECH is decreased by increasing the level of intracellular heme[6]. EPP exhibits both recessive and dominant patterns of inheritance and a high degree of allelic heterogeneity with incomplete penetrance. Most heterozygotes are asymptomatic. Symptoms do not occur unless FECH activity is less than 30% of normal, but such low levels are not present in a majority of patients[7]. Recently a new pattern of EPP has been described related to gain-of-function mutations in the aminolevulinic acid synthase 2 gene[8].

Cells which synthesize heme are predominantly erythroblasts/reticulocytes in the bone marrow (80%) and hepatocytes (20%). Deficiency of *FECH* results in increased release of protoporphyrin, which binds to albumin in plasma and subsequently undergoes hepatic extraction. Normally, most protoporphyrin in hepatocytes is secreted into bile; the remainder undergoes transformation into heme. Some protoporphyrin in bile is returned to the liver as a consequence of the enterohepatic circulation; the remaining protoporphyrin in the intestine undergoes fecal excretion. Protoporphyrin is insoluble and hence unavailable for renal excretion. In EPP, subnormal biotransformation of protoporphyrin into heme results in accumulation of protoporphyrin in hepatocytes[9].

FECH deficiency is associated with increased concentrations of protoporphyrin in erythrocytes, plasma, skin and liver. Retention of protoporphyrin in skin predisposes to acute photosensitivity. As a result of absorption of ultraviolet light (400 nm) by protoporphyrin in plasma and erythrocytes when blood circulates through the dermal vessels, free radicals are formed, erythrocytes become unstable and injury to the skin is induced[10]. A significant increase in the hepatobiliary excretion on protoporphyrin can damage the liver through both cholestatic phenomena and oxidative stress[9] predisposing to hepatobiliary disease of varying degrees of severity[11-13].

DIAGNOSIS

EPP is generally suspected by the presence of acute photosensitivity of the skin and can be confirmed by detection of a plasmatic fluorescence peak at 634 nm. It is also useful finding increased levels of protoporphyrin in feces and the demonstration of an excess of free protoporphyrin in erythrocytes[12]. Screening for *FECH* mutation on one allele or aminolevulinic acid synthase 2 gain-of-function mutation in selected family members may be useful, especially in genetic counseling. If one parent is affected with EPP with a *FECH* allele mutation, the risk of the offspring developing EPP is less than 2.5%, therefore, screening for the presence of the *FECH* IVS3-48C allele in the other parent may be helpful to estimate the probability for the offspring[12,14,15].

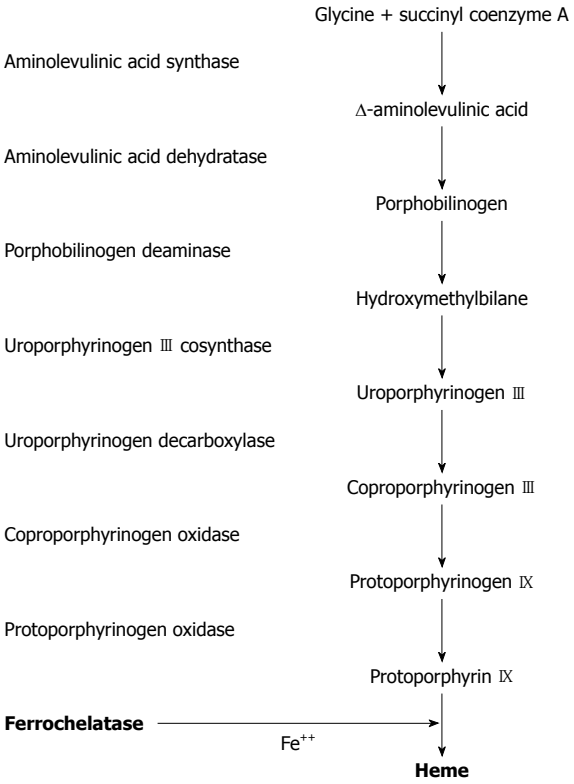

Figure 1 The heme biosynthetic pathway. In erythropoietic protoporphyria there is a deficiency in the activity of ferrochelatase which catalyzes the insertion of the ferrous iron into the protoporphyrin Ⅸ ring to form heme.

Liver biopsy

Liver biopsy confirms hepatic disease in EPP by the presence of protoporphyrin deposits in the hepatocytes that can be observed as a brown pigment within the biliary canaliculi and the portal macrophages. Macroscopically, the cirrhotic liver can have a black color due to protoporphyrin deposits. Using polarized light the characteristic Maltese cross shape of birefringent crystalline pigment deposits is found. The examination of liver tissue under a Wood's lamp reveals a red fluorescence due to protoporphyrin. Liver biopsy is not helpful for estimation of prognosis of liver disease[11,13].

HEPATOBILIARY DISEASE

EPP usually presents in childhood, but there are a few reports of cases presenting in adults[16,17]. The commonest mode of presentation is acute photosensitivity of the skin. It affects areas exposed to the sun and tends to be intractable. A few minutes of exposure to the sun induces pruritus, erythema, swelling and pain. Longer periods of exposure may induce second degree burns. After repeti-

tive exposure, patients may present with lichenification, hypopigmentation, hyperpigmentation and scarring of the skin[10,16,18].

Clinical findings suggestive of liver disease appear in approximately 5%-20% of patients[11-13,19]. The susceptibility of individual patients with EPP to protoporphyrin-induced liver damage is highly variable. The host factors responsible for this variable susceptibility are unknown. The spectrum of hepatobiliary disease associated with EPP is wide. It includes cholelithiasis, mild parenchymal liver disease, progressive hepatocellular disease and end-stage liver disease[12].

Pathophysiology

Liver damage in EPP has been attributed to precipitation of insoluble protoporphyrin in bile canaliculi and to protoporphyrin-induced oxidative stress. The latter arises as a consequence of excess unmetabolized protoporphyrin interacting with the hepatocellular membrane and inducing impaired function of the Na^+/K^+-ATPase pump within the membrane. The accumulation of excess protoporphyrin, that does not undergo biliary excretion, exacerbates cholestasis and further reduces the excretion of protoporphyrin[20]. These pathophysiological phenomena may result in hepatic inflammation, progressive hepatocellular disease, hepatic fibrosis, and, eventually, cirrhosis[11-13].

Clinical manifestations

Cholelithiasis: Insoluble protoporphyrin in bile may act as nuclei for stone formation. Cholelithiasis is frequent in EPP (10%-20%), due to the accumulation of free protoporphyrin and increased biliary protoporphyrin concentration. Clinical manifestations of cholelithiasis and choledocolithiasis are similar to lithiasis by cholesterol or bilirubin[5]. Many patients with EPP and gallstones undergo cholecystectomy. Subsequent analysis of the stones reveals that they are birefringent and contain high concentrations of protoporphyrin. Some authors believe that EPP should be suspected when cholelithiasis presents in childhood[21].

Mild parenchymal liver disease: Most patients (20%) present with a mild liver disease, characterized by increased levels of aminotransferases and/or cholestatic enzymes. Typically, in patients with mild disease, there are no symptoms. Patients can also present with splenomegaly and hepatomegaly. Liver biopsy in such patients may reveal features of appreciable hepatocellular injury[5,13,22-24].

Progressive hepatocellular disease: Symptoms include upper abdominal pain and jaundice. There may be associated rapid deterioration of photosensitivity because of decreased secretion of protoporphyrin into bile, secondary to cholestasis and hemolysis[12].

It is rare for the initial presentation of EPP to be manifestations of progressive hepatocellular disease[25]. When jaundice is clinically evident, hepatocellular disease is advanced and hepatic clearance function is appreciably reduced. Blood protoporphyrin levels increase further, but fecal protoporphyrin excretion decreases[14].

End-stage liver disease: Only 5% of liver damage presents as an acute liver insufficiency. Progressive hepatocellular disease ultimately leads to cholestatic hepatocellular failure, which often has an acute onset, and a rapidly progressive, irreversible course. Progressive hepatocellular disease in EPP is usually fatal within months if liver transplantation is not undertaken (see below)[25-42].

SURVEILLANCE AND TREATMENT OF PATIENTS WITH EPP AND LIVER DISEASE

Surveillance

There is no consensus regarding optimal surveillance for patients with EPP. Liver biopsy is the gold standard to assess the degree of hepatic damage. Results of non-invasive methods, such as serum biochemical liver tests, do not correlate closely with the degree of hepatic injury. In a review, Anstey and Hift[11] proposed the following indications for liver biopsy in patients with EPP: (1) Presence of null mutations or autosomal recessive disease; (2) Family history of EPP-related liver disease; (3) Presence of risk factors for the development of liver disease, such as markers of viral hepatitis, factors suggestive of non-alcoholic fatty liver disease, and alcohol abuse; (4) Abnormal results of serum biochemical liver tests; (5) Evidence of hepatocellular decompensation; and (6) To relieve a patient's anxiety or to comply with a patient's preference.

The optimal frequency of blood tests to monitor patients with EPP has not been established. Some authors advocate serum biochemical liver tests every 6 mo; others prefer to have these tests done annually up to the age of 20 years and then biennially[43,44].

Treatment approaches

A variety of non-surgical therapeutic approaches have been adopted for the management of EPP associated with progressive hepatocellular disease. However, none of these has been shown to be unequivocally efficacious. Nevertheless, some may have a place in preparing patients for liver transplantation[45]. The pathophysiology of this disease suggests several potential therapeutic targets (Table 1). Such targets include attempts to induce bile flow, to render bile less toxic, to reduce protoporphyrin production in the bone marrow, to reduce the circulating pool of protoporphyrin, to promote hepatocellular metabolism and transport of protoporphyrin, to protect hepatocytes from toxic damage, and to interrupt the enterohepatic circulation[11-13,46].

Ursodeoxycholic acid: This bile acid is administered to promote biliary secretion of protoporphyrin. Results of its use in EPP are controversial. However, it is known to alter the composition of bile, to protect hepatocytes from the cytotoxic effect of hydrophobic bile acids, and to stimulate biliary secretion by several distinct mechanisms[46,47].

Table 1 Pathogenic mechanisms and therapeutic approaches of erythropoietic protoporphyria

Pathogenic mechanism	Treatment
Induce bile flow	Ursodeoxycholic acid
Reduce protoporphyrin production	Parenteral iron
	Transfusion of erythrocytes
	Infusions of hematin
Reduce protoporphyrin levels	Plasmapheresis
	Extracorporeal albumin dialysis
Interrupt enterohepatic circulation	Cholestyramine
	Activated charcoal
Protect hepatocytes from toxic damage	N-acetyl cysteine
Remove the principal source of protoporphyrin	Bone marrow transplant
Erythropoietic protoporphyria-related liver failure	Liver transplant

Parenteral iron and transfusion of erythrocytes: The objective of administering iron and/or erythrocytes is to suppress erythropoiesis and, hence, reduce the protoporphyrin level. In theory, iron therapy should not work since it stimulates heme synthesis *via* 5-aminolevulinate synthase. However, results of this approach in patients with EPP are contradictory. It has been reported that iron therapy may exacerbate hepatic dysfunction[48,49] whereas in some case reports the correction of iron deficiency has improved EPP[50,51]. Nevertheless, the mechanism of this favorable response to iron therapy remains unknown, so more studies with a significant number of patients are necessary to clarify the role of iron therapy in the medical treatment of EPP. Because of the lack of definitive clinical data, the contradictory reports and the theoretical possibility of exacerbating hepatic dysfunction, the decision to use this therapy should be individualized.

Infusions of hematin: Hematin appears to reduce excess protoporphyrin production in the bone marrow. It has been administered to patients with EPP (3-4 mg/kg iv) who develop a crisis after liver transplantation (see below)[52].

Plasmapheresis: Circulating levels of protoporphyrin can be decreased by plasma exchange[53].

Extracorporeal albumin dialysis: This type of dialysis is used to decrease circulating levels of albumin-bound toxins. Accordingly, in patients with EPP, damage to the hepatobiliary system may be reduced by using this approach to reduce plasma concentrations of protoporphyrin. It may also reduce levels of protoporphyrin in erythrocytes, when protoporphyrin subsequently diffuses out of these cells into the plasma. A greater reduction in erythrocyte protoporphyrin levels following treatment with a molecular adsorbent recirculating system (9.1%) than following plasmapheresis (0.8%) or treatment with the Prometheus system (5.9%) has been documented in a case study of a patient with EPP and liver disease[25,54].

Cholestyramine: This orally administered resin reduces circulating levels of protoporphyrin by binding to protoporphyrin in the intestine and, hence, interrupting the enterohepatic circulation. It is usually used in combination with other treatment approaches[55].

Activated charcoal: Like cholestyramine, activated charcoal also binds to protoporphyrin in the intestine and prevents its absorption. It is cheap and readily available. It seems to be effective in reducing circulating protoporphyrin levels[56,57].

N-acetyl cysteine: In liver diseases free radicals are increased, thereby damaging the hepatic tissue[58]. In addition, nitric oxide has deleterious effects in the presence of reactive oxygen species, participating in the pathophysiology of different liver diseases[59,60].

N-acetyl cysteine (NAC) modulates the expression of inducible oxide synthase in hepatocytes[61], and this action could be effective in the attenuation of oxidative and nitrosative stress in liver injury. Based on these findings, it is suggested that antioxidant therapy might be beneficial in the treatment of liver damage of different etiologies[62]. There is little experience with NAC in EPP, only clinical reports such a 32-year-old man with EPP who developed progressive hepatocellular disease and was treated with NAC 300 mg/kg body weight per day IV infusion for 3 wk. This treatment was associated with an improvement in hepatocellular function, in particular, serum levels of hepatic enzymes normalized[63].

Bone marrow transplantation: The purpose of this approach is to remove the tissue primarily responsible for the overproduction of protoporphyrin. It is a frequently discussed option, but the incidence of associated adverse events has limited its use as a treatment for EPP. However, some authors advocate bone marrow transplantation as a complementary treatment in an attempt to avoid liver re-transplantation in patients who have undergone liver transplantation for EPP-associated liver disease (see below)[26].

Liver transplantation: More than 40 patients with EPP and hepatocellular disease, who have undergone liver transplantation, have been reported in the world literature[12]. Liver transplantation does not correct the constitutional deficiency of FECH. Consequently, there is a risk of recurrence of liver disease after liver transplantation as a result of the continuing overproduction of protoporphyrin. A review of 20 transplanted cases of EPP in the USA led the authors to recommend that bone marrow transplantation should be considered in liver allograft recipients to prevent recurrence of hepatic disease[45]. Patients with EPP are prone to certain perioperative complications of liver transplantation. Management involves adopting appropriate precautions or treatment options. Phototoxic abdominal burns may be induced; the mechanism is analogous to that responsible for skin photosensitivity. Such burns can be avoided by fitting filters to lamps

in the operating theater[64]. Acute neuropathy is a major complication. It is associated with severe abdominal pain, an acute deterioration in hepatocellular function, and an increase in erythrocyte protoporphyrin levels. Treatment options include hematin infusions and/or plasmapheresis[25]. Acute protoporphyrin-mediated damage to the liver allograft may occur secondary to high circulating levels of protoporphyrin at the time of transplantation. This complication can be prevented by taking short-term measures to reduce levels of protoporphyrin at the time of surgery[54,65]: (1) Cholecystectomy; (2) Vaccines against viral hepatitis; and (3)Avoid hepatotoxic drugs. Avoid drugs that might develop a drug induced liver injury.

CONCLUSION

In EPP there is a mutation of the gene that encodes FECH, the enzyme that catalyzes the insertion of ferrous iron into the protoporphyrin IX ring to form heme. EPP presents both recessive and dominant patterns of inheritance. Recently, a new pattern of EPP has been described related to gain-of-function mutations in the aminolevulinic acid synthase 2 gene. FECH deficiency is associated with increased concentrations of protoporphyrin in erythrocytes, plasma, skin and liver. A significant increase in the hepatobiliary excretion of protoporphyrin can damage the liver through both cholestatic phenomena and oxidative stress predisposing to a wide spectrum of hepatobiliary disease of varying degrees of severity that includes cholelithiasis, mild parenchymal liver disease, progressive hepatocellular disease and end-stage liver disease. The susceptibility of individual patients with EPP to protoporphyrin-induced liver damage is highly variable. There is no consensus regarding optimal surveillance for patients with EPP. Liver biopsy is the gold standard to assess the degree of hepatic damage. A variety of non-surgical therapeutic approaches have been adopted for the management of EPP associated with progressive liver disease, but none of these has been shown to be unequivocally efficacious. Liver transplantation does not correct the constitutional deficiency of FECH and, as a result of the continuing overproduction of protoporphyrin, there is a risk of recurrence of liver disease after liver transplantation.

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ORIGINAL ARTICLE

Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome

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Abstract

AIM: To investigate the correlations between self-reported symptoms of irritable bowel syndrome (IBS) and the gastrointestinal (GI) microbiota composition.

METHODS: Fecal samples were collected from a total of 44 subjects diagnosed with IBS. Their symptoms were monitored with a validated inflammatory bowel disease questionnaire adjusted for IBS patients. Thirteen quantitative real-time polymerase chain reaction assays were applied to evaluate the GI microbiota composition. Eubacteria and GI bacterial genera (*Bifidobacterium*, *Lactobacillus and Veillonella*), groups (*Clostridium coccoides/Eubacterium rectale*, *Desulfovibrio desulfuricans*) and distinct bacterial phylotypes [closest 16S rDNA sequence resemblance to species *Bifidobacterium catenulatum*, *Clostridium cocleatum*, *Collinsella aerofaciens* (*C. aerofaciens*), *Coprococcus eutactus* (*C. eutactus*), *Ruminococcus torques* and *Streptococcus bovis*] with a suspected association with IBS were quantified. Correlations between quantities or presence/absence data of selected bacterial groups or phylotypes and various IBS-related symptoms were investigated.

RESULTS: Associations were observed between subjects' self-reported symptoms and the presence or quantities of certain GI bacteria. A *Ruminococcus torques* (*R. torques*)-like (94% similarity in 16S rRNA gene sequence) phylotype was associated with severity of bowel symptoms. Furthermore, among IBS subjects with *R. torques* 94% detected, the amounts of *C. cocleatum* 88%, *C. aerofaciens*-like and *C. eutactus* 97% phylotypes were significantly reduced. Interesting observations were also made concerning the effect of a subject's weight on GI microbiota with regard to *C. aerofaciens*-like phylotype, *Bifidobacterium* spp. and *Lactobacillus* spp.

CONCLUSION: Bacteria seemingly affecting the symptom scores are unlikely to be the underlying cause or cure of IBS, but they may serve as biomarkers of the condition.

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Key words: Irritable bowel syndrome; Self-reported symptoms; Gastrointestinal microbiota; Quantitative real-time polymerase chain reaction

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional bowel disorder, with an estimated worldwide prevalence of 10%-20% among adults and adolescents. IBS is characterized by pain or discomfort, distorted bowel habits and altered stool characteristics[1]. Although the prognosis of IBS is good, the syndrome results in a reduced quality of life. Subjects with IBS report significantly more comorbidities, including dyspepsia, asthma and head- and backache, as well as anxiety, depression and insomnia, than the general population[2]. The exact etiology of IBS is likely to be multifactorial; moreover, patients diagnosed with the disorder may well be experiencing bowel symptoms due to different causes.

Much attention has recently been focused on the impact of gastrointestinal (GI) microbiota on this disorder. Changes observed in the fecal microbiota composition[3-5], high incidence of IBS after GI infections[6], alterations seen in IBS patients' GI immune systems[7], as well as ability of probiotics to alleviate the symptoms of IBS[8-10], all suggest that microbes play a key role in IBS. However, a genetic basis for IBS has also been presented. In twin studies, a greater likelihood for a twin to develop IBS if the other sibling already had the disorder was observed among monozygotic twins compared to dizygotic twins[11]. Upregulation of genes involved in mucin production has been described to take place in IBS patients[12]. Downregulation of protease-activated receptor 1 expression and upregulation of protease-activated receptor 2 ligand mast cell tryptase in diarrhea-predominant IBS (IBS-D) are involved in visceral hypersensitivity; a change in the expression ratio of these two protease-activated receptors appears to take place in the context of IBS-D[13]. Similarly to ulcerative colitis, colonic mucosal 5-HT (serotonin) concentrations are significantly lowered in IBS patients compared with the levels observed in healthy controls, suggesting the existence of a shared mechanism underlying the symptoms[14].

In this study, we examined whether the presence or absence of certain microbes previously linked to either IBS or healthy controls' microbiota correlated with the symptoms experienced by IBS patients. Our results suggest that a connection between IBS-related microbiota and severity of self-reported symptoms exists.

MATERIALS AND METHODS

Subjects

Subjects fulfilling the Rome I criteria for IBS[15] were recruited from the district of Kuopio in Eastern Finland by the Kuopio University Hospital and Harjula Hospital during years 2004-2005. The participants (n = 44; 11 men, 33 women) were 20-72 years old and their general condition was confirmed as good by medical experts (see Table 1 for subject characteristics). Exclusion criteria for participation included presence of organic GI diseases, inadequately treated hypertension or pharmaceutically treated diabetes. Use of statins, pharmaceutically treated hypertension or coronary artery disease were not considered exclusion criteria if medication had been used for at least six months prior to the study with no changes in dosage.

Clinical studies and laboratory tests

Participants were subjected to a standard medical examination and laboratory tests, including blood cell counts (B-Leuk, B-Trom, B-Eryt), hemoglobin (B-Hb), hematocrit (B-Hcr), erythrocyte mean cell volume (E-MCV), mean cell hemoglobin (E-MCH) and mean corpuscular hemoglobin concentration (E-MCHC), erythrocyte sedimentation rate, glycosylated hemoglobin (B-GHb$_{A1c}$) and blood lipids (fS-Chol, fS-Chol-HDL, S-Trigly). Lactose absorption was ensured with a DNA test for a mutation in the lactase gene. The participants were also tested for the presence of IgA antibodies against gliadin, followed by a duodenal biopsy if celiac disease was suspected. Subjects over 45 years were examined for the presence of carcinoembryonic antigen (CEA). In addition, presence of occult blood in the feces was evaluated.

Questionnaires

Participants filled in a questionnaire regarding their quality of life and symptoms. The survey was based on an internationally approved and validated questionnaire for inflammatory bowel disease questionnaire (IBDQ)[16]. The questions in the IBDQ were adapted to suit IBS patients. The query contained 30 questions clustered into four groups, comprising "bowel symptoms", "systemic symptoms", "social function" and "emotional function".

Table 1 Characteristics of the study subjects

Characteristic	mean ± SD	Median (range)
Age (yr)	48.4 ± 11.9	49.0 (20-72)
Body mass index (kg/m^2)	26.3 ± 5.1	26.0 (19.4-45.6)
Systolic blood pressure (mmHg)	129.2 ± 19.1	129.5 (95-175)
Diastolic blood pressure (mmHg)	83.0 ± 10.2	83.5 (61-100)
Hemoglobin (g/L)	139.0 ± 12.0	139.0 (108-165)
B-GHb$_{a1c}$ (%)	5.6 ± 0.38	5.6 (4.8-6.4)
Total cholesterol (mmol/L)	5.1 ± 0.73	5.0 (3.8-7.3)
Sedimentation rate (mm/h)	7.4 ± 7.1	5.0 (2-43)

The seven alternative answers for each question were assigned numerical values from 1 to 7 (1 = no problem at all, 7 = very severe problem). Two questions regarding the form of feces and the frequency of defecation were treated as separate variables. The participants also answered two questions about prior antibiotic treatments and GI infections.

Analysis of fecal bacterial microbiota

Each subject gave a fecal sample for bacteriological studies. The samples were stored at -80℃ prior to analysis. Bacterial DNA was isolated from 1 g of fecal material by removing the undigested particles from the fecal material with three rounds of low-speed centrifugation, collection of the bacterial cells with high-speed centrifugation, enzymatic and mechanical cell lysis and DNA extraction and precipitation[17]. DNA concentrations were measured with the multilabel plate reader Victor3TM (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). With the method, the average yield of DNA was 342 μg/g of fecal material (median: 290 μg/g, SD: 167 μg/g). Quantitative real-time polymerase chain reaction (qPCR) was used to assess the amounts of selected GI bacteria or bacterial phylotypes (Table 2) in the fecal samples.

In total, 13 qPCR assays were performed to analyze the GI microbiota in fecal samples (Table 2). The applied assays targeted quantitatively IBS-associated human GI bacteria (*Lactobacillus* spp., *Clostridium coccoides*/*Eubacterium rectale*-group, *Veillonella* spp. and *Bifidobacterium* spp.)[5] and bacterial phylotypes [*Collinsella aerofaciens*-like, *Clostridium cocleatum* 88%, *Coprococcus eutactus* 97%, *Ruminococcus torques* 91% and *Ruminococcus torques* (*R. torques*) 94%][4,18] or bacteria associated with IBS in semi-quantitative sequence data analyses (*Bifidobacterium catenulatum*/*Pseudocatenulatum*-like)[4] or with intestinal disturbances according to the literature (*Desulfovibrio desulfuricans*-group)[19]. The iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) in conjunction with the iCycler Optical System Interface software (version 2.3; Bio-Rad) were used to analyze the samples as described previously[4,5]. Two technical replicates were used for the samples and standard reactions. Depending on the assay, 0.5 or 50 ng of fecal DNA was applied in the reactions.

Statistical tests

Basic statistical analysis of the data was performed using the SPSS program, version 14.0 (SPSS Inc., Chicago, IL, USA). R program, version 2.8.0[20] and the package Rcmdr, version 1.4-10[21] were used to perform the principal component analysis (PCA), to describe the categorical data and to statistically test this data. Linear models were used to describe the relationship between variables and were applied to each bacterial genus and phylotype quantified.

The health-related quality of life questionnaire yielded ordinal data; thus, non-parametric statistical methods were used for analysis. However, PCA analysis was performed for the questionnaire data. Results are presented as medians and interquartile ranges. The χ^2 test was applied to compare nominal data, and the Mann-Whitney *U* test to compare continuous data when two patient groups were analyzed.

Logarithmic transformation was performed on the bacterial data prior to further analyses. Within the data, undetected abundances possibly caused by technical limitations instead of the absence of the target phylotype were imputed with the mean values obtained from the qPCR runs with the same primer applied to water. If these also were undetected for a certain assay, the minimum of all detected water runs was used. Bacterial qPCR data were also inspected to ascertain the presence or absence of certain phylotypes in the patient samples. Binary data were then used to evaluate whether the phylotypes had any relationship with various traits of the study subjects.

Ethical issues

The study protocol was approved by the Kuopio University Hospital Ethical Committee. Participation in the study was voluntary, and patients were allowed to withdraw at any point without giving an explanation.

RESULTS

Characteristics of IBS patients

Characteristics of the IBS patients are listed in Table 1. In general, the clinical studies revealed no major issues regarding the health status of participants. However, of the 44 subjects studied, 7/11 men and 15/33 women had a body mass index (BMI) value above 25, which is considered borderline between normal weight and slightly overweight[22]. Presence of *Helicobacter* had been confirmed previously in 8 patients; interestingly, in some cases, treatment of the infection had remained incomplete (data not shown). *Helicobacter* infection or the way it had been treated was not, however, reflected in the symptoms (data not shown). Origin of IBS as a result of GI inflammation was not given support by this study, as only one subject recalled suffering from gastroenteritis but could not remember whether the onset of IBS occurred before or after the ailment.

Bacterial analyses and correlation of symptom scores with microbiota composition

The modified IBDQ symptom questionnaire consisted

Table 2 Targets, assay conditions and primers of quantitative real-time polymerase chain reaction assays

Target bacterial group/phylotype	Positive control strain or clone	$MgCl_2$ (mmol/L)	Detection (℃)	Annealing (℃)	Primer sequences (F: forward, R: reverse)
Bifidobacterium catenulatum/ Pseudocatenulatum-like[4]	AM277302	3	87	68	F: 5'-ACTCCTCGCATGGGGTGTC-3' R: 5'-CCGAAGGCTTGCTCCCGAT-3'
Bifidobacterium spp.[30]	*Bifidobacterium longum* DSM20219	3	85	58	F: 5'-TCGCGTC(C/T)GGTGTGAAAG-3' R: 5'-CCACATCCAGC(A/G)TCCAC-3'
Clostridium coccoides/Eubacterium rectale-group[30]	*Ruminococcus productus* DSM2950	4	85	55	F: 5'-CGGTACCTGACTAAGAAG-3' R: 5'-AGTTT(C/T)ATTCTTGCGAAC-3'
Clostridium cocleatum 88%[4]	AM275477	4	80	60	F: 5'-AATACATAAGTAACCTGGCRTC-3' R: 5'-CGTAGCACTTTTCATATAGAGTT-3'
Collinsella aerofaciens-like[4]	AM276364	4	89	67	F: 5'-CCCGACGGGAGGGGAT-3' R: 5'-CTTCTGCAGGTACAGTCTTGAC-3'
Coprococcus eutactus 97%[4]	AM278899	2	83	63	F: 5'-AGCTTGCTCCGGCYGATTTA-3' R: 5'-CGGTTTTACCAGTCGTTTCCAA-3'
Desulfovibrio desulfuricans-group[30]	*Desulfovibrio desulfuricans* ATCC7757	4	85	58	F: 5'-GGTACCTTCAAAGGAAGCAC-3' R: 5'-GGGATTTCACCCCTGACTTA-3'
Eubacterial 16S[31]	*Bifidobacterium longum* DSM20219	3	80	50	F: 5'-TCCTACGGGAGGCAGCAGT-3' R: 5'-GGACTACCAGGGTATCTAATCCTGTT-3'
Lactobacillus-group[32,33]	*Lactobacillus acidophilus* ATCC4356	2	85	58	F: 5'-AGCAGTAGGGAATCTTCCA-3' R: 5'-CACCGCTACACATGGAG-3'
Ruminococcus torques 91%[4]	AM276624	5	82	62	F: 5'-TGCTTAACTGATCTTCTTCGGA-3' R: 5'-CGGTATTAGCAGTCATTTCTG-3'
Ruminococcus torques 94%[4]	AM277929	2	85	65	F: 5'-AATCTTCGGAGGAAGAGGACA-3' R: 5'-ACACTACACCATGCGGTCCT-3'
Streptococcus bovis-like[4]	AM276479	5	80	60	F: 5'-TTAGCTTGCTAAAGTTGGAA-3' R: 5'-ATCTACTAGTGAAGCAATTGCT-3'
Veillonella spp.[30]	*Veillonella parvula* ATCC10790	3	85	62	F: 5'-A(C/T)CAACCTGCCCTTCAGA-3' R: 5'-CGTCCCGATTAACAGAGCTT-3'

of 28 questions divided into the four categories of bowel symptoms, systemic symptoms, social function and emotional function (Table 3). High median values along with a narrow interquartile range can be considered characteristic of the questions central for ascertaining symptoms of IBS (Table 3). Correlations between the four categories were all significant (Table 4).

Abundance and prevalence of the 13 qPCR target bacteria or phylotypes in patient samples are shown in Tables 5 and 6, respectively. An association between *R. torques* 94% phylotype and symptom scores (emotional function, social function, systemic symptoms, bowel symptoms) was observed in a PCA visualization of the results as they correlated significantly with the same dimension, whereas a weaker negative association was observed for *Coprococcus eutactus* (*C. eutactus*) 97%, *Bifidobacterium* spp., *Veillonella* spp. and *Desulfovibrio desulfuricans* (*D. desulfuricans*)-group and the symptom scores (Figure 1A). When the bowel symptoms (bloating, passing gas, increased need to defecate or need to defecate when bowel is empty, abdominal cramps, abdominal pain, soiling) were analyzed in a PCA, a similar effect was observed: *R. torques* 94% and all bowel symptoms except soiling correlated with the same dimension, whereas *Collinsella aerofaciens* (*C. aerofaciens*)-like, *C. eutactus* 97%, *Veillonella* spp., *Bifidobacterium* spp., and *Lactobacillus* spp. were negatively associated (Figure 1B).

In linear modeling of continuous data, *R. torques* 94% was associated with an increase in self-reported bowel symptoms [analysis of variance (ANOVA), $P < 0.05$]. When the IBS subjects were grouped according to whether *R. torques* 94% was detected in their fecal samples (Table 7), self-reported bowel symptoms tended to be higher among subjects with *R. torques* 94% present (ANOVA, $P = 0.056$). Interestingly, presence of *R. torques* 94% had a negative effect on the abundance of *C. eutactus* 97% ($P < 0.01$), *C. aerofaciens*-like ($P < 0.05$) and *C. cocleatum* 88% ($P < 0.05$) phylotypes.

No other bacterial associations with symptom scores could be verified. Although in particular the *C. aerofaciens*-like phylotype had a negative association with *R. torques* 94%, its relationship with self-reported symptoms remained obscure. The phylotype was, however, associated with lower BMI values (Mann-Whitney test for present-absent data; $P < 0.01$) and lower blood pressure (Mann-Whitney test for present-absent data for systolic and diastolic blood pressure; $P < 0.01$ and 0.01, respectively), and the data also suggested a link to lower blood sugar levels ($P = 0.06$). The *C. aerofacien*-like phylotype was less frequently observed in subjects with BMI above 25 (Table 6) and none of the six subjects with BMI values over 30 had the *C. aerofaciens*-like phylotype in their feces. Similary to *C. aerofaciens*, *C. eutactus* 97% was also associated with lower blood pressure ($P < 0.05$ and 0.05 for diastolic and systolic blood pressure, respectively). A positive association was present between the presence of *C. aerofaciens* and amounts of *C. eutactus* 97% ($P < 0.01$), whereas a strong negative effect on *R. torques* 94% was observed ($P < 0.001$). In addition, when the IBS subjects were categorized according to their BMI, subjects with a BMI value over 25 had more bifidobacteria than normal-

Table 3 Symptom questionnaire results

Symptom groups	Median (interquartile range)	Minimum value	Maximum value
Bowel symptoms			
Increased frequency of defecation	3 (2)	1	6
Abdominal cramps	3 (1)	1	5
Passing gas	4 (2)	1	7
Abdominal bloating	5 (2)	1	7
Feeling a need to defecate	3 (2)	1	7
Soiling	1 (1.25)	1	5
Stomach pain	3 (1)	1	6
Systemic symptoms			
Tired and worn out	3.5 (1)	1	6
Nausea	3 (2)	1	6
Generally unwell	3 (1.25)	1	5
Sleep disturbance	4 (2)	1	7
Weight problems	3 (2.25)	1	7
Social function			
Work/school activities	1 (0)	1	7
Cancel social engagements	1 (1)	1	7
Leisure/sports activity	3 (3)	1	7
Avoid events lacking toilet	1 (1)	1	4
Sexual activity	2 (2)	1	7
Emotional function			
Frustrated/impatient	3 (1)	1	6
Energy	3 (2)	2	6
Fear of not finding a toilet	2 (2)	1	4
Depressed/discouraged	3 (1)	1	5
Worried about illness	3 (1.25)	1	7
Relaxed/free from worries	5 (1)	2	7
Tearful or upset	2 (1.25)	1	5
Angry	3 (2)	1	7
Irritable	3 (2)	1	5
Lack of understanding by others	2 (1)	1	6
Satisfaction with personal life	4 (2)	1	6

Table 4 Mean values and Spearman correlations (r_s) of symptom groups

Variable	mean (SD)	Bowel symptoms r_s	Systemic symptoms r_s	Social symptoms r_s
Bowel symptoms	24.0 (6.5)	-	-	-
Systemic symptoms	16.4 (8.1)	0.58[b]	-	-
Social function	9.3 (4.3)	0.60[b]	0.34[a]	-
Emotional function	39.9 (8.1)	0.71[b]	0.76[b]	0.58[b]

[a]$P < 0.05$, [b]$P < 0.001$.

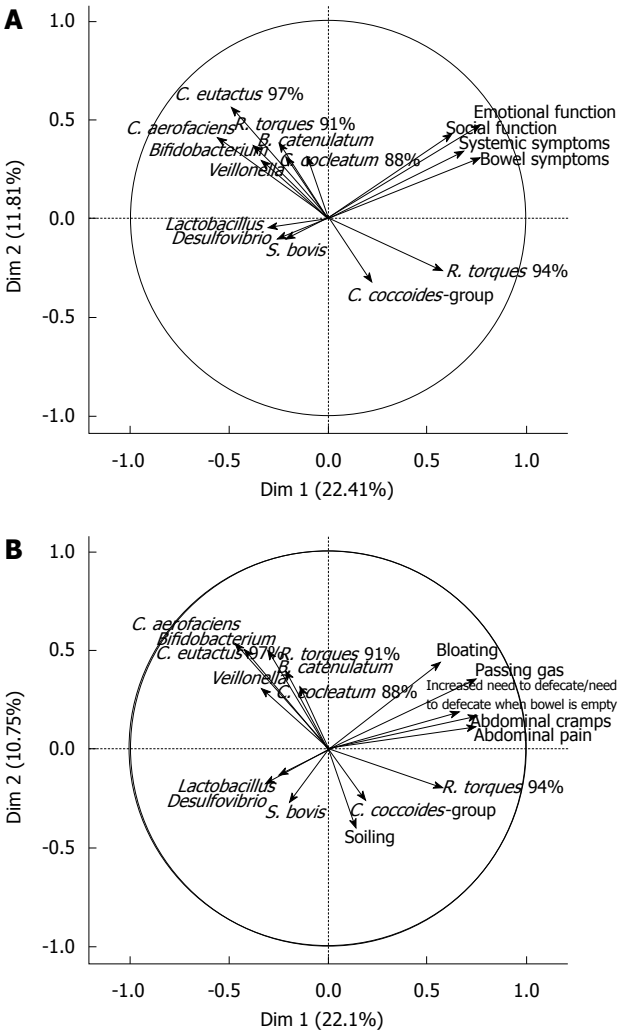

Figure 1 Principal component analysis of bacterial data and patient symptom scores. A: Dimension 1 explains 20.4% of the observed variation among test subjects when bacterial data and symptom groups are studied. The four symptom groups have correlations with Dimension 1 of 0.77-0.63, all with $P < 0.001$, and *Ruminococcus torques* 94% has a correlation of 0.58, with $P < 0.001$. *Coprococcus eutactus* 97%, *Bifidobacterium* spp., *Veillonella* spp. and *Desulfovibrio desulfuricans*-group also present significant, albeit smaller, negative correlations with Dimension 1; B: When the bowel symptom sum is studied question-wise, individual symptoms (except for soiling) as well as *Ruminococcus torques* 94% show a significant correlation of 0.57-0.75, with $P < 0.001$, with Dimension 1. Negative associations are observed for *Collinsella aerofaciens*-like, *Coprococcus eutactus* 97%, *Veillonella* spp., *Lactobacillus* spp. and *Bifidobacterium* spp.

weight subjects, but less lactobacilli in an almost significant manner (Table 5).

DISCUSSION

Self-reported symptoms and GI microbiota composition of IBS patients were analyzed to investigate putative biomarkers for the disorder. Interesting associations between GI microbiota composition and symptom severity were observed.

To measure IBS patients' symptoms, we applied the IBDQ, designed for assessing the quality of life of IBD patients[16], with some modifications for IBS patients. According to the Spearman's correlations calculated for each question and symptom group, the questions generally described best the group in which they were included (data not shown). Generally, the range observed for each question contained the entire available scale, indicating that the patients formed a heterogeneous group regarding the severity of individual symptoms (Table 3). However, between symptom groups, there were high correlations, indicating that although the questions measured severity of specific issues, the groups themselves actually measured the same health issues (Table 4). This is understandable since IBS patients' physical and mental symptoms reflect their well-being at a given point of observation[23]. Bearing in mind that the questionnaire was intended for IBD

Table 5 Number of 16S rRNA gene copies detected in 50 ng of fecal DNA

qPCR assay	All (n = 44)	BMI < 25[1] (n = 17)	BMI > 25 (n = 22)	P[2]
Bifidobacterium catenulatum/Pseudocatenulatum-like	4.8 (1.6)[3]	5.4 (0.7)	4.3 (1.8)	0.366
Bifidobacterium spp.	5.8 (1.0)	5.5 (1.0)	6.2 (0.9)	0.009
Clostridium coccoides/Eubacterium rectale-group	7.3 (0.2)	7.3 (0.1)	7.3 (0.2)	0.630
Clostridium cocleatum 88%	5.6 (1.1)	5.5 (1.4)	5.6 (1.0)	0.483
Collinsella aerofaciens-like	5.6 (1.1)	5.3 (1.2)	5.8 (0.9)	0.294
Coprococcus eutactus 97%	5.3 (1.5)	5.5 (1.7)	5.4 (1.3)	0.639
Desulfovibrio desulfuricans-group	3.9 (0.8)	3.8 (0.6)	4.0 (0.9)	0.403
Eubacterial 16S	6.0 (0.3)	5.9 (0.4)	6.0 (0.3)	0.630
Lactobacillus-group	3.9 (0.9)	4.2 (0.7)	3.7 (0.9)	0.060
Ruminococcus torques 91%	4.6 (0.8)	4.4 (0.8)	4.8 (0.7)	0.461
Ruminococcus torques 94%	3.8 (1.0)	4.0 (1.3)	3.6 (0.8)	0.409
Streptococcus bovis-like	2.7 (1.5)	3.2 (2.1)	2.5 (1.2)	0.732
Veillonella spp.	3.4 (1.0)	3.5 (1.1)	3.4 (1.1)	0.745

[1]Body mass index (BMI) data missing for 5 subjects; [2]Calculated with Mann-Whitney *U*-test; [3]Values are presented as log10 averages with standard deviation in parentheses. qPCR: Quantitative real-time polymerase chain reaction.

Table 6 Prevalence of target 16S rRNA genes detected for each quantitative real-time polymerase chain reaction assay

qPCR assay	All (n = 44)	BMI < 25[1] (n = 17)	BMI > 25 (n = 22)	P[2]
Bifidobacterium catenulatum/Pseudocatenulatum	12	3	7	0.315
Bifidobacterium spp.	44	17	22	ND
Clostridium coccoides/Eubacterium rectale-group	44	17	22	ND
Clostridium cocleatum 88%	33	13	15	0.568
Collinsella aerofaciens-like	29	15	12	0.024
Coprococcus eutactus 97%	16	7	8	0.759
Desulfovibrio desulfuricans-group	29	11	15	0.819
Eubacterial 16S	44	17	22	ND
Lactobacillus-group	44	17	22	ND
Ruminococcus torques 91%	38	15	18	0.582
Ruminococcus torques 94%	29	9	16	0.202
Streptococcus bovi-like	33	11	18	0.225
Veillonella spp.	41	16	20	0.709

[1]Body mass index (BMI) data missing for 5 subjects; [2]Calculated with pearson χ^2 test. ND: Not determined; qPCR: Quantitative real-time polymerase chain reaction.

patients, the results should be interpreted with caution. For example, reasons underlying weight problems are different in IBS patients than in IBD patients, who may experience problems with either loss or gain of weight, depending on the status of their disease[24]. In our study, weight problems were correlated with a higher BMI and can thus be considered a measure of problems with weight. Division of the patients into two groups according to the BMI values resulted in a significant divergence ($P < 0.01$) in the systemic symptom scores between these two groups, with the patients having a BMI in excess of 25 experiencing more symptoms than leaner subjects. This observation may suggest that some variables other than severity of IBS might be affected by BMI, as seemed to be the case for lactobacilli and bifidobacteria (Table 5). In addition, as some of the participants were treated for hypertension, any connections between blood pressure and GI microbiota should be observed with extreme caution.

C. aerofaciens-like phylotype had interesting associations with patient characteristics. We have previously observed a reduction in the amount of *C. aerofaciens* in the fecal samples of IBS patients compared with healthy controls[4]. Recently, Mäkivuokko *et al*[25] reported that elderly subjects using non-steroidal anti-inflammatory drugs (NSAIDs) had reduced amounts of *C. aerofaciens* present in their feces relative to healthy young subjects and elderly subjects without NSAIDs. A link between anti-inflammatory drugs and the absence of *C. aerofaciens* was suggested by the authors. In this present study, we observed a negative correlation between the presence (or amounts) of *C. aerofaciens* and the BMI value of test subjects. Notably, obese (BMI > 30) subjects were negative for *C. aerofaciens*, but contradictory to our results, Turnbaugh *et al*[26] have found *C. aerofaciens* to be more prominent in obese than lean twins and their mothers. Obesity has been associated with a low-grade systemic inflammation in which the GI microbiota may be involved[27]; this could explain the negative association observed for *C. aerofaciens* and BMI values. It was difficult to conclude whether *C. aerofaciens* could have any role in IBS; in general, overweight (BMI > 25) subjects reported more systemic symptoms than normal-weight subjects.

Table 7 Association of *Ruminococcus torques* 94% phylotype with bowel symptoms and other fecal bacterial phylotypes

Variable	All samples	*Ruminococcus torques* 94%	
		Detected	Undetected
Symptom sums[1]			
All symptoms	83.6 (20.0)	87.6 (19.6)	75.9 (19.1)
Bowel symptoms	24.1 (6.5)	25.4 (6.5)	21.5 (5.8)[2,3]
Systemic symptoms	16.4 (4.8)	17.0 (4.7)	15.2 (4.8)
Social function	9.3 (4.3)	10.0 (4.5)	7.8 (3.6)
Emotional function	33.9 (8.1)	35.2 (8.0)	31.5 (7.9)
qPCR assays[4]			
Bifidobacterium catenulatum/ Pseudocatenulatum	2.5 (1.7)	2.5 (1.7)	2.5 (1.6)
Bifidobacterium spp.	5.8 (1.0)	5.8 (1.0)	5.9 (1.1)
Clostridium coccoides /Eubacterium rectale-group	7.3 (0.2)	7.3 (0.2)	7.3 (0.2)
Clostridium cocleatum 88%	3.9 (2.8)	3.1 (3.0)	5.4 (1.7)[5]
Collinsella aerofaciens-like	3.0 (3.8)	1.6 (4.0)	5.6 (0.7)[5]
Coprococcus eutactus 97%	1.9 (2.8)	0.9 (2.2)	3.7 (3.0)[6]
Desulfovibrio desulfuricans-group	1.9 (3.0)	1.4 (3.1)	2.7 (2.6)
Eubacterial 16S	6.0 (0.3)	5.9 (0.3)	6.0 (0.4)
Lactobacillus-group	3.9 (0.9)	3.8 (0.8)	4.1 (1.0)
Ruminococcus torques 91%	4.1 (1.5)	4.1 (1.6)	3.9 (1.3)
Ruminococcus torques 94%	2.7 (1.8)	3.8 (1.0)	-
Streptococcus bovis-like	1.9 (1.9)	1.6 (1.6)	2.4 (2.3)
Veillonella spp.	3.0 (1.7)	3.2 (1.7)	3.0 (1.8)

[1]Values are presented as averages with standard deviation in parentheses; [2]All statistical comparisons made between samples with *Ruminococcus torques* 94% detected and undetected; [3]Significant difference ($P < 0.05$) using analysis of variance (ANOVA) test with continuous data; [4]The values are presented as log10 averages with standard deviation in parentheses; [5]Significant difference ($P < 0.05$) using ANOVA test with binary data; [6]Significant difference ($P < 0.01$) using ANOVA test with binary data.

This leads to a problem in interpretation of the results; as the measured symptom groups correlate strongly with each other, it is difficult to determine whether a rise observed in one symptom group is actually caused by a rise in another symptom group rather than by IBS itself.

The phylogenetically most similar species to *R. torques* 94% which has previously been associated with IBS-D[18] is a known mucin degrader[28], and the reported increase of mucin in the context of IBS could explain the observed link between this phylotype and the symptoms. The negative association of *R. torques* 94% with *C. cocleatum* 88%, *C. aerofaciens*-like and *C. eutactus* 97%, observed for this phylotype could thus also be due to sample characteristics (i.e. abundance of mucus and human cells in the samples) and is in correlation with previous results, as these phylotypes have been associated with healthy controls' GI microbiota in comparison with that of IBS subjects[4]. However, with our knowledge being restricted to the 16S ribosomal DNA sequence of the phylotype, all suggestions about the functions of these bacteria should be considered merely speculative.

Regarding *R. torques* 94%, an association with BMI values was lacking, while a role for this phylotype in IBS was suggested (Figure 1A and B). Of the bacterial genera and phylotypes here negatively associated with symptom scores or bowel symptoms (Figure 1A and B), *Lactobacillus* spp., *Bifidobacterium* spp., *D. desulfuricans*-group, *C. aerofaciens*-like and *C. eutactus* 97% have previously been detected in lowest quantities among IBS-D patients in comparison to other IBS symptom subtypes and healthy control subjects[5,18]. *Veillonella* spp. has previously been associated with constipation-predominant IBS subjects[5] but was not found to correlate with self reported IBS symptoms in this study.

The observed higher abundance of *Bifidobacterium* spp. in overweight subjects has previously been reported in a large metagenomic study[26]. Interestingly, an energy-restricted diet has been shown to reduce *C. coccoides*-group, *Bifidobacterium longum* and *Bifidobacterium adolescentis* counts and increase *Bacteroides fragilis*- and *Lactobacillus*-group counts in originally overweight adolescents, with the effect being more pronounced among subjects who had lost more weight[29]. However, as Santacruz *et al*[29] concluded, it may well be the proportional amounts of various bacterial groups within the GI tract rather than their absolute numbers that play a role or react in complex events within the GI tract; they found the *Bifidobacterium* to *C. coccoides*-group ratio to increase in correlation with weight loss. In our study, the *C. coccoides/E. rectale*-group levels were the same in normal-weight and overweight subjects (Table 5).

In conclusion, our findings indicate that certain bacterial phylotypes might serve as markers of symptom severity in IBS. While the presence of *R. torques* 94% was associated with an increase in symptom severity, some other phylotypes seemed to act in the opposite direction. These microbes are, however, not found in all individuals and they may also be present in healthy subjects' samples[4]; therefore it is unlikely that their presence or absence in the GI tract would be the underlying cause of IBS.

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COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional gastrointestinal (GI) disorder and results in a reduced quality of life. Alterations in the human GI microbiota have been detected among patients suffering from the syndrome. The abnormalities in the GI microbiota are suggested to contribute to IBS symptoms.

Research frontiers

The role of GI microbiota in IBS has been under investigation and studies suggest that microbes associated with IBS possess potential as non-invasive biomarkers. Since the majority of the GI bacteria are uncultivable, molecular methods are crucial in this field and have enabled a deeper study of the dis-

turbed microbiota. The authors examined whether the quantities, or presence or absence, of certain microbes previously linked to either IBS or healthy microbiota, correlated with the symptoms experienced by IBS patients.

Innovations and breakthroughs

Alterations in the overall microbiota and certain microbial phylotypes have been detected in IBS. The results of this study suggest that there is a connection between IBS-related microbiota and severity of self-reported symptoms.

Applications

The findings in this study indicate that certain bacterial phylotypes are associated with symptom severity in IBS. These bacteria may serve as biomarkers of the course of the condition.

Terminology

Human intestinal microbiota is the ensemble of all microbes in the gastrointestinal tract. The term bacterial phylotype stands for an operative taxonomic unit determined by the 16S rRNA gene sequence similarity. Quantitative real-time polymerase chain reaction (qPCR) is a method that enables quantification of target DNA molecules in a sample. In this study 16S rDNA sequences of known bacterial genera and of bacterial phylotypes were quantified using qPCR.

Peer review

This paper represents a large amount of work. Although the results are largely negative, they should be published, since IBS is such an important issue.

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ORIGINAL ARTICLE

Lowered HGK expression inhibits cell invasion and adhesion in hepatocellular carcinoma cell line HepG2

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Abstract

AIM: To investigate the effects of RNA interference targeting hepatocyte progenitor kinase-like kinase (HGK) in the invasion and adhesion of hepatocellular carcinoma (HCC) cell line HepG2.

METHODS: Three paired insert DNA fragments specific to HGK gene and one negative control DNA fragment were synthesized and inserted into RNAi-Ready pSIREN-RetroQ-ZsGreen vector. Western blotting assay and real-time reverse transcriptase polymerase chain reaction (RT-PCR) were used to screen the vector with a highest inhibitory rate. The vector was used to generate recombinant retrovirus specific to HGK. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) assay was used to examine cell growth; wound closure assay and cell adhesion assay were employed to investigate cell migration and adhesion respectively; and transwell assay and three-dimensional culture invasion assay were used to detect cell invasion. The expressions of matrix metalloproteinase (MMP)-2, MMP-9 and nuclear factor (NF)-κB were detected by Western blotting assay.

RESULTS: The real time RT-PCR and Western blotting assay showed that cells transfected with retrovirus mediating RNAi targeting of HGK (RV-shHGK)-1 vector had the strongest inhibition of HGK protein, with an inhibition rate of 76%, and this vector was used to generate recombinant retrovirus RV-shHGK-1. Cell adhesion assay and MTT assay found that cell adhesion and growth of the cells infected with RV-shHGK-1 were significantly lower than those of the control cells ($P < 0.05$). Wound closure assay, transwell assay and three-dimensional culture invasion assay showed that the cell invasiveness was significantly less in HGK knockdown cells than in the control cells ($P < 0.05$). The expressions of MMP-2, MMP-9 and NF-κB were inhibited in HepG2 cells infected with RV-shHGK-1.

CONCLUSION: Down-regulation of HGK can obviously inhibit the migration and invasion of HepG2 cells *in vitro*. HGK may be a new therapeutic target for treatment of HCC.

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Key words: Hepatocellular carcinoma; Hepatocyte progenitor kinase-like kinase; RNA interference; Invasion; Metastasis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most fatal cancers due to delayed diagnosis and lack of effective treatment options[1]. However, little is known of its pathogenesis by the currently available methods[2]. Hepatocyte progenitor kinase-like kinase (HGK) is a member of the mammalian STE20/MAPK family that is widely overexpressed in certain types of human tumors, including pancreatic carcinoma, prostatic carcinoma, lung cancer and HCC[3-6]. Recent studies suggest that HGK plays an important role in cell migration and invasiveness of prostate and breast cancer cells[3,5,6]. However, the role of HGK overexpression in HCC has been scarcely studied. In this study, we constructed a retrovirus vector mediating RNAi targeting HGK (RV-HGK shRNA). The efficacy of RV-HGK shRNA vectors in interference with HGK was confirmed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting assay. We observed that retrovirus mediating RNAi targeting of HGK (RV-shHGK)-1 suppressed invasion by markedly decreasing the expression of HGK, matrix metalloproteinase (MMP)-2, MMP-9 and nuclear factor (NF)-κB in HepG2 cells, but RV-shHGK-C (control) showed no effect in HepG2 cells. Based on these results, HGK may be a new therapeutic target for treatment of HCC[4,6].

MATERIALS AND METHODS

Cells and reagents

HCC cell line HepG2 was purchased from American Type Culture Collection; RNAi-Ready pSIREN-RetroQ-ZsGreen vector, PT-67 retrovirus packaging cells and Retro-XTM qRT-PCR titer kit from Clontech (Clontech Laboratories, Palo Alto, CA); DMEM high glucose medium and fetal calf serum from Hyclone (Hyclone Laboratories, Logan, Utah); cell lysis solution trizol reagent, liposome lipofectamine™ 2000 and Opti-MEM culture medium from Invitrogen Corporation (Carlsbad, CA, USA); RIPA lysis buffer and real-time RT-PCR kit from Takara Company (Takara Suzo, Kyoto, Japan); HGK and MMP-2 polyclonal antibodies from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA); NF-κB and MMP-9 polyclonal antibodies from Cell Signaling Technology (Beverly, MA); HRP-labeled goat anti-rabbit IgG from Wuhan Boster Company; matrigel from the BD (BD Biosciences, San Jose, CA); fibronectin (FN), laminin (LN) and collagen Ⅳ from Sigma (St. Louis, MO); transwell chambers from Corning (Coming Glass Works, Corning, NY, USA); and Western luminescent detection kit [electrochemiluminescence (ECL)] from Pierce Company (Rockford, Illinois, USA). The DNA sequences used in this study were all synthesized in Jiangsu Nantong Bio-Technology Co. Ltd. Other chemicals were obtained in their commercially available highest purity grade.

Generation of recombinant retrovirus

To target the HGK protein for down-regulation by siRNA, we established the RNAi-Ready pSIREN-RetroQ-ZsGreen vector (Clontech) of the RNA interference retrovirus vector specific to HGK gene. The retrovirus vector contains a CMV-driven ZsGreen reporter, which makes it easy to detect the transfection efficiency, and a U6 promoter upstream of the cloning restriction sites (*Bam*H Ⅰ and *Eco*l Ⅰ). Three paired insert DNA fragments according to the coding regions of HGK (NM_145686.2), starting at 393, 985 and 2224, were designed using software offered by Qiagen (Valencia, CA). Three vectors were named RV-shHGK-1, RV-shHGK-2 and RV-shHGK-3, respectively (Table 1). RNAi-Ready pSIREN-RetroQ-ZsGreen control vector was constructed according to the manufacturer's instructions (Clontech), named RV-shHGK-C. All the DNA sequences were synthesized in a pattern as *Bam*H Ⅰ -sense DNA-loop (TTCAAGACG)-antisense DNA-*Eco*l Ⅰ. The most effective vector was selected to produce recombinant retrovirus. Recombinant retrovirus vectors were transfected into PT-67 packaging cells (Clontech) by liposome lipofectamine™ 2000 (Invitrogen, USA). After 48 h, the supernatant containing the viral particles was collected, filtered through the 0.45 μm low protein binding syringe filter and the titer of viruses was determined in 293T cells by real time RT-PCR (Takara).

Recombinant retrovirus infection

HepG2 cells were cultured and plated into 6-well culture plates at 5×10^5 cells/well in DMEM high glucose culture medium with 100 mL/L fetal bovine serum (FBS) at 37℃ in a saturated humidified atmosphere containing 50 mL/L CO_2. After 24 h, the cells were infected with different viral supernatants, respectively, at a multiplicity of infection (MOI) of 4 transducing units/cell for 24 h[7].

Real-time RT-PCR

The total cellular RNA was isolated from the collected cells by Trizol. The HGK mRNA copies were quantified using the real-time RT-PCR Kit (Takara) on a Bio-Rad iQ5 Sequence Detection System (Bio-Rad, Hercules, CA, USA). The primers of HGK and β-actin were as follows: forward primer 5'-GAGCAGTGCTGAAGGCCAAAG-3', reverse primer 5'-ACTAAAGTCCTGTGGCGATGGAA-3' and forward primer 5'-CACCAACTGGGACGACAT-3', reverse primer 5'-ATCTGGGTCATCTTCTCGC-3'. The housekeeping gene β-actin was amplified to normalize the HGK mRNA expression. The copy numbers of β-actin and HGK were determined according to each standard curve. Relative HGK mRNA levels were determined by comparing the PCR cycle thresholds between the cDNA of HGK and that of β-actin.

Western blotting assay

The cellular total proteins were extracted by RIPA Lysis Buffer (Takara) and 50 μg was added to each well for 100 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After semi-dry electric transfer at 45 V for 1 h, polyvinylidine fluoride membrane was sealed with 50 g/L skim milk powder at room temperature (15-25℃) for 6 h, and incubated at 4℃ overnight with rabbit anti-human HGK, NF-κB, MMP-2 and MMP-9 polyclonal antibodies

Table 1 Three vectors specifically interfering with hepatocyte progenitor kinase-like kinase expression and a control vector

Name	Paired DNA fragment
RV-shHGK-1	5'-GGATCCTTACAGACCTTGTGAAGAATTCAAGACGTTCTTCACAAGGTCTGTAATTTTTGAATTC-3'
	5'-GAATTCAAAAATTACAGACCTTGTGAAGAAGGTGTTGAATTCTTCACAAGGTCTGTAAGGATCC-3'
RV-shHGK-2	5'-GGATCCGAAGAAGAGAGGCGAGAAAATTCAAGACGTTTCTCGCCTCTCTTCTTCTTTTTGAATTC-3'
	5'-GAATTCAAAAAGAAGAAGAGAGGCGAGAAACGTCTTGAATTTTCTCGCCTCTCTTCTTCGGATCC-3'
RV-shHGK-3	5'-GGATCCGAGCAATGGTGAAACGGAATTCAAGACGTTCCGTTTCACCATTGCTCTTTTTTGAATTC-3'
	5'-GAATTCAAAAAGAGCAATGGTGAAACGGAACGTCTTGAATTCCGTTTCACCATTGCTCGGATCC-3'
RV-shHGK-C	5'-GGATCCGGCCAGAGGTTGAAAGTGATTCAAGACGTCACTTTCAACCTCTGGCCTTTTTGAATTC-3'
	5'-GAATTCAAAAAGGCCAGAGGTTGAAAGTGACGTCTTGAATCACTTTCAACCTCTGGCCGGATCC-3'

RV-shHGK: Retrovirus mediating RNAi targeting of hepatocyte progenitor kinase-like kinase.

(1:500) and mouse anti-human β-actin (1:1000) monoclonal antibody, respectively. After being washed, it was incubated at 37℃ for 2 h with second-antibody (1:1000) and colored by ECL. It was scanned for the relative value of protein expression in gray scale by Image-Pro plus software 6.0.

Cell viability assays

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) assay in 96-well micro-culture plates. In brief, HepG2 cells (1×10^4/well) were routinely cultured in DMEM with 100 mL/L FBS for 12 h. After infection for 1, 2, 3 and 4 d, 10 μL of 5 g/L MTT (Sigma) was added into each well and incubated for another 4 h. The supernatant was then discarded and 0.1 mL dimethyl sulfoxide was added into each well. After oscillated for 5 min, the absorbance of 96-well culture plate was read by a Bio-Rad 550 Microplate Reader (Hercules, CA) at a wavelength of 490 nm. There were two groups in this study: HepG2 cells infected with RV-shHGK-1 and RV-shHGK-C. Each group had 3 parallel wells to replicate the test 3 times. A L02 cell sample of gradiently diluted parental HepG2 cells was used to draw the standard curve of cell number.

Cell adhesion assay

HepG2 cells (5×10^4/well) infected with RV-shHGK-1, RV-shHGK-C and parental HepG2 cells were suspended and added into 96-well micro-culture plates coated with FN, LN, and collagen Ⅳ (3 μg/well), respectively. After cultured in the incubator at 37℃ for 1 h, the cells were allowed to adhere, and then washed three times with PBS, fixed with 40 g/L formaldehyde, stained with 5 g/L crystal violet in 200 mL/L methanol/water and viewed under microscope. The amount of bound cells was estimated by reading the absorbance at a wavelength of 490 nm[8]. Triplicate determinations were done for each group.

Wound closure assay

The 6-well culture plates were coated with PBS containing 10 g/L collagen Ⅳ. HepG2 cells trypsinized with 2.5 g/L trypsin, were recultured (5×10^5 cells/well) in DMEM high glucose medium containing 100 mL/L FBS for 6 h to get adherent monolayer growth state. The cells in 6-well culture plates were scratched with the tip of 200 μL pipet, and recultured in the incubator at 37℃ for 24 h. At the time points of 0, 12 and 24 h after scratched, four visions of each well were photographed under microscope and observed for the scratch healing.

Transwell assay

The transwell chamber (Corning) containing an 8-μm pore size polycarbonate membrane filter was coated with a matrigel (Sigma) and inserted in a 24-well culture plate. Cells collected after infection for 48 h were adjusted to a density of 1×10^9 cells/L with serum-free DMEM high glucose culture medium. The cell suspension of 200 μL was added into the upper transwell chamber and 500 μL DMEM high glucose medium containing 200 mL/L FBS was added into the lower transwell chamber. After recultured with 50 mL/L CO_2 at 37℃ for 24 h, the transwell chambers were inverted and stained with hematoxylin and eosin. Five fields were randomly selected and the number of trans-membrane cells was counted.

Three-dimensional culture invasion assay

Matrigel 100 μL (Sigma) was dropped onto 8 mm × 8 mm coverslips in a 24-well culture plate. The matrigel was allowed to polymerize for 30 min at room temperature. HepG2 cells were trypsinized and re-suspended in complete medium (DMEM + 100 mL/L FBS) at 2.5×10^7 cells/L, and 500 μL of the cell suspension was dropped onto the matrigel to analyze the ability of cell invasion. After 24 h incubation at 37℃, cells were observed under inverted microscope[5,9].

Statistical analysis

SPSS 13.0 software was used for statistical analysis, and *t* test was used in the comparison between two groups. One-way analysis of variance was used for multiple comparisons. There was statistical significance when *P* value was less than 0.05.

RESULTS

Retrovirus mediated knockdown of HGK by RNA interference

Three plasmids containing shHGK (1-3) and plasmid shHGK-C were transfected into HepG2 cells, respectively. The expression of reporter ZsGreen in HepG2 cells was observed under a fluorescent microscope 48 h after

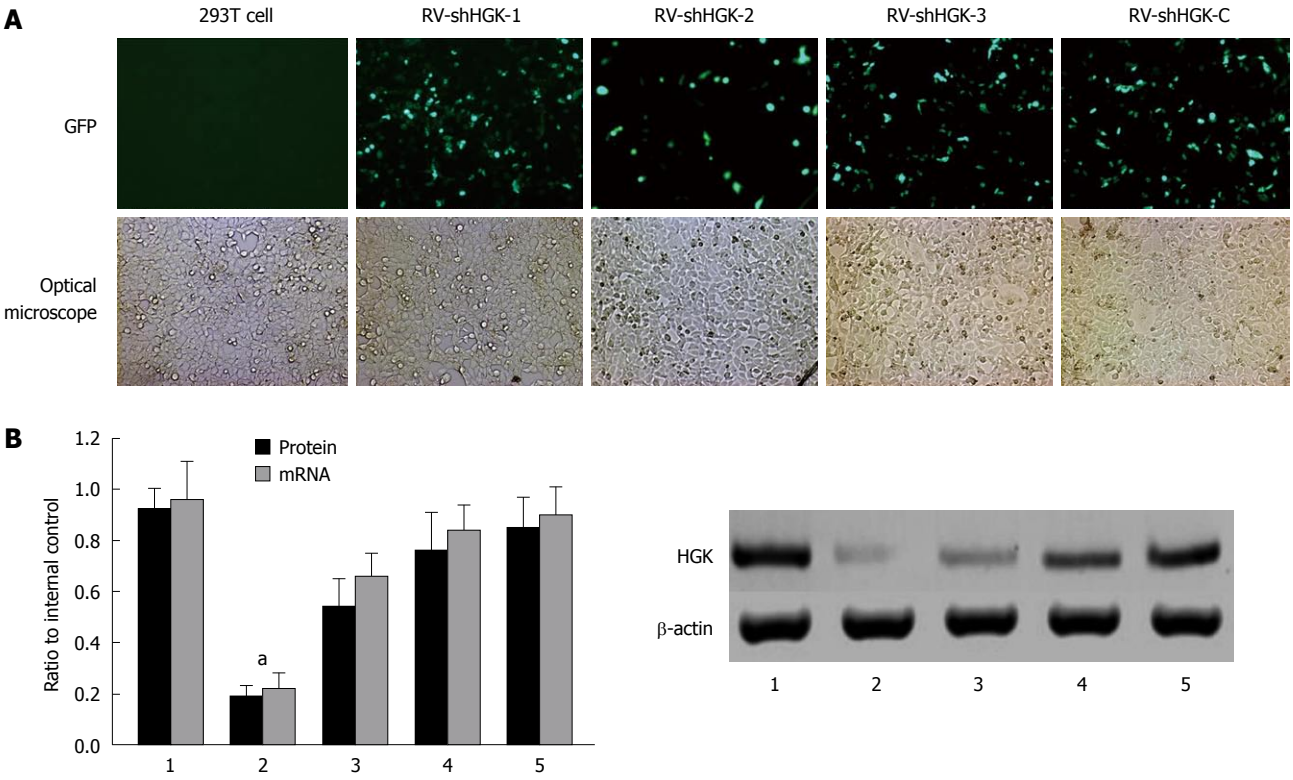

Figure 1 Selection of the most effective hepatocyte progenitor kinase-like kinase specific shRNA expression vector in 293T cells. A: Phase contrast and GFP expression under a fluorescent microscope after 48 h in 293T cells; B: Protein mRNA and hepatocyte progenitor kinase-like kinase (HGK) levels after HepG2 cells were treated with different vectors detected by real-time reverse transcriptase polymerase chain reaction and Western boltting assay. The vector retrovirus mediating RNAi targeting of HGK (RV-shHGK)-1 significantly inhibited HGK expression in HepG2 cells, $^{a}P < 0.05$ *vs* HepG2 cells and HepG2 cells transfected with RV-shHGK-C vector. 1: 293T cells; 2: Transfection of RV-shHGK-1 vector in 293T cells; 3: Transfection of RV-shHGK-2 vector; 4: Transfection of RV-shHGK-3 vector; 5: Transfection of RV-shHGK-C vector (original magnification × 200).

transfection with plasmids containing shHGK (1-3) and shHGK-C (Figure 1A). To evaluate the interfering effects of vectors in the expression of HGK, real-time quantitative RT-PCR and Western-blotting were performed. The relative ratios of HGK mRNA and protein of the HepG2 cells transfected with vectors were analyzed (Figure 1B). The results showed that vector shHGK-1 could significantly suppress the expression of HGK. It was the most effective RNA interference vector. In the following experiment, vectors shHGK-1 and shHGK-C were used to produce retrovirus RV-shHGK-1 and RV-shHGK-C, the virus titers were approximately 1×10^{11} v.p./L. To evaluate the effect of RV-shHGK-1 in the expression of HGK, real-time quantitative RT-PCR and Western-blotting were performed to determine the mRNA and protein level in HepG2 cells infected by RV-shHGK-1 at an indicated MOI (MOI = 4). The HGK expression was significantly suppressed in cells infected with RV-shHGK-1 compared with RV-shHGK-C ($P < 0.05$, Figure 2A and B), showing the markedly inhibitory effect of RV-shHGK-1 system on HGK expression in HepG2 cells.

Down-regulation of HGK inhibits HepG2 cell growth

To validate the HGK functions in cell growth regulation, cell proliferation was monitored for 4 d after HepG2 cells were infected with RV-shHGK-1 and RV-shHGK-C. At day 4, the growth of HepG2 cells was reduced to 45% (Figure 3A), indicating that the suppression of HGK expression apparently reduces the growth of HepG2 cells.

Down-regulation of HGK inhibits HepG2 cell adhesion to extracellular matrix proteins

To verify the effects of HGK expression in adhesion to extracellular matrix (ECM) proteins in HCC, HepG2 cells infected with RV-shHGK-1, RV-shHGK-C and parental HepG2 cells were examined by cell adhesion assay. As shown in Figure 3B, down-regulation of HGK could inhibit HepG2 cell adhesion to FN, LN and collagen Ⅳ. The results indicated that HGK participated in cell adhesion.

Down-regulation of HGK inhibits HepG2 cell invasion

In order to confirm whether HGK is involved in the process of HepG2 cell motility and invasiveness, the wound closure assay and transwell assay were used to determine the impact of RV-shHGK-1 on HepG2 cell invasion. Cells infected with RV-shHGK-1 migrated more tardily and filled in the wound more slowly than RV-shHGK-C infected cells ($P < 0.05$, Figure 4A). Consistent with the data of wound closure assay, the results of transwell assay showed that the invasiveness through matrigel was significantly decreased in RV-shHGK-1 infected HepG2 cells, compared with that in RV-shHGK-C infected cells ($P < 0.05$, Figure 4B).

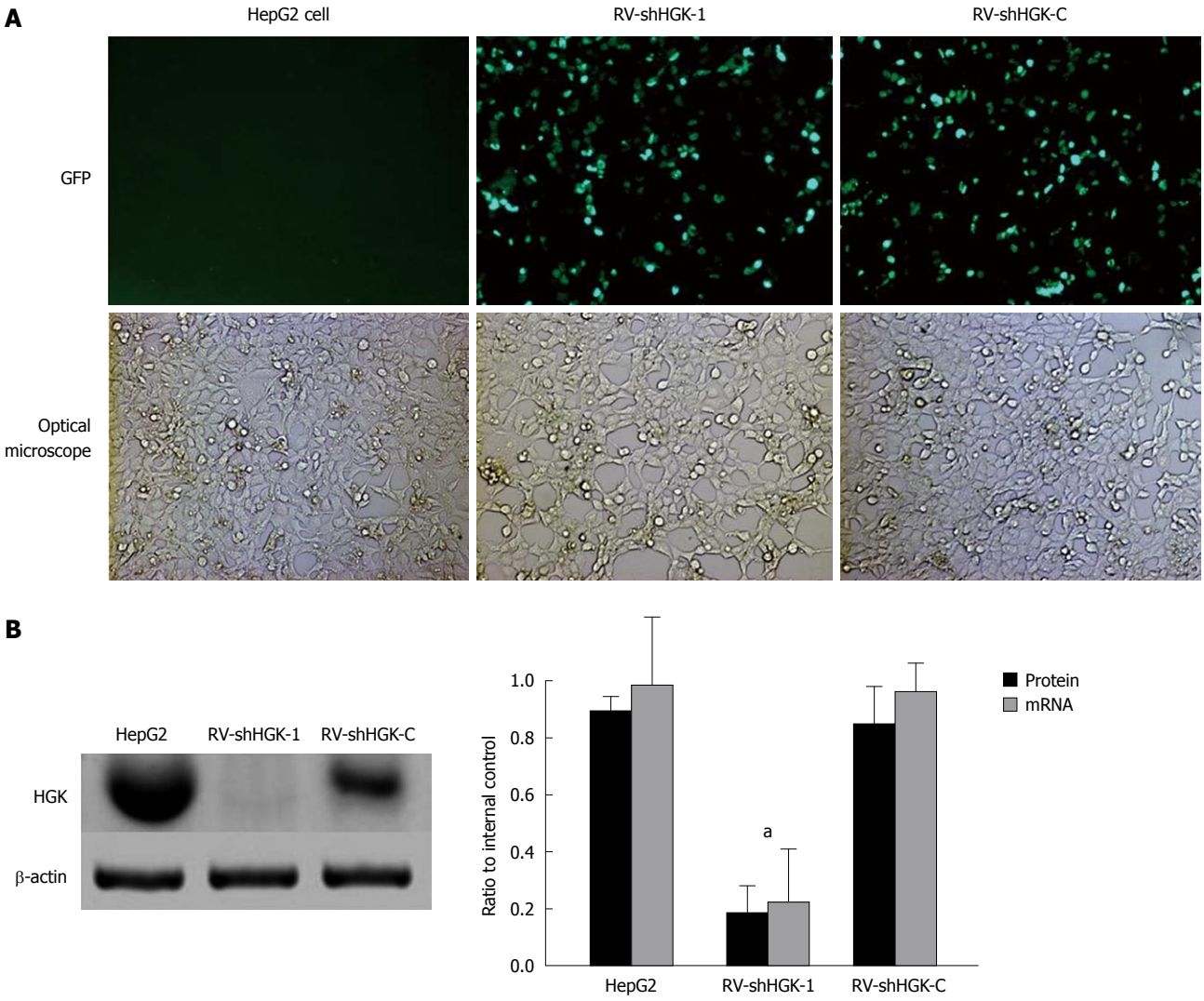

Figure 2 Hepatocyte progenitor kinase-like kinase expression suppressed by RV-shHGK-1 retrovirus in HepG2 cells. A: HepG2 cells infected with retrovirus mediating RNAi targeting of hepatocyte progenitor kinase-like kinase (RV-shHGK)-1 or RV-shHGK-C (multiplicity of infection = 4), GFP expression and the phase contrast images after 48 h (original magnification × 200); B: Protein and mRNA levels of hepatocyte progenitor kinase-like kinase (HGK) after HepG2 cells were treated with different retrovirus detected by real-time reverse transcriptase polymerase chain reaction and Western boltting assay. The retrovirus RV-shHGK-1 significantly inhibited HGK expression in HepG2 cells, $^{a}P < 0.05$ *vs* HepG2 cells and HepG2 cells infected with RV-shHGK-C retrovirus.

The effects of HGK on tumor metastasis and invasion in HCC were further examined in HepG2 cells under three-dimensional cell culture. As shown in Figure 4C, the structure patterned network of interconnected loops was not found in RV-shHGK-1 infected HepG2 cells under three-dimensional cell culture, but present in RV-shHGK-C infected cells. These results indicated that RV-shHGK-1 system could dramatically suppress the aggressive ability of HepG2 cells.

Down-regulation of HGK inhibits MMP-2, MMP-9 and NF-κB expression in HepG2 cells

Because MMPs and NF-κB are known to play important roles in HCC cell invasion and metastasis, and down-regulation of HGK can inhibit HepG2 cell invasion ability, Western blotting assay was performed to investigate whether the down-regulation of HGK in HepG2 cells affects MMPs and NF-κB expression levels. The results showed that the expressions of MMP-2, MMP-9 and NF-κB were inhibited in HepG2 cells infected with RV-shHGK-1 compared with that in control cells (Figure 5A and B).

DISCUSSION

Mitogen-activated protein (MAP) kinases are cellular regulators that play significant roles in various diverse processes as apoptosis, differentiation, and proliferation[10-13]. Their activation can be mediated by many upstream kinases that regulate the downstream MAP kinases[14]. Sterile 20 kinases were identified in the past few years. One of the protein kinase families can act upstream of MAP kinases[15]. These kinases can be divided into two structural classes, the p21-activated protein kinases and the germinal center protein kinases[16,17]. HGK is a member of the germinal center protein kinases. More recently, increasing data have shown that HGK can control cel-

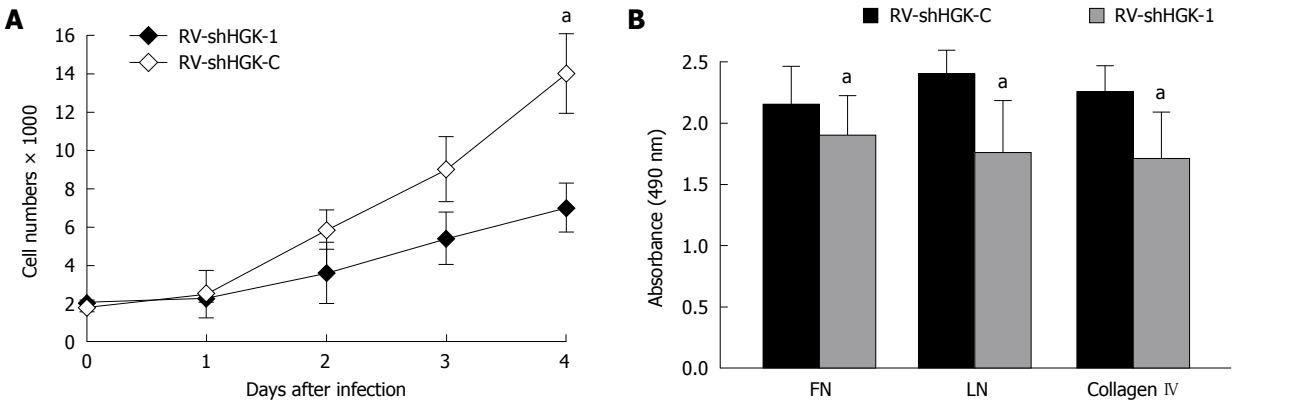

Figure 3 Down-regulation of hepatocyte progenitor kinase-like kinase inhibits HepG2 cell growth and adhesion (methyl thiazolyl tetrazolium assay). A: HepG2 cell growth was significantly suppressed by retrovirus mediating RNAi targeting of hepatocyte progenitor kinase-like kinase (RV-shHGK)-1 *vs* RV-shHGK-C group; B: RV-shHGK-1 inhibited HepG2 adhesion to fibronectin (FN), laminin (LN) and collagen Ⅳ. $^{a}P < 0.05$ *vs* control.

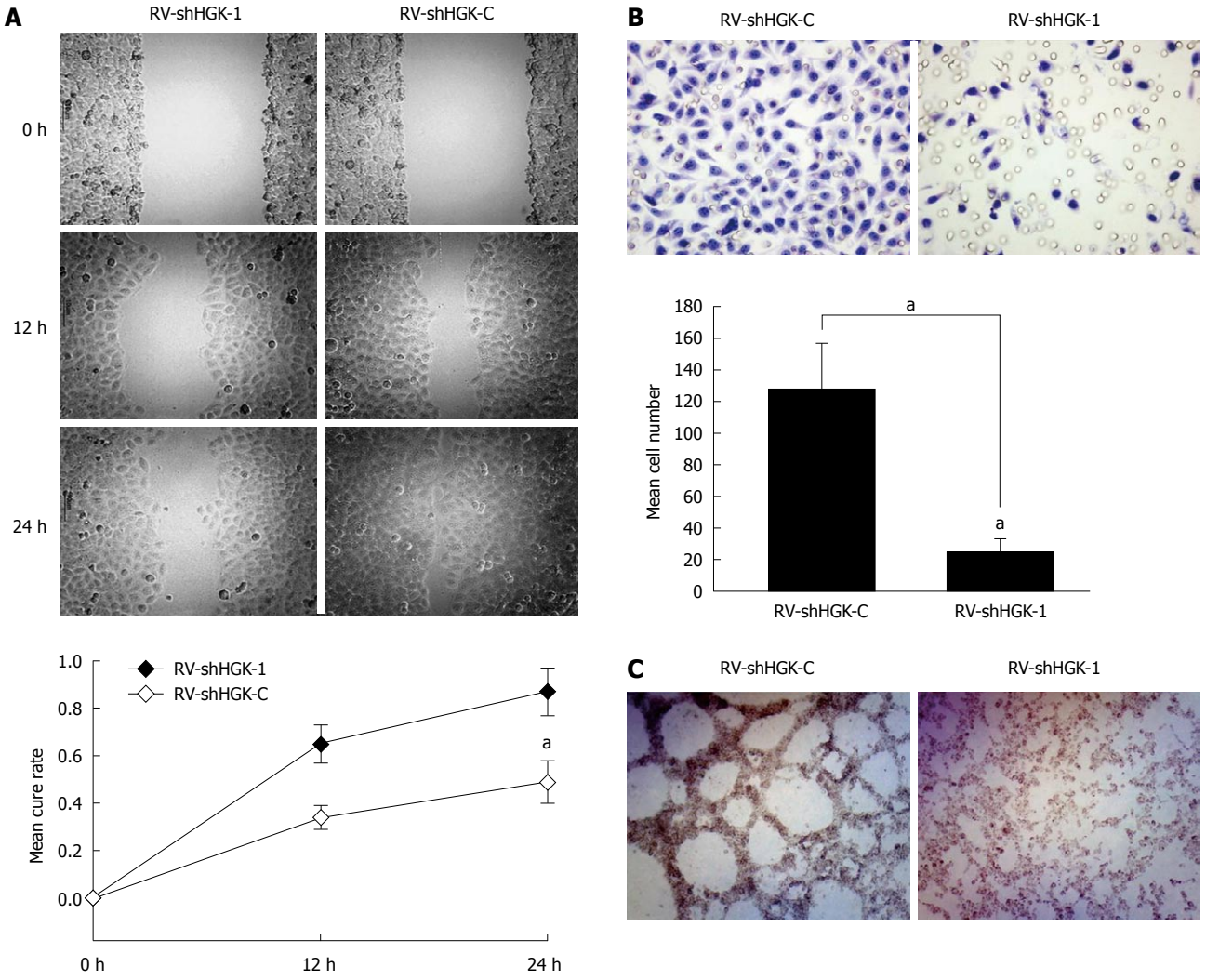

Figure 4 Down-regulation of hepatocyte progenitor kinase-like kinase inhibits HepG2 cell invasion. A: HepG2 cell invasiveness was significantly suppressed by retrovirus mediating RNAi targeting of hepatocyte progenitor kinase-like kinase (RV-shHGK)-1 *vs* RV-shHGK-C group (original magnification × 200, $^{a}P < 0.05$); B: The blue-stained cells are those invading the ECMatrix and migrating through the polycarbonate membrane to the lower surface of the membrane (original magnification × 200, $^{a}P < 0.05$ *vs* HepG2 cells infected with RV-shHGK-C); C: HepG2 cells infected with RV-shHGK-1 do not form the structure patterned network of interconnected loops (original magnification × 200).

lular events ranging from cell motility, cell adhesion and invasion in some cancer cells, including breast cancer, lung cancer, pancreatic cancer and colon carcinoma[5,6,18]. Thus, the potentiality of regulating cancer cell invasion

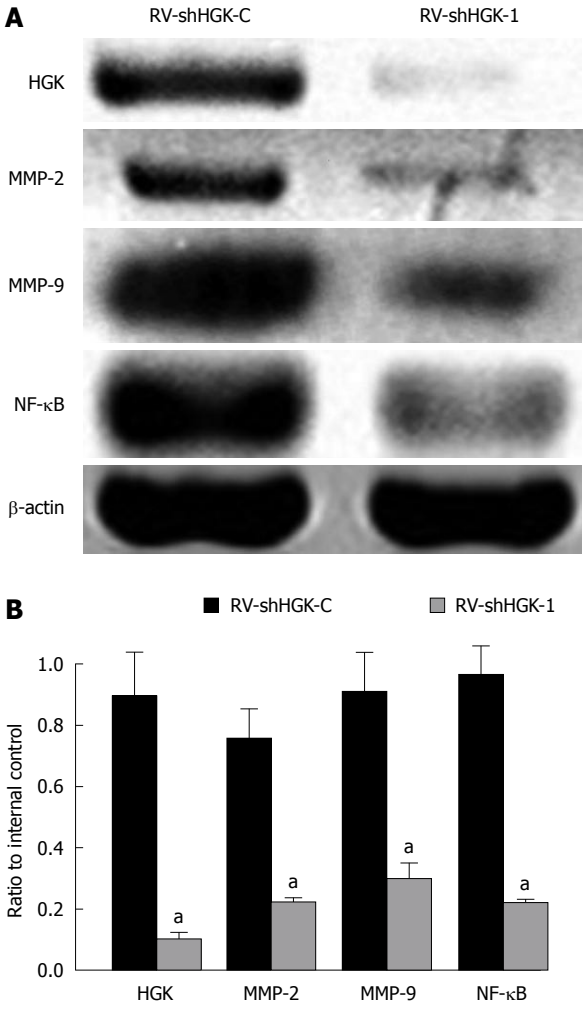

Figure 5 Hepatocyte progenitor kinase-like kinase, matrix metalloproteinase-2, matrix metalloproteinase-9 and nuclear factor-κB expressions in HepG2 cells (Western blotting). A: Retrovirus mediating RNAi targeting of hepatocyte progenitor kinase-like kinase (RV-shHGK)-1 significantly inhibited matrix metalloproteinase (MMP)-2, MMP-9 and nuclear factor (NF)-κB in HepG2 cells. MMP-2 protein expression in each group (Western blotting); B: Densitometric analysis hepatocyte progenitor kinase-like kinase (HGK), MMP-2, MMP-9 and NF-κB protein expression in HepG2 cells infected with RV-shHGK-1 or RV-shHGK-C, respectively. $^{a}P < 0.05$ *vs* HepG2 cells infected with RV-shHGK-C.

and metastasis is to control HGK expression. However, the effect of HGK in HCC is unclear.

To evaluate the functional role of HGK in HCC, knockdown experiments were performed in HepG2 cells. Recently, RNAi has been widely used to silence the expression of many targets. In this study, three RNA interference retrovirus vectors targeting HGK were designed and synthesized, and the most effective vector was selected to generate the recombinant retrovirus named RV-shHGK-1. Meanwhile, the control retrovirus was produced and named RV-shHGK-C. Because the inhibitory effect of RNAi is related to the specificity to its target sequence, real-time RT-PCR and Western blotting assay were used to confirm the effect of RV-shHGK-1 in HepG2 cells. The results showed that the expressions of HGK mRNA and protein were visibly blocked, indicating that RV-shHGK-1 can down-regulate the expression of HGK effectively.

Metastasis is known to be the biggest problem to oncologists and the main cause of death in cancer patients[19]. The metastatic process involves a series of interdependent events, including cancer cell growth, invasion and adhesion[20]. In this study, the effects of RV-shHGK-1 on HepG2 cell growth and adhesion were investigated by MTT assay and cell adhesion assay. The results indicated that the growth and the adhesion of HepG2 cells were inhibited by RV-shHGK-1. Notably, the down-regulation of cell adhesion and the disassembly of basement membrane (BM) are the key elements of cell metastasis[21,22]. FN, LN and collagen Ⅳ are known to be the major components of BM[23]. In cell adhesion assay, the adhering ability to BM of HepG2 cells infected with RV-shHGK-1 was inhibited compared with HepG2 cells infected with RV-shHGK-C. The results revealed that HGK may correlate with cell adhesion. In addition, the ability of cell invasion was examined by transwell assay and three-dimensional invasion assay. The results showed that down-regulation of HGK could inhibit the invasiveness of HepG2 cells. To further investigate the involvement of HGK in cell invasion, whether the down-regulation of HGK in HepG2 cells affects MMPs and NF-κB expression level was also observed by Western blotting assay. MMPs and NF-κB are considered to be important in cancer cell invasion by degrading components of the BMs and ECM[24,25]. The effect of HGK in regulation of cell adhesion and invasion may be related to regulation of the HGK expression.

Overall, these observations are in agreement with recent reports that examined the effects of HGK in other cancer cells[5,26]. These findings have laid a foundation for further investigation into the manipulation of HGK in the treatment of HCC.

COMMENTS

Background

Hepatocyte progenitor kinase-like kinase (HGK) is a member of the mammalian STE20/MAPK family that is overexpressed in human hepatocellular carcinoma (HCC) compared with normal liver tissues.

Research frontiers

Recent studies suggest that HGK plays an important role in cell migration and invasiveness of prostate and breast cancer cells. However, the role of HGK overexpression in HCC has been scarcely studied. To explore the role of HGK in HCC, the authors investigated the effects of HGK through RNA interference mediated by retrovirus.

Innovations and breakthroughs

The effects of HGK in regulation of cell adhesion and invasion may be related to regulation of the HGK expression. HGK may be a new therapeutic target for treatment of HCC.

Applications

The down-regulation of the HGK expression appears to inhibit the invasiveness and HCC cell migration. HGK may be an important factor promoting HCC progression. Therefore, it can be used as a new therapeutic target for treatment of HCC.

Terminology

HGK is a member of the germinal center protein kinases, which has been found to control cellular events ranging from cell motility, cell adhesion and invasion in some cancer cells, including breast cancer, lung cancer, pancreatic cancer and colon carcinoma.

Peer review

The authors have knocked-down the expression of HGK in HepG2 using RNAi

and investigated the effect on cell proliferation, migration and invasion *in vitro*. The results have shown that decreasing the expression of HGK inhibited cellular proliferation, invasiveness and migratory capability using different assay systems. Expression of matrix metalloproteinase (MMP)-2, MMP-9 and nuclear factor-κB were decreased with HGK knocked-down. All the experiments were well conducted and controlled. The conclusions made are valid.

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ORIGINAL ARTICLE

Infantile hepatic hemangioendothelioma: A clinicopathologic study in a Chinese population

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Abstract

AIM: To investigate whether the clinicopathologic features of infantile hemangioendothelioma (IHE) of the liver in a Chinese population are similar to the features observed in other races.

METHODS: The clinical data, radiological findings, histopathological changes and outcome of 12 cases of IHE diagnosed by the Department of Pathology, West China Hospital over the last 10 years were analyzed retrospectively. Immunohistochemical studies were carried out using antibodies against CD31, CD34, Factor Ⅷ, cytokeratin 8 and cytokeratin 18.

RESULTS: The 12 patients were aged from fetal to 5 years (three males and nine females). The tumor was presented with different clinical manifestations, mainly as an asymptomatic, palpable, upper abdominal mass, except for the two fetuses who were detected antenatally by ultrasound. In one patient, this presentation was accompanied by an initial severe pneumothorax. No symptoms of congestive heart failure were present and neither congenital abnormalities nor vascular tumors in the skin or other organs were found. Laboratory abnormalities included leukocytosis (40%), anemia (60%), thrombocytosis (60%), hyperbilirubinemia (16.7%), abnormal liver function (50%) and increased α-fetoprotein (80%). Based on radiological findings and gross specimens, the tumor presented as a solitary lesion or a multifocal space-occupying lesion. The tumor size ranged from 5.0 cm × 3.5 cm × 2.0 cm to 13.8 cm × 9.0 cm × 7.7 cm, and the 0.2-1.1 cm nodules were diffusely distributed within the multifocal tumor. Seven cases were surgically resected, three cases underwent biopsy and the two fetuses were aborted. Histologically, nine cases were classified as type Ⅰ and three as type Ⅱ, presenting aggressive morphologic features, immature vessels, active mitosis and necrosis. An inflammatory component, predominantly eosinophilic granulocytes, sometimes obscured the nature of the tumor. Ten patients are alive after a follow-up of 1-9 years. Based on immunohistochemistry, the endothelial cells in all cases were positive for CD31, CD34 and polyclonal factor Ⅷ antigen, whereas the scattered hyperplasia bile ducts were positive for cytokeratin 8 and cytokeratin 18.

CONCLUSION: The clinical manifestations of IHE are non-specific. There is no significant correlation between histological type and prognosis. The clinicopathologic features of IHE in Chinese patients may provide a clue to further evidence-based studies.

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Key words: Infantile haemangioendothelioma; Pediatrics; Hepatic neoplasm; Pathological diagnoses; Chinese

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INTRODUCTION

Infantile hemangioendothelioma (IHE) of the liver is a rare mesenchymal tumor, but it is the most common benign vascular tumor of the liver in infancy. IHE has been described as a "tumor malformation" when it occurs in the uterus, and patients usually manifest symptoms or syndromes prior to the first year of life. Kunstadter *et al*[1] was the first to review and describe IHE in 1933. Since then, a series of cases have been reported in the literature. Previously, IHE was known as "multinodular hemangiomatosis of the liver"[2]. To date, most cases reported in the literature are IHEs mainly in the Caucasians. However, diseases affecting individuals of the Mongoloid race often present with unique characteristics. The purpose of this study was to examine the clinical and pathologic features of IHE in Chinese patients and to correlate these features with the treatment and prognosis. Some reports have suggested that a few IHEs may be metastatic and undergo malignant degeneration into angiosarcoma[3], while most of the cases undergo involution and regression. These findings make the treatment controversial. In addition, we have reviewed adequately documented reports of similar tumors from the literature and compared them with our findings. This is the first and largest population of patients examined from a single institution in China in a period of 10 years.

MATERIALS AND METHODS

The records of 12 infants and children (including two aborted fetuses) with IHE of the liver seen at West China Hospital over the last 10 years between 2000 and 2010 were retrieved from the files of the Department of Pathology. West China Hospital is a Medical Center with the largest number of beds in China. The clinical data, laboratory examinations, radiographic findings, surgical findings, treatment, histopathological changes and outcome of these cases were retroactively reviewed. The surgical specimens from partial hepatectomies and biopsies were taken for histopathological evaluation and the morphological characteristics were reviewed according to the standard of diagnosis described by Dehner *et al*[4]. Follow-up information was available for all patients. Immunohistochemistry (IHC) using antibodies against CD31, CD34, Factor Ⅷ, cytokeratin 8 and cytokeratin 18 was performed on paraffin-embedded tissue specimens. IHC was performed using an Envision kit on a Dako automatic stainer (Dako, Carpinteria, CA). Positive (internal) controls were included. Sequential tissue sections treated with sera from the same species as the primary antibody were used as negative controls (Table 1).

Table 1 Immunostains used in the study

Antibody	Clone/PAD	Company and Cat. No.	Staining pattern	Dilution used
CD31	JC70A	Dako, M0823	Membrane	1:50
CD34	QBEnd/10	Zymed, ZM-0046	Membrane	1:25
Factor Ⅷ	Polyclonal serum	Zymed, ZA0111	Cytoplasmic	1:50
CK8	C51	Zymed, ZM-0310	Cytoplasmic	1:100
CK18	DC-10	Zymed, ZM-0073	Cytoplasmic	1:100

RESULTS

Clinical data

The clinical features of the IHE are listed in Table 2. The age of the 12 patients ranged from fetal to 5 years. The patients were referred to our hospital for different clinical manifestations. Ten (83.33%) were seen at diagnosis, prior to 6 mo of age, with a mean age of 10 mo at presentation. There were three males and nine females, with a ratio of 1:3. Eight had asymptomatic palpable abdominal masses detected during a routine physical examination. Additional symptoms included abdominal distention and pain caused by a large tumor, jaundice, dyspnea, neonatal pneumonia and fever. A 5-year-old girl presented with repeated abdominal pain combined with a pneumothorax for 2 mo. No congenital defect was observed and no vascular tumor was found in the skin or other organs. No symptoms of congestive heart failure (CHF) were present in any of the patients. The primary physical finding was an upper abdominal mass or hepatomegaly, with some extending across the midline, with a smooth surface and sharp edges. No family history was reported in any case. No proof of maternal exposure to dangerous environmental factors during pregnancy was ascertained. The fetuses with IHE had no chorioangioma or intrauterine growth retardation detected antenatally using ultrasound.

Laboratory examination

Laboratory data, including hemograms and liver function tests were available for all patients, except for the aborted fetuses. Leukocytosis, anemia and thrombocytosis were found. One of six cases had hyperbilirubinemia and displayed jaundice. An elevated aspartate aminotransferase level was seen in five cases. The serum α-fetoprotein (AFP) concentration was abnormal in four cases. The serum hepatitis virus markers and blood coagulation system were normal in all patients. The laboratory examinations are summarized in Table 2.

Table 2 Clinical presentations

Case	Sex/age	Presenting features	Solitary or multicentric lesion; site/size (cm)	Leuko cytosis	Anemia	Thrombo cytosis	Hyperbiliru binemia	Abnormal liver function	Increased AFP	Histological type	Treatment	Follow-up (yr)
1	M/2 mo	AM	S; LL; 9.0 × 9.0 × 7.5	No	Yes	Yes	No	No	NA	I	CR	Alive, 9
2	F/17 d	Abdominal distention	S; LL and RL; 11.0 × 8.0 × 6.5	No	No	No	Yes	Yes	NA	I	BO	Alive, 6.75
3	F/2 mo	AM, neonatal pneumonia	S; LL; 5.0 × 3.5 × 2.0	Yes	No	Yes	NA	Yes	Yes	I	CR	Alive, 6.25
4	F/6 mo	AM	S; LL; 11.0 × 10.0 × 8.0	No	Yes	Yes	NA	No	NA	I	CR	Alive, 5
5	F/5 yr	Abdominal pain, dyspnea	M; LL and RL; Oblique diameter of right hepatic: 9.7	Yes	No	Yes	No	Yes	NA	II	BO	Alive, 4
6	M/5 mo	AM	S; LL; 10.5 × 7.5 × 7.0	Yes	Yes	Yes	NA	No	NA	II	CR	Alive, 3.75
7	F/6 mo	AM	S; RL; 10.0 × 10.0 × 8. 0	No	Yes	No	No	No	Yes	I	CR	Alive, 3.5
8	F/3 yr	Jaundice, fever	S; LL, RL and CL; 13.8 × 9.0 × 7.7	Yes	Yes	No	NA	Yes	No	II	CR	Alive, 2.25
9	F/5 mo	AM	S; RL; 6.2 × 6.0 × 3.5	No	No	No	No	No	Yes	I	CR	Alive, 1.5
10	F/2 mo	AM	S; CL; 6.0 × 5.0 × 4.0	No	Yes	Yes	No	Yes	Yes	I	BO	Alive, 0.25
11	F/AF	AM	S; RL; 8.2 × 6.5 × 7.8	NA	NA	NA	NA	NA	NA	I	Aborted	
12	M/AF	AM	S; LL; 7.6 × 7.4 × 7.2	NA	NA	NA	NA	NA	NA	I	Aborted	

AF: Aborted fetuses; AM: Abdominal mass; S: Solitary lesion; M: Multicentric lesion; LL: Left lobe; RL: Right lobe; CL: Caudate lobe; NA: Not available; CR: Complete resection; BO: Biopsy only; AFP: α-fetoprotein.

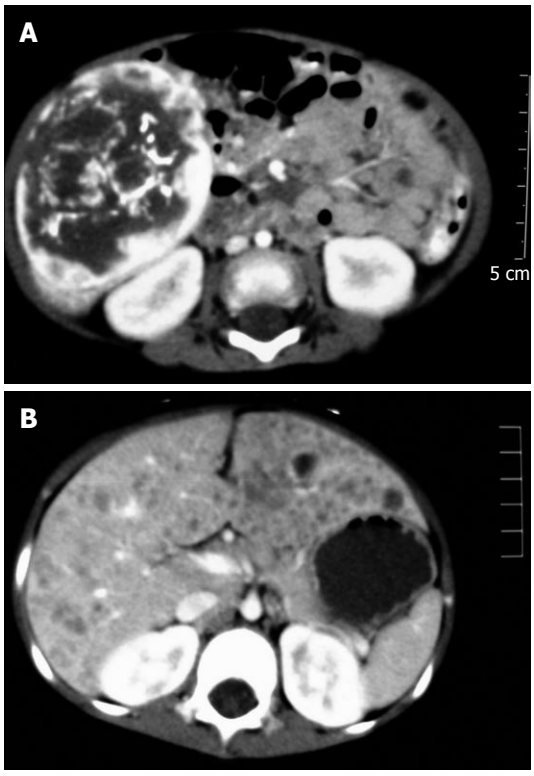

Figure 1 Contrast-enhanced arterial phase computed tomography. A: Solitary hypoattenuated mass with a well-defined contour containing calcifications in a fine-speckled pattern (case 7); B: Diffuse polycystic changes with variable sizes and an ill-defined region in both hepatic lobes (case 5).

Radiographic findings

Abdominal ultrasonography, computed tomography (CT), contrast-enhanced arterial phase CT and magnetic resonance imaging scans demonstrated different features in the solitary lesions and multiple lesions (Figure 1). Local calcification was prevalent in this tumor and presented as a fine-speckled pattern. In the thoracic CT scans, the pneumothorax (case 5) appeared as lung consolidation, the left lung was compressed by air, and the mediastinum moved to the right.

Surgical findings and treatment

Nine cases presented with a solitary lesion in one lobe. The lesion was restricted to the right or left lobe of liver in five and three patients, respectively; one case had the tumor in the caudate lobe. Case 2 and case 8 had a huge mass arising from the right lobe that extended to the left and caudate lobe. The size of solitary lesions ranged from 5.0 cm × 3.5 cm × 2.0 cm to 13.8 cm × 9.0 cm × 7.7 cm and the lesion presented as a large, well-circumscribed, red-brown tumor, with a smooth and glittery appearance and a capsule containing abundant blood vessels of different sizes. On the cut surface, the tumor was more or less homogeneous, red-brown and partially ill-circumscribed (Figure 2). Sometimes the tumor had partially stranded vessels and a central fibrosis or infarction. The lesion in case 5 was diffuse and multifocal, and both lobes were involved. Extensive multiple masses in the swelling liver made the surface

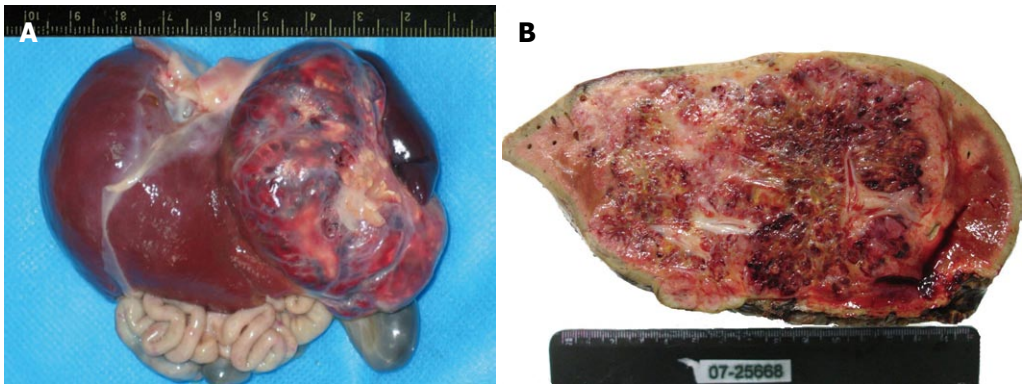

Figure 2 Appearance of a solitary mass. A: A solitary mass in the liver from an autopsy case (case 12); B: Cut surface of a solitary mass in liver demonstrating homogeneous, red-brown and partially ill-circumscribed tumor (case 8).

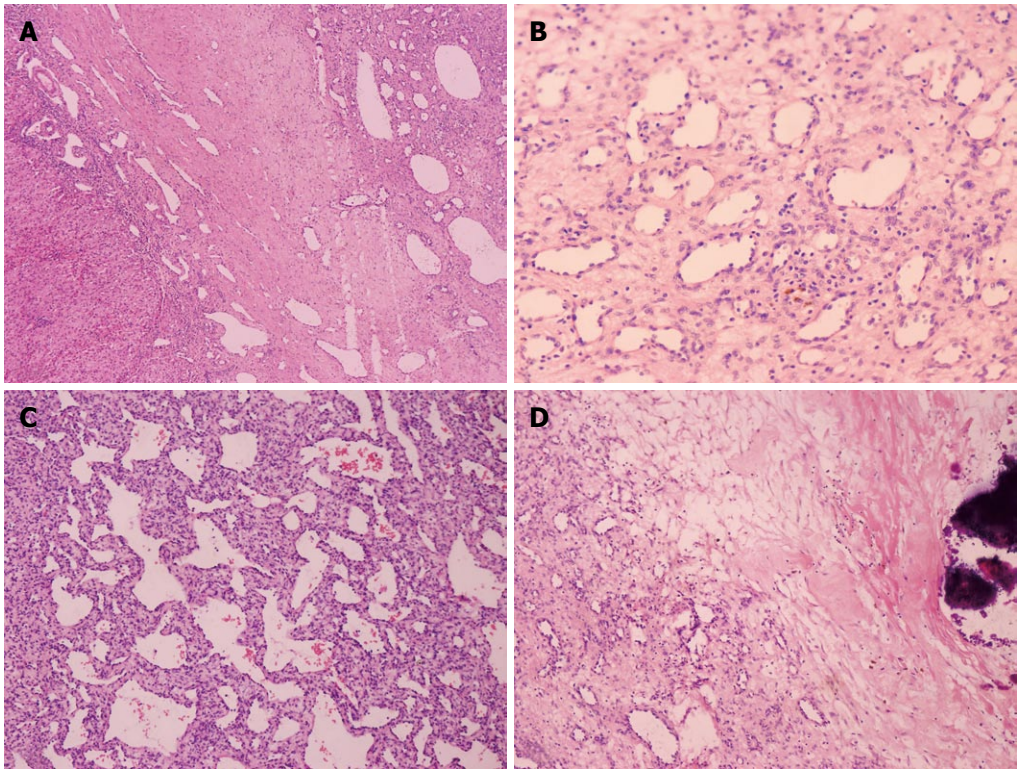

Figure 3 Histopathological features of type Ⅰ lesions. A: Mature vascular channels (case 7, HE, × 60); B: Plump endothelial cells with a bland cytological appearance (case 7, HE, × 200); C: Cavernous structures (case 7, HE, × 100); D: Calcification foci (case 7, HE, × 100).

irregular, and the nodules were too numerous to count. The maximum diameter of the nodules was 1.1 cm. On the cut surface, the liver was replaced by diffuse multiple, solid or cystic, reddish-gray masses partially surrounded by a thin rim but without evidence of hemorrhage.

All patients underwent surgical interventions. The tumor was completely removed surgically in seven patients, whereas segmental resection or left/right lobectomy was performed according to the size and location of the tumor. Three exploratory laparotomies with liver biopsies were carried out for the patients with multifocal and solitary lesions, but subsequent therapies were not accepted by their parents. In our series, the two aborted fetuses were delivered following induced labor.

Histopathological features

Because of the broad variety of histological features, the growth pattern of the tumors was subdivided into two subtypes: type Ⅰ and type Ⅱ. The nine cases of type Ⅰ consisted of vascular channels with some variations in structure and a single layer of endothelial cells without polymorphisms. Dilated capillaries were interspersed among loose, edematous, myxoid connective tissues. Disorganized elongated branching bile ducts and scanty cavernous structures were also observed in the lesion. A lesion with infiltrating inflammatory cells mimicked granulation tissues. Infarction, extramedullary hematopoiesis and foci of calcification were more prominent in the type Ⅰ cases (Figure 3). No bile plug or thrombus hemorrhage was observed.

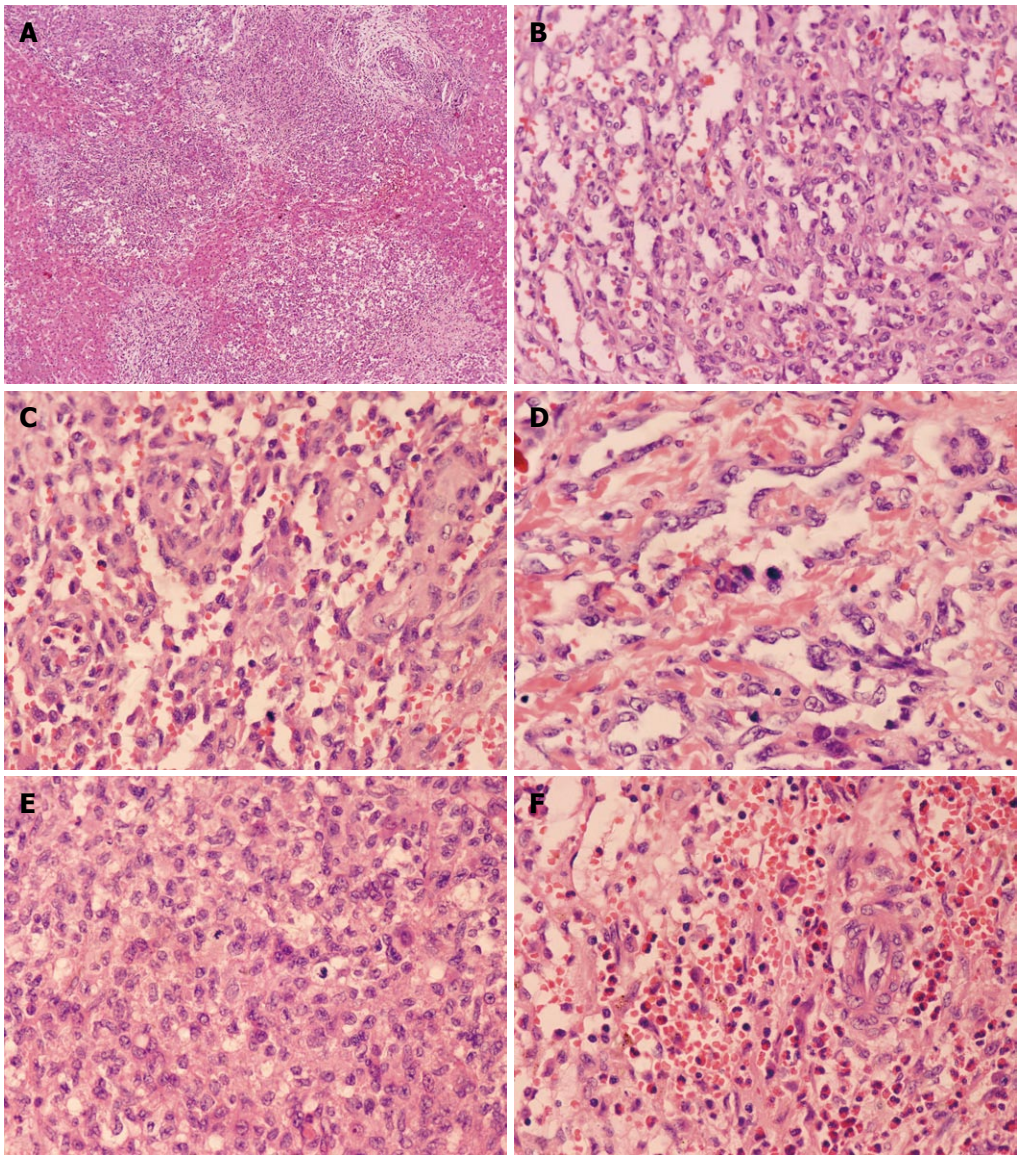

Figure 4 Histopathological features of type Ⅱ lesions. A: Multifocal nodule without encapsulation. Tumor nodules intermixed with the hepatic plate (case 5, HE, × 60); B: Anastomosing vascular structures (case 8, HE, × 300); C: A papillary structure with atypical endothelial cells (case 5, HE, × 400); D: Hyperchromatic and pleomorphic endothelial cells with mitotic cells (case 8, HE, × 400); E: Activated mitotic cells in a dense area (case 8, HE, × 400); F: Eosinophilic granulocytes were the predominant inflammatory component in one case (case 5, HE, × 400).

Three cases were classified as type Ⅱ. Although two cases appeared as a solitary lesion, based on their histological morphology, they were classified as type Ⅱ. The remaining case was a multifocal lesion that diffusely spread throughout the hepatic parenchyma. The multifocal and sinusoidal parts in these lesions were more prominent than in type Ⅰ. At the periphery, the nodules were not encapsulated. The tumor nodules intermixed with the hepatic plate, and the normal hepatic parenchymal cells surrounding the nodules were compressed and infiltrated by projections of the tumor. The vascular spaces were twisting with irregular budding, branching and anastomosing structures. The endothelial cells were plump and more hyperchromatic and pleomorphic than those in type Ⅰ cases. Mitosis in the endothelial cells demonstrated a high rate of proliferation. Most of the bile ducts were located in the central region of the nodules. The presence of eosinophilic granulocytes as the predominant inflammatory component in case 5 resulted in an initial misdiagnosis as a parasite infection (Figure 4).

IHC

IHC revealed that the dilated vessels were vascular spaces. The endothelial nature of the cells surrounding the vascular spaces was supported by the expression of the vascular markers CD34 (Figure 5), CD31 and Factor Ⅷ. Cytokeratin 8 and cytokeratin 18 staining verified the presence of scattered bile ducts.

Outcome

All of our patients, except for the aborted fetuses, are alive and have presented with no symptoms suggestive

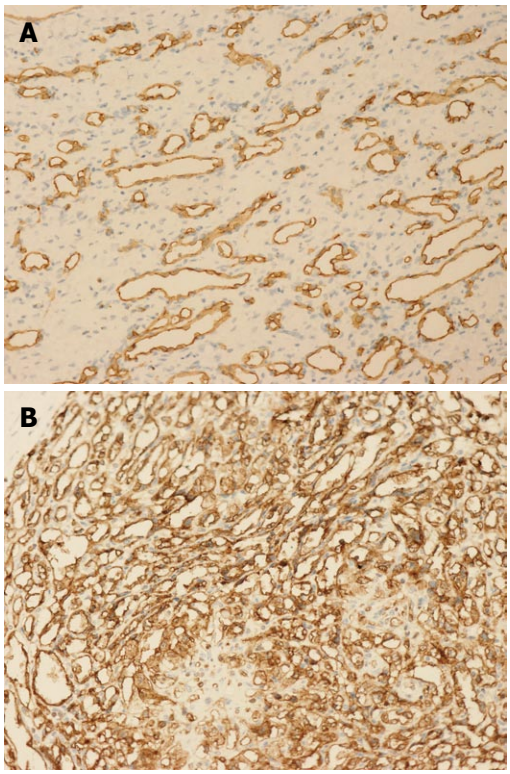

Figure 5 Immunohistochemical results. A: Expression of CD34 in endothelial cells in a type Ⅰ lesion (case 7, immunohistochemistry, × 200); B: CD34 expression in endothelial cells in a type Ⅱ lesion (case 8, immunohistochemistry, × 200).

of recurrence during the follow-up of 1-9 years. Follow-up ultrasonographic evaluation of the tumor volume and Doppler examination of tumor perfusion were performed in several cases; however, no evidence of tumor recurrence has been detected. The three patients (cases 2, 5 and 10) who underwent surgical biopsy only and without subsequent treatment were closely followed, and a regression in tumor size was noted by ultrasonography without complications.

DISCUSSION

In recent years, there have been important advances in the conceptual understanding of vascular tumors and malformations of the skin in infants. However, the study of hepatic IHE has lagged far behind skin lesions, in part because of its low incidence. Although IHE is the third most common type of hepatic tumor and the most common benign vascular tumor of the liver in infants, hepatic tumors are relatively rare in children, accounting for 2%-3% of all pediatric tumors[5]. Many diagnostic tests have been established for hepatic tumors in adults, but there are significant differences in pediatric tumors[6-8]. Over the last 10 years, we have diagnosed 12 cases of IHE (including two cases diagnosed at autopsy), which accounted for 38.8% of liver tumors and 80% of benign liver tumors in children in our institution. Pediatric hepatic vascular tumors are usually divided into IHEs and cavernous hemangiomas. IHE of the liver has been recognized for over 70 years, and to date, most cases reported were in Caucasians. The current study for the first time details the characteristics of hepatic IHE in a Chinese population.

The patients in our series ranged in age from fetal to 5 years. Most (83.33%) of the lesions were diagnosed within 6 mo of birth[9,10]. The sex ratio was 1:3 (male: female), consistent with other studies that demonstrated a clear female preponderance [9]. An asymptomatic abdominal mass or hepatomegaly found in a routine physical examination has been by far the most frequent symptom observed. Cutaneous and mucosal hemangiomas were not detected. The observed symptoms in Chinese individuals correct the misconceptions regarding the previously described associations with IHE, namely the classic triad of hepatomegaly, high-output CHF and cutaneous hemangioma[11]. There were no instances of CHF resulting from an arteriovenous shunt as other cases described in Mongoloid individuals[12]. Some previously described cases also had a variety of associated congenital abnormalities or hypothyroidism[13,14]. In our group, a 5-year-old girl had prominent signs of dyspnea caused by a pneumothorax, which is an unusual combined lesion. As similar reports have not been reported to date, we are not sure about the correlation between the pneumothorax and IHE. Our assumption is that IHE (hepatomegaly) caused a portion of the right lung to become compressed, which led to the compensatory expansion of the left lung. This in turn resulted in a local pneumothorax.

The serum AFP level, which often increases in primary hepatic malignancies, has been used as an important tumor marker for hepatoblastoma, hepatocellular carcinoma and some germ cell tumors. In this study, the serum AFP level was increased in four of five infants (80%), although it is seldom elevated in IHE[15,16]. Based on the data from laboratory examinations, we conclude that laboratory tests are neither nonspecific nor indicative of the diagnosis. As these lesions were excised or regressed, all the abnormal data subsided. These phenomena could be helpful in monitoring the recurrence of the tumor.

Imaging studies showed the presence of a space-occupying lesion in the liver and may provide a diagnosis or a differential diagnosis[17]. However, some cases undergoing involution may contain calcifications, fibrosis and vascular channel deficiencies which make it difficult to differentiate from other hepatic neoplasms. Therefore, biopsy of the mass is necessary for a definitive diagnosis. IHE presented as a solitary mass or multicentric nodules based on the radiographic findings and gross specimens. The former was more common in our study. It is noteworthy that the two solitary tumors in this group presented features of type Ⅱ lesions, whereas in the literature, the diffuse multifocal lesions were all classified as type Ⅱ. Therefore, solitary tumors are not exclusively type Ⅰ, and it is necessary to determine the histological type using microscopy. There seems no close correlation between the size of the tumor, solitary or multinodular appearance and poor prognosis in this study.

IHE must be histologically distinguished from cavernous hemangioma, epithelioid hemangioendothelioma, angiosarcoma and mesenchymal hamartoma[4]. Cavernous hemangioma is less common than IHE in children. In cavernous hemangioma, the capillary and sinusoidal portions are invisible and are replaced by widely dilated nonanastomotic thin-walled vascular spaces lined with flat endothelial cells and supported by fibrous issues. Epithelioid hemangioendothelioma is regarded in the World Health Organization (WHO) classification as an intermediate grade tumor, composed of epithelioid or spindle cells growing in myxoid stroma. This tumor often infiltrates extensively and forms intracellular vascular lumina that may contain erythrocytes. Angiosarcoma is an extremely rare malignant tumor during childhood. The dilated vascular channels are lined by pseudopapillary processes, and the disrupted liver cells act as scaffolding for the pleomorphic, neoplastic endothelial cells. Giant cell formation, solid sarcomatous foci, intrasinusoidal spread and invasion of portal or hepatic veins within the liver are often observed in angiosarcomas. A history of exposure to toxins, age, immature vascular differentiation, malignant cell morphology, pathologic karyokinesis and vascular infiltration all suggest a diagnosis of angiosarcoma. The differential diagnosis between IHE and mesenchymal hamartomas is difficult. The latter is a mixture of bile ducts, mesenchymal tissue and blood vessels. Mesenchymal tissue presents as myxomatous stroma with loosely arranged stellate cells, and the bile ducts display a ductal plate malformation. In case 5, eosinophilic granulocytes were the predominant inflammatory component. This concealed the vascular nature of the lesion and led to the misdiagnosis of a parasite infection. The formation of vascular channels, the absence of exposure to pathogens and the absence of antibodies to parasites could be considered to avoid such a misdiagnosis.

Based on the results of our study, it is critical to remind pathologists that it is not possible to predict the clinical behavior of these tumors based on morphologic criteria alone as there was no significant correlation between the histological type (type Ⅰ or type Ⅱ) and prognosis. Dehner *et al*[4] subdivided the tumor histologically into type Ⅰ and Ⅱ[4,18]. Type Ⅰ tumors are composed of capillary, sinusoidal and cavernous parts lined by plump endothelial cells with a bland cytological appearance[19]. Type Ⅱ tumors have areas composed of papillate tufting vascular channels that are lined by larger pleomorphic and hyperchromatic cells. Type Ⅱ tumors were thought to have a poorly formed and more aggressive microscopic appearance, but the presence of extensive endothelial cell proliferation, active mitosis and an infiltration margin do not indicate malignant characteristics compared with other pediatric tumors. Selby explained the cellular pleomorphism as a degenerative phenomenon in the setting of IHE, which is supported by other encouraging follow-up data[9]. The outcome in case 5, case 6 and case 8 in our study, which were classified as type Ⅱ tumors, demonstrated that histological type Ⅱ was not a significant indicator of poor prognosis, and the histological divisions have not been mentioned by the WHO classification[20]. In contrast, fibrosis, calcification and the formation of cavernous hemangiomatous foci are representative of regression or maturation. We propose to classify IHE into solitary and multifocal lesions rather than into type Ⅰ and type Ⅱ. However, to test the validity of this classification, a large number of cases and long-term follow-up will be needed.

IHE has been regarded as a benign vascular tumor by most authors, although a few have reported cases in which the IHE was malignant and developed into an angiosarcoma with eventual metastasis (especially type Ⅱ)[3,21,22]. All of the IHE cases in our study, except for the aborted fetuses, were associated with a good prognosis. In the natural course of our patients, the tumors grew rapidly for a period after they first appeared and then spontaneously involuted in the ensuing years. The natural evolution of this tumor, which is similar to that of cutaneous hemangioma during infancy, indicates a possible homogeneous histogenesis and functional differentiation. The prognosis is considered excellent except for some patients with severe complications, such as thrombocytopenia, hemolytic anemia, intravascular consumption coagulopathy and CHF that substantially contribute to the mortality associated with the tumor. However, these adverse risk factors are corrected once the tumor is resected. The more complications that occur, the poorer the prognosis will be. Therefore, pediatricians should pay a close attention to the complications to determine the best treatment strategy.

Because IHE frequently coexists and shares some biological features with cutaneous hemangioma, they may be related diseases. We presume that the simultaneous occurrence of hemangiomas in other organs can be attributed to a multicentric origin rather than metastasis[2,9,23]. "Kaposiform hemangioendothelioma", "retiform hemangioendothelioma", "composite hemangioendothelioma" and "epithelioid hemangioendothelioma" all demonstrate an intermediate or malignant clinical course. We propose that IHE should be designated "infantile capillary hepatic hemangioma". This name reflects its benign nature, which is better than "hemangioendothelioma" that often refers to the intermediate vascular tumor.

The treatment of IHE is controversial and the effects of the various forms of therapy are diverse and inconclusive[24]. The high survival rate in the IHE reported in this study, in contrast to the high mortality rates reported in the literature, illustrates the significant improvement made in the management of these tumors[4]. Although the tumor can spontaneously regress, it is necessary to guard against potentially life-threatening complications that may become a therapeutic challenge. Based on our experience, if IHE is discovered as a solitary asymptomatic mass and is a large lesion in a suitable location, partial hepatectomy should be the first line therapy[25]. There is no need for over-treatment of asymptomatic multicentric

lesions. Supportive care, controlling severe complications and close follow-up are all necessary for the treatment of these lesions. The resolution of the hemangioma is best monitored by ultrasound. Additionally, there are reports of treating diffuse lesions with steroids, interferon or vincristine, but these treatments may have adverse effects. If there is severe heart failure, digitalis and diuretics, transfusion and steroids should be used before surgical intervention. The use of therapeutic radiation and cyclophosphamide still remains controversial[26]. Transplantation is not recommended, unless the IHE is complicated with severe liver failure[27]. In a word, integrating the clinical manifestations with pathological features is appropriate in the treatment of tumors.

In summary, this retrospective study covered 12 cases of IHE of the liver seen over the past 10 years. All of the patients with a solitary lesion remained alive following surgery, and the multifocal cases underwent spontaneous regression without additional treatment. All of the IHE cases in our study were benign vascular tumors and were associated with a good prognosis despite some aggressive histological appearances. There was no correlation between the clinical behavior and the morphologic features in these cases. This is the first and largest population of patients with IHE examined from a single institution in China over a period of 10 years. A rigorous, evidence-based study in the future will guide in the selection of therapies for this disease.

COMMENTS

Background

Infantile hemangioendothelioma (IHE) of the liver is a rare mesenchymal tumor, but it is the most common benign vascular tumor of the liver in infancy. Since the first article to review and describe IHE in 1933, a series of cases have been reported in the literature.

Research frontiers

To date, most cases reported in the literature have been IHEs mainly in the Caucasians. However, diseases affecting individuals of the Mongoloid race often present with unique characteristics. The purpose of this study was to examine the clinical and pathologic features of IHE in Chinese individuals and to correlate these features with the treatment and prognosis.

Innovations and breakthroughs

This is the first and largest population of patients with IHE examined from a single institution in China over a period of 10 years. All of the IHE cases in this study were benign vascular tumors and were associated with a good prognosis despite some aggressive histological appearances.

Applications

The clinicopathologic features of IHE in Chinese patients may provide a clue to further evidence-based studies and guide the selection of therapies.

Terminology

IHE of the liver is a rare mesenchymal tumor in infancy. Type Ⅰ and type Ⅱ are subtypes of IHE classified according to the histological characteristics. CD34, CD31 and factor Ⅷ are antibodies that are expressed mainly in vascular tumors. CK8 and CK18 are antibodies that are expressed in epithelial cells, including bile ducts.

Peer review

In this study, the authors investigated the clinicopathological features of 12 IHE cases. They describe that this is the largest series of IHE from a single institution of China. The prognoses of their cases were fairly good; this is a different result from that of previous reports.

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BRIEF ARTICLE

High prevalence of viable *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's disease

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Abstract

AIM: To examine the detection rate of viable *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in patients with inflammatory bowel disease [Crohn's disease (CD) and ulcerative colitis (UC)].

METHODS: Thirty patients with CD (15 with at least one *NOD2/CARD15* mutation), 29 with UC, and 10 with no inflammatory bowel disease (IBD). were tested for MAP by polymerase chain reaction (specific IS900 fragment) and blood culture.

RESULTS: MAP DNA was detected in all original blood samples and 8-wk blood cultures (CD, UC and non-IBD). Positive MAP DNA status was confirmed by dot blot assays. All 69 cultures were negative by acid-fast Ziehl-Neelsen staining. Viable MAP, in spheroplast form, was isolated from the 18-mo blood cultures of all 30 CD patients, one UC patient, and none of the non-IBD controls. No association was found between positive MAP cultures and use of immunosuppressive drugs or CD-associated single nucleotide polymorphisms.

CONCLUSION: MAP is widely present in our area and MAP DNA can be recovered from the blood of CD, UC and non-IBD patients. However, MAP spheroplasts were only found in CD patients.

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Key words: *Mycobacterium avium* subspecies *paratuberculosis*; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Polymerase chain reaction; Genetic susceptibility

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two

related chronic remitting and relapsing inflammatory diseases of the gastrointestinal tract, commonly known as inflammatory bowel disease (IBD). Although the causes of IBD are unknown, it is thought that inflammation results from inappropriate chronic activation of the innate and adaptive mucosal immune systems in a genetically susceptible host, and that enteric microflora play a central role in initiation and maintenance of disease[1]. Some investigators have postulated that some mycobacterial infections are involved in development of CD, based on the similarities between CD and intestinal tuberculosis[2]. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has the specific ability to cause chronic bowel inflammation of a number of histopathological types in many animals, including primates[3,4]. Systematic review and meta-analysis of research from many laboratories have shown a significant and specific association between MAP infection and chronic bowel inflammation of the CD type in humans[5,6]. Recently, Naser *et al*[7] have found viable MAP in the peripheral blood from 50% of CD patients.

Several findings suggest that the presence of certain bacteria in the face of permissive *NOD2/CARD15* mutations is necessary for development of CD and provides evidence for a pathogen-host interaction[8-10]. A recent study has demonstrated an association in IBD with a coding variant of *ATG16L*, *IL-23R*[11] and *IRGM*[12] genes, thereby implicating the autophagy pathway that is crucial in inhibiting *Mycobacterium tuberculosis* survival in infected macrophages[13].

The aim of this study was to examine, after stratifying CD patients based on the presence or absence of the well-established *NOD2/CARD15* mutations, the culture detection rate of viable MAP in peripheral blood from patients with IBD (CD and UC) and healthy controls (non-IBD).

MATERIALS AND METHODS

Study population

Sixty-nine subjects were recruited into the study: 30 CD patients in clinical remission (of whom 15 carried at least one *NOD2/CARD15* mutant; these patients were matched one to one with patients with no *NOD2/CARD15* mutants based on the following criteria: time since IBD diagnosis, age at diagnosis, disease location, behavior and prior surgery); 29 UC patients in clinical remission; and 10 healthy controls (non-IBD), members of the staff of the Department of Clinical Microbiology, Hospital Clinico San Carlos de Madrid, Spain. Diagnosis of CD and UC was based on standard clinical, radiographic, endoscopic, and histological criteria[14], and all patients were recruited at an IBD unit of a single referral center in Madrid, Spain. Phenotypic details were obtained by review of clinical records and personal interview with the patients.

The protocol was approved by the Ethics Committee of Hospital Clinico San Carlos, Madrid, and all patients were recruited into the study after giving informed consent.

Samples and cultures

Two venous blood samples (10 mL) were taken from all patients and controls and drawn into sterile K2-EDTA vacutainer tubes.

Genomic DNA extraction and blood cultures were done using culture methods previously reported by Naser *et al*[7], except that 10 mL of whole blood was used for each culture. Cultures were incubated at 37℃ for 8 wk (bottle Ⅰ) or 18 mo (bottle Ⅱ).

DNA extraction and sequencing

Total DNA was prepared by two different methods. DNA was obtained from original samples and 8-wk cultures using the QIAamp DNA Blood Kit (Qiagen). Total DNA from 18-mo cultures was obtained using an EasyMag magnetic extractor (bioMerieux) according to the manufacturer's recommendations.

Polymerase chain reaction assay

Amplification of IS900 was conducted basically as described by Naser *et al*[7] with minor alterations: after the first round of nested polymerase chain reaction (PCR), amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and then diluted 1:100 with sterile water.

Dot blot assays

To confirm PCR results, DNA that had been extracted from original samples and 8-wk cultures was analyzed by dot blot hybridization using the DIG System (Roche Molecular Biochemicals). *M. avium* subs. *paratuberculosis* ATCC 43 544 was used as a positive control. One *Mycobacterium fortuitum* and one *M. avium* subs. *avium* were used as negative controls in dot blot assays.

Staining methods for microscopy

Smears were prepared by placing one drop from 18-mo cultures on a microscope slide. Smears were heat-fixed and stained using the Ziehl-Neelsen and phenolic acridine orange techniques to detect mycobacterial bacilli and/or spheroplasts[15].

Genotyping

Genotyping of rs2241880 (*ATG16L1*), rs4958847 (*IRGM*), rs7517847 (*IL23R*) and *NOD2/CARD15* polymorphisms was performed as previously described[16-18].

Statistical analysis

This was a case-control study. Numerical variables were summarized by the mean, median, and range. Nominal variables were summarized based on their frequency distribution.

RESULTS

The group of 30 CD patients consisted of 11 men and 19 women. Median age at diagnosis was 27 years (mean: 31, range: 14-48 years). Median follow-up duration was 7 years (mean: 8, range: 6-9 years). Table 1 shows the characteristics of the 30 CD and 29 UC patients enrolled into this study.

Table 1 Characteristics of patients with crohn's disease and ulcerative colitis *n* (%)

Phenotypic characteristics	CD *CARD15/NOD2* (+) (*n* = 15)	CD patients *CARD15/NOD2* (-) (*n* = 15)	UC patients (*n* = 29)
Male/female	5 (33.4)/10 (66.7)	6 (49.1)/9 (50.9)	15 (51.7)/14 (47.3)
Median age at diagnosis (range, yr)	27 (14-48)	27 (14-48)	
A1, < 40	14 (93.4)	14 (93.4)	
A2, ⩾ 40	1 (6.6)	1 (6.6)	
Family history	4 (26.7)	3 (21.4)	1 (3.4)
Disease behavior			
Nonstricturing, nonpenetrating (B1)	6 (40)	6 (40)	
Stricturing (B2)	3 (20)	3 (20)	
Penetrating (B3)	6 (40)	6 (40)	
Perianal disease	7 (46.6)	7 (46.6)	
Disease location			
Terminal ileum (L1)	6 (40)	6 (40)	
Colon (L2)	3 (20)	3 (20)	
Ileocolon (L3)	5 (33.4)	5 (33.4)	
Upper gastrointestinal (L4)	1 (6.6)	1 (6.6)	
Pancolitis			15 (51.7)
Left side			14 (48.3)
Treatment			
Surgery	6 (40)	6 (40)	1 (3.4)
Infliximab	8 (53.3)	6 (40)	0 (0)
Immunosuppressants	7 (46.6)	7 (46.6)	10 (34.5)
No immunosuppressive therapy	5 (33.4)	5 (33.4)	19 (65.5)
Genotype characteristics			
At least one *CARD15/NOD2* mutation	15 (100)	0 (0)	4 (13.8)
ATG16L1 (rs2241880) AA/GA/GG (*n*)	0/9/5	1/9/4	2/11/13
IRGM (rs4958847) AA/GA/GG (*n*)	0/5/8	1/4/9	0/7/15
IL23R (rs7517847) GG/TG/TT (*n*)	2/6/3	2/5/7	3/10/13

CD: Crohn's disease; UC: Ulcerative colitis.

Table 2 Results of samples and cultures in patients and controls (positive) %

	Original blood samples MAP DNA	BACTEC culture 8 wk of incubation (Bottle Ⅰ)		BACTEC culture 18 mo of incubation (Bottle Ⅱ)		
		BACTEC MGTI[1]	Dot-blot MAP	BACTEC MGTI[1]	Acid-fast Ziehl-Neelsen staining	Spheroplasts
CD patients (*n* = 30)	100	0	100	0	0	100
UC patients (*n* = 29)	100	0	100	0	0	3.4
Healthy controls (*n* = 10)	100	0	100	0	0	0

[1]Automatically detected; CD: Crohn's disease; UC: Ulcerative colitis; MAP: *Mycobacterium avium* subspecies *paratuberculosis*.

MAP DNA was detected in all original blood samples. No PCR internal control was positive for MAP DNA, which indicated no laboratory contamination. Nucleotide sequencing of purified MAP DNA fragments was also positive in the second round of nested PCR, which confirmed amplification of the IS900 nucleotide sequence (Table 2).

After 8 wk incubation (bottle Ⅰ), no mycobacterial growth was automatically detected in the 69 BACTEC MGTI cultures.

Dot blot assays confirmed the positive MAP status of all original blood samples and 8-wk cultures.

After 18 mo incubation (bottle Ⅱ), no mycobacterial growth was automatically detected in the 69 BACTEC MGTI cultures. All 69 buffy coat cultures were negative by acid-fast Ziehl-Neelsen staining. However, all of the 30 18-mo cultures from CD patients were positive by phenolic acridine orange staining, which suggested the presence of wall-deficient cells or spheroplasts (Figure 1).

Thus, 18-mo blood cultures were MAP-positive in all CD patients. No association could be found between positive cultures and use of tumor necrosis factor (TNF)-α antibodies and thiopurine drugs. No correlation was seen between MAP-positive blood cultures and *CARD15/NOD2*, *ATG16L1*, *IGRM* or *IL-23R* CD-associated single nucleotide polymorphisms (SNPs).

DISCUSSION

In this study, nested IS900-specific PCR showed that MAP DNA is widespread in our environment. Original blood samples and 8-wk cultures from all CD and UC patients and non-IBD controls were PCR-positive. However, viable MAP spheroplasts (cell-wall-deficient forms) were only found in the 18-mo blood cultures from all CD and one UC patient, but in none of the non-IBD

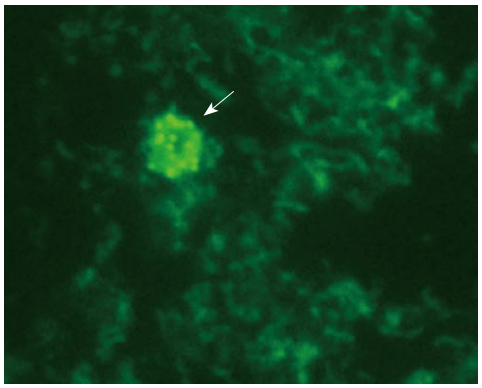

Figure 1 Microscopic examination of *Mycobacterium avium* subspecies *paratuberculosis* cultures isolated from blood of patients with Crohn's disease. Phenolic-acridine orange techniques to detect cell-wall-deficient *Mycobacterium avium* subspecies *paratuberculosis* [spheroplasts (arrow)].

controls. The observation that MAP could be cultured from CD patients was not correlated with use of immunosuppressive drugs (TNF-α antibodies and thiopurine drugs), *NOD2/CARD15* mutations, or other studied genes, which implicated the autophagy pathway of the innate immune system (*ATG 16L1*, *IRGM*, *IL-23R*).

The equal circulation of MAP DNA in patients with and without IBD lends support to the contention that environmental exposure to MAP is widespread, possibly from water, milk, or other sources[2]. Our group of subjects with no IBD was recruited from the staff of the Department of Microbiology, Hospital Clinico San Carlos de Madrid, Spain. These healthy subjects were most likely colonized by MAP, but none of them showed detection of viable MAP in 18-mo blood cultures. This result supported the findings in the study by Naser *et al*[7], where no viable MAP was subsequently cultured from any of the IS900-positive samples from healthy controls. The same occurred with the blood cultures from UC patients, as viable MAP was detected in only one of them. Thus, in our area, MAP is an ubiquitous environmental organism that does not usually cause disease unless the host is predisposed to infection, as occurs with other members of the *M. avium* complex.

Previous studies have shown that reliable and reproducible detection of *MAP* by PCR tests applied directly to DNA extracted from human tissue and other samples is extremely difficult. Use of suboptimal sample processing procedures results in false-negative results[19]. Results of MAP detection using nucleic-acid-based techniques have recently been reported in two meta-analyses that have suggested that there is adequate evidence for the presence of MAP in the bowel of CD patients, regardless of whether these patients are compared to subjects with no IBD or those with UC[5,20]. However, this association remains controversial and inconclusive. PCR data can be criticized on the grounds that the procedure assesses DNA that might come from live bacteria or merely be scattered debris from killed organisms, and therefore of questionable biological consequence[21].

The gold standard for detection of MAP is based on isolation of the organism using culture methods. However, this method is time-consuming because of the fastidious nature and slow growth, and pleomorphic, variably acid-fast and spheroplast-like organisms. Chiodini *et al*[15] have demonstrated that MAP strains isolated from CD tissue first appeared as cell-wall-deficient forms (spheroplasts), and have suggested that MAP is present in CD tissue in a spheroplast-like form[15,22]. We could not detect viable MAP by acid-fast Ziehl-Neelsen staining, but all cultures from CD patients were shown to contain spheroplasts. Naser *et al*[7] have reported that the MAP-positive cultures of the buffy coat were negative by acid-fast Ziehl-Neelsen staining during the early weeks of culture incubation but were positive by acridine orange (spheroplast), but this observation has not been confirmed by other studies[23-25]. Therefore, MAP spheroplasts might play a role in development of CD, as well as in paucibacillary forms of Johne's disease in other species. It has not been determined whether the presence of MAP in CD is related to infection, colonization, or a defect in the intestinal barrier/microbial killing. We did not study MAP in intestinal tissue, nor explore the possibility of increased bowel permeability. However, detection of viable MAP in the blood of CD patients could be due to the inability of macrophages in CD to kill MAP[26].

No association was found between a positive MAP culture from the blood of CD patients and *CARD15/NOD2*, *ATG16L1*, *IRGM* or *IL-23R* CD-associated SNPs, but our sample was small and a type Ⅱ error cannot be excluded.

The most irrefutable evidence that a microbial agent causes a disease is long-term remission of clinical manifestations and a change in the natural history of disease after clearance of infection. Recently, a large, well-designed, randomized, placebo-controlled trial of clarithromycin, rifabutin, and ethambutol failed to show a sustained response in CD patients, although a short-term benefit of antibiotics at 16 wk, additional to the effect of corticosteroid therapy, was reported[27]. A recent study[28] has shown that antimycobacterial and thiopurine drugs used in concert might have an interactive effect. The apparently bacteriostatic effects of 6-mercaptopurine on *M. paratuberculosis* renders the organism less susceptible to the bactericidal effects of antibiotics.

An argument against a role of MAP in CD is that if CD were a chronic mycobacterial infection, immunosuppressive therapies would be associated with increased rates and severity of mycobacterial disease, rather than with improvement[29]. We were not able to show any association between occurrence of MAP bacteremia and use of immunosuppressive drugs, because all CD patients showed positive MAP cultures. Viable MAP could not be cultured from UC patients who received immunosuppressive therapy. This might indicate that use of immunosuppressive therapy has no influence on the presence of viable MAP in blood.

In conclusion, MAP is widely present in our area and MAP DNA can be recovered from the blood of CD and UC and healthy controls. Spheroplasts were only found in

the blood cultures from CD patients. However, the pathogenetic role of MAP remains controversial and inconclusive. However, even if MAP is not causally related to CD, the presence of viable MAP in the blood might have secondary clinical implications.

COMMENTS

Background

The hypothesis postulating that *Mycobacterium avium paratuberculosis* (MAP) is the cause of Crohn's disease (CD) has been circulating for many years. Advances in molecular techniques, such as PCR and culture methods, have allowed researchers to demonstrate an association between MAP and CD.

Research frontiers

MAP is a recurrent candidate as the cause of CD for several reasons: MAP induces epidemic chronic colitis in cattle and other species, including primates; it is reportedly detectable in the intestinal tissues and blood of many CD patients; MAP antibodies are often associated to the disease; and in some cases, antimycobacterial drugs improve the disease. In this study, authors demonstrated that MAP spheroplasts were cultured from the peripheral blood of CD patients only, but not from patients with ulcerative colitis (UC) or normal controls.

Innovations and breakthroughs

MAP is widely present in Spain, and MAP DNA may be recovered from the blood of CD patients, UC patients, and healthy controls, but in this study MAP spheroplasts were only found in the 18-mo blood cultures from all CD patients. The observation that MAP could be cultured from CD patients was not correlated with the use of immunosuppressive drugs or mutations associated with CD patients.

Applications

The status of viable MAP spheroplasts might play a role in development of CD, as well as in paucibacillary forms of Johne's disease in other species.

Terminology

CD is a chronic remitting and relapsing inflammatory disease of the gastrointestinal tract. MAP is a bacterium that is a member of the *M. avium* complex. *M. avium* strains are widely distributed in the environment and also occur in normal animal and human intestines. Spheroplasts are cell-wall-deficient forms of MAP.

Peer review

This is a small case-control study that attempted to correlate the presence of MAP with inflammatory bowel disease. Although this is not an entirely original study and the number of patients is relatively small, the authors report some interesting findings.

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BRIEF ARTICLE

Risk factors associated with the development of ischemic colitis

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Abstract

AIM: To ascertain the role of cardiovascular risk factors, cardiovascular diseases, standard treatments and other diseases in the development of ischemic colitis (IC).

METHODS: A retrospective, case-control study was designed, using matched data and covering 161 incident cases of IC who required admission to our hospital from 1998 through 2003. IC was diagnosed on the basis of endoscopic findings and diagnostic or compatible histology. Controls were randomly chosen from a cohort of patients who were admitted in the same period and required a colonoscopy, excluding those with diagnosis of colitis. Cases were matched with controls (ratio 1:2), by age and sex. A conditional logistic regression was performed.

RESULTS: A total of 483 patients (161 cases, 322 controls) were included; mean age 75.67 ± 10.03 years, 55.9% women. The principal indications for colonoscopy in the control group were lower gastrointestinal hemorrhage (35.4%), anemia (33.9%), abdominal pain (19.9%) and diarrhea (9.6%). The endoscopic findings in this group were hemorrhoids (25.5%), diverticular disease (30.4%), polyps (19.9%) and colorectal cancer (10.2%). The following variables were associated with IC in the univariate analysis: arterial hypertension (P = 0.033); dyslipidemia (P < 0.001); diabetes mellitus (P = 0.025); peripheral arterial disease (P = 0.004); heart failure (P = 0.026); treatment with hypotensive drugs (P = 0.023); angiotensin-converting enzyme inhibitors; (P = 0.018); calcium channel antagonists (P = 0.028); and acetylsalicylic acid (ASA) (P < 0.001). Finally, the following variables were independently associated with the development of IC: diabetes mellitus [odds ratio (OR) 1.76, 95% confidence interval (CI): 1.001-3.077, P = 0.046]; dyslipidemia (OR 2.12, 95% CI: 1.26-3.57, P = 0.004); heart failure (OR 3.17, 95% CI: 1.31-7.68, P = 0.01); peripheral arterial disease (OR 4.1, 95% CI: 1.32-12.72, P = 0.015); treatment with digoxin (digitalis) (OR 0.27, 95% CI: 0.084-0.857, P = 0.026); and ASA (OR 1.97, 95% CI: 1.16-3.36, P = 0.012).

CONCLUSION: The development of an episode of IC was independently associated with diabetes, dyslipidemia, presence of heart failure, peripheral arterial disease and treatment with digoxin or ASA.

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Key words: Ischemic colitis; Diabetes mellitus; Dyslip-

idemia; Acetylsalicylic acid; Peripheral arterial disease; Digoxin

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INTRODUCTION

Ischemic colitis (IC) is the most frequent form of intestinal ischemia (70%) and arises in cases where the colon is transitorily deprived of vascular flow. IC may be the result of an occlusive or non-occlusive disease. Clinically, IC is classified as gangrenous or non-gangrenous. Non-gangrenous IC affects the mucosa and submucosa, and is responsible for 80%-85% of cases[1]. Non-gangrenous forms have been subclassified into transitory reversible forms with mild damage, and chronic irreversible forms, which include chronic colitis and stenosis and imply more severe impairment[1]. Development of IC has been associated with a series of diseases and risk factors. In general, any condition or factor that reduces the blood flow to the colon can generate IC. In this respect, a higher incidence of IC has been described in patients who undergo vascular surgery (aortic surgery or coronary bypass in particular)[2,3] or have cardiovascular[4-6] or hematological diseases[7,8]. IC has also been described among patients with microvascular disease, such as systemic lupus erythematosus[9,10], with chronic renal failure that requires dialysis[4] and with use/abuse of certain medications or drugs, such as oral contraceptives, vasoconstrictors, psychotropic drugs, interferon-α, nonsteroidal anti-inflammatory drugs (NSAIDs), 5-HT$_3$ receptor antagonists and cocaine[11,12].

In recent years, different population-based studies have been published which have sought to detect risk factors associated with the development of IC[11,13-15]. Note should be taken of the relationship observed between diagnosis of irritable bowel syndrome and IC, both in case-control and cohort studies[11,13,14]. Similarly, population-based studies of cohorts with chronic obstructive pulmonary disease (COPD) have reported an increased risk of developing an episode of IC among such patients[11].

The prevalence of cardiovascular risk factors, cardiovascular diseases and related medication in cohorts of patients requiring admission due to an episode of IC is high. Thus, in two series published recently in this country, the prevalence of arterial hypertension, dyslipidemia and diabetes was in the order of 57%-66%, 24%-26% and 20%-22%, respectively. In these series, a high prevalence of cardiovascular disease was detected, whether ischemic or hypertensive heart disease (18%-21% and 15.4%, respectively), cerebral vascular disease (12%-20%) or peripheral arterial disease (8%-14%). Lastly, there was a high rate of patients treated with acetylsalicylic acid (ASA) (32%), hypotensive drugs (54%) and diuretics (33.7%-20.7%)[16,17]. Although such prevalence might be linked to the advanced mean age of the cohorts described, there are no population-based studies published that have assessed the possible causal effect of cardiovascular risk factors on the development of IC.

This study sought to: assess whether cardiovascular risk factors, such as hypertension, diabetes, hypercholesterolemia, smoking and cardiovascular diseases, are linked to the development of IC; confirm the relationship between COPD and development of IC in Spain; and finally, assess the effect that standard drug treatments have on the development of IC.

MATERIALS AND METHODS

Study design

This was a retrospective, observational, case-control study using clinical history data matched in a ratio of 1:2.

Selection of cases

We selected incident cases of IC requiring admission to the Ourense Hospital Complex (Galicia, Spain) during the period January 1998 through March 2003. To detect such cases, endoscopic and pathological anatomy records were searched for diagnoses of IC, segmentary colitis and/or indeterminate colitis, and the pertinent clinical histories were then reviewed. Diagnosis of IC was based on endoscopic findings and/or diagnostic or compatible histology. Individuals with a diagnosis of colitis of any other origin (infectious, inflammatory, diverticulitis, associated with antibiotics or NSAIDs) were excluded from this cohort, as were individuals with IC who did not require admission. IC cases that were prevalent during the study period were likewise excluded. Finally, a total of 161 individuals were included. Their endoscopic location and endoscopic and histological findings are shown in Table 1.

Selection of controls

Controls were selected from the cohort of 1555 patients who, during the same time period, required admission to the Ourense Hospital Complex and underwent a colonoscopy. Individuals with diagnosis of colitis of any origin were excluded from this cohort.

Variables collected

Demographic data (age, sex) were collected. Insofar as personal histories were concerned, information was gathered regarding the presence of the following diseases: arterial hypertension; hypercholesterolemia; diabetes mellitus; smoking; COPD; chronic renal failure; heart disease (of hypertensive, ischemic or valvular etiology, heart failure, atrial fibrillation); peripheral arterial disease; abdominal aortic aneurysm surgery; and cerebrovascular disease. With respect to usual medication, we examined whether patients were receiving one or more of the following drugs at date of

Table 1 Diagnosis of ischemic colitis

	n (%)
Endoscopic location	
Pancolitis	3 (1.9)
Right colon	10 (6.5)
Transverse colon	34 (21)
Splenic angle	36 (23.2)
Descending colon	69 (44.5)
Sigmoid colon	106 (68.4)
Rectum	38 (24.5)
Endoscopic findings	
Hyperemia	98 (65.3)
Petechiae	37 (24.7)
Intramucosal bleeding	30 (20)
Fibrin	43 (26.5)
Ulcers	84 (56)
Active bleeding	9 (6)
Necrosis	12 (8)
Estenosis	12 (8)
Histologic findings	
Crypt loss	17 (14.7)
Epithelium loss	13 (8)
Edema	68 (58.6)
Inflamatory infiltrate	87 (75)
Capillar thrombosis	17 (14.7)
Necrosis	40 (34.5)
Hemorrhage	1 (2.5)

Location, endoscopic and histologic findings are summarized. Variables are expressed as absolute number and percentage.

admission: ASA; NSAIDs; acenocumarol; digoxin (digitalis); diuretics; antidepressants; beta blockers; calcium channel antagonists; angiotensin-converting enzyme inhibitors; angiotensin Ⅱ receptor antagonists; and antidepressants. Lastly, in the control group we recorded both the reason for a colonoscopy being requested and the endoscopic findings.

Sample size

Sample size was ascertained on the basis of data on the prevalence of cardiovascular factors known to be present in our cohort of IC patients[17], with the cardiovascular risk factor with the lowest prevalence being used to calculate the sample size. Hence, bearing in mind that smoking registered a prevalence of 10.1%, and accepting an alpha risk of 0.05 and a beta risk of 0.20 in a two-sided test for detection of an odds ratio (OR) of 3160 individuals were required in the case group and 320 in the control group. Cases were matched 1:2 with controls, by age and sex. The EPIDAT 3.1 computer software program was used to calculate sample size and randomization of controls.

Ethical aspects

This study was formally authorized by the Galician Clinical Research Ethics Committee (code 2008/374) on 15th December 2008.

Statistical analysis

As a first step, we conducted a descriptive analysis of the variables covered by the study. Univariate and multivariate analyses were then performed using conditional logistic regression. In the multivariate analysis, account was taken of those variables that had proved statistically significant in the univariate analysis and those that were deemed to be clinically relevant. The goodness-of-fit of the models generated was ascertained using the pseudo R^2 coefficient of determination and the area under the curve (AUC), with an alpha error = 0.05 and beta error = 0.2 being considered in all tests. The statistical analysis was performed using the free R software (www.r-project.org). Odds ratios and their confidence intervals (CIs) were graphically depicted using the Graph Pad Prism 5.0 program.

RESULTS

Demographic characteristics

A total of 483 individuals, 161 cases and 322 controls, were included in the study; mean age 75.67 ± 10.03 years (cases: 75.42 ± 10.58, controls: 75.79 ± 9.75, $P = 0.56$), 55.9% women (cases: 55.9%, controls: 55.9%, $P = 0.5$). The reasons given for performing a colonoscopy in controls were: lower gastrointestinal hemorrhage, 114 (35.4%); ferropenic anemia, 109 (33.9%); abdominal pain, 64 (19.9%); diarrhea syndrome, 31 (9.6%); intestinal rhythm disorder, 22 (6.8%); and melena, 13 (4%). The endoscopic findings detected in the controls were: diverticular disease, 98 (30.4%); hemorrhoids, 82 (25.5%); polyps, 64 (19.9%); colorectal cancer, 33 (10.2%); and angiodysplasias, 24 (7.5%).

Descriptive analysis of patients

The clinical history and standard pharmacologic treatments of cases and controls are shown in Tables 2 and 3, respectively, along with the pertinent ORs, CIs and significance levels in the univariate conditional logistic regression.

Multivariate conditional logistic regression

In the multivariate analysis, a statistically significant relationship was found between diagnosis of IC and the following variables: diabetes mellitus ($P = 0.029$); hypercholesterolemia ($P = 0.007$); heart failure ($P = 0.034$); peripheral arterial disease ($P = 0.015$); and continued treatment with digoxin ($P = 0.018$) or ASA ($P = 0.012$). To control for the effect of heart disease, a model was then fitted including the significant variables adjusted for diagnosis of heart disease of any origin. The results yielded by this model can be seen in Table 4 and Figure 1. The pseudo R^2 coefficient of determination was 0.086. Figure 2 depicts the receiver operator characteristic curve of the model and its AUC.

DISCUSSION

Although development of IC has been associated with diverse phenomena that reduce mesenteric perfusion, there are no studies that have assessed the relationship between IC and cardiovascular risk factors and cardiovascular diseases. In one case-control study of autopsies, a statistically significant relationship was found between diagnosis of fatal IC and presence of heart failure, valvular heart disease, acute myocardial infarction and previous surgery[18].

Table 2 Clinical characteristics present in cases and controls

	Case (%)	Control (%)	*P* value	OR	95% CI
Arterial hypertension	56.5	46.6	0.034	1.543	1.035-2.301
Dyslipidemia	19.8	12.1	0.025	1.793	1.075-2.992
Diabetes mellitus	26.7	12.7	< 0.001	2.427	1.506-3.911
Smoking habit	10.5	9.9	0.825	1.075	0.564-2.049
Chronic renal failure	4.3	4.6	0.877	0.93	0.372-2.33
Valve heart disease	7.4	6.8	0.806	1.094	0.535-2.234
Ischemic heart disease	18	14.9	0.381	1.254	0.756-2.08
Hypertensive heart disease	15.5	13.4	0.516	1.195	0.698-2.044
Atrial fibrillation	14.3	14	0.923	1.028	0.585-1.808
Heart failure	10.5	5	0.026	2.253	1.103-4.604
Peripheral arteriopathy	8.1	2.2	0.004	4.658	1.643-13.21
Abdominal aorta surgery	1.2	0	0.096		
Cerebrovascular disease	12.4	9.6	0.339	1.348	0.731-2.483
COPD	18	18.6	0.864	0.956	0.576-1.587

Variables are expressed as percentages. Differences are considered statistically significant when $P < 0.05$. COPD: Chronic obstructive pulmonary disease; OR: Odds ratio; CI: Confidence interval.

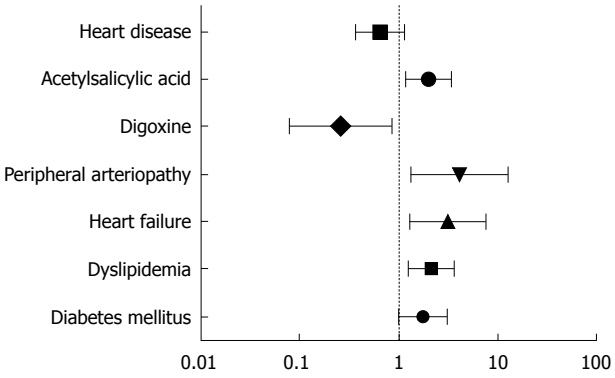

Figure 1 Odds ratio graph. Odds ratio and 95% confidence interval of variables included in model B.

Table 3 Medications taken by cases and controls

	Case (%)	Control (%)	*P* value	OR	95% CI
Antihypertensives	51.5	41	0.023	1.595	1.067-2.383
Beta-blockers	5.6	5.6	1	1	0.443-2.26
Ca^{2+} channel antagonists	22.3	14.6	0.027	1.771	1.065-2.946
ACE inhibitors	27.3	18.3	0.018	1.776	1.102-2.861
Nitrates	10.5	9.3	0.67	1.143	0.617-2.117
ARB	1.2	4	0.109	0.358	0.102-1.259
NSAIDs	10.5	9.9	0.823	1.077	0.561-2.07
Digoxin	3.1	7.4	0.060	0.383	0.141-1.042
Antidepressants	3.7	6.2	0.222	0.540	0.201-1.453
Diuretics	26.1	26.1	1	1	0.656-1.525
ASA	31.6	18	< 0.001	2.153	1.371-3.382
Acenocumarol	5.6	5.6	1	1	0.443-2.26

Variables are expressed as percentages. Differences are considered statistically significant when $P < 0.05$. ACE: Angiotensin converting enzyme; ARB: Angiotensin Ⅱ blockers; NSAIDs: Non-steroidal anti-inflammatory drugs; ASA: Acetylsalicylic acid; OR: Odds ratio; CI: Confidence interval.

Table 4 Multivariant conditional logistic regression model

Variables	*P* value	Coefficient	OR	95% CI
Diabetes mellitus	0.04600	0.5671	1.7632	1.0101-3.0776
Dyslipidemia	0.00437	0.7550	2.1277	1.2658-3.5762
Heart failure	0.01064	1.1539	3.1705	1.3079-7.6853
Peripheral arteriopathy	0.01461	1.4109	4.0997	1.3211-12.7219
Digoxin	0.02651	-1.3173	0.2679	0.0836-0.8576
Acetylsalicylic acid	0.01219	0.6808	1.9755	1.1600-3.3640
Heart disease	0.14541	-0.4224	0.6554	0.3711-1.1575

P value, beta coefficient, odds ratio (OR) and 95% confidence interval (CI) of variables included in model B.

The results of this study are limited, however, in that it covered a series of autopsies in which serious forms of IC were diagnosed. In the majority of patients, IC develops as a transitory or self-limited form[16,17,19,20]. In our study, both mild or transitory and severe forms were included. We detected an independent relationship between development of IC and previous diagnoses of cardiovascular risk factors (diabetes mellitus, hypercholesterolemia) and cardiovascular diseases (peripheral arterial disease, heart failure). However, no relation was found with smoking habit. In fact, the prevalence of smoking habit was low. The most recent data available in Spain show that the proportion of the population over 65 years who are actively smoking is low: 8.5%[21]. Our findings thus support the hypothesis of IC as a disease associated with a reduction in mesenteric blood flow.

Moreover, we detected an independent relationship between IC and treatment with ASA or digoxin. NSAIDs in general, and ASA in particular, are known to have a harmful effect on the gastrointestinal tract. NSAIDs-related colitis has been described in connection with both short-[12,22] and long-term treatments[23]. Different clinical observations and case-control studies have linked the use of NSAIDs or salicylates to diagnosis of acute diarrhea syndrome[24] and exacerbation of inflammatory colitis (ulcerative colitis or Crohn's disease)[23]. Insofar as the relationship with the development of IC is concerned, there is little evidence. There is one clinical report in which the use of meloxicam is associated with the development of IC[12]. In the case-control study published by Collin *et al*[25] a statistically significant relationship was observed between use of NSAIDs and development of segmentary non-gangrenous colitis. In our study, an independent relationship was detected between use of ASA and development of IC. This did not apply to continued use of NSAIDs, once ASA had been excluded. The mechanism associated with tissue damage has not been fully elucidated. Studies based on a model of colitis associated with trinitrobenzene sulfonic acid have confirmed the ability of NSAIDs to exacerbate colitis[26]. This effect is brought about by inhibition of cycloxygenase-2 (COX-2)[26]. COX-2 is also the principal agent responsible for the process that leads to resolution of the inflammation. Prostaglandin D2 acts as a termination signal, by reducing granulocyte infiltration[27]. Similarly, inhibition of COX-2 and prostaglandins with their vasodilatory effect could increase vascular resistance in the splanchnic circulation[22]. As regards the protective

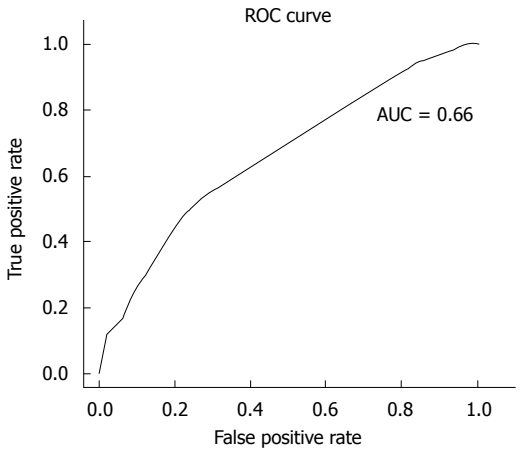

Figure 2 Receiver operator characteristic curve. Receiver operator characteristic (ROC) curves and area under the curve (AUC) of the model developed in the case-control study.

effect of treatment with digoxin observed in our study, little has been published. Indeed, only one case-control study has been reported, in which use of digoxin was found to have a harmful effect[25]. Nevertheless, this study failed to analyze confounding variables, such as diagnoses of heart failure and heart disease, which we introduced into our analysis. The protective effect could be linked to the positive inotropic effect associated with digoxin treatment, which would generate an increase in splanchnic blood flow.

Our patients with IC had an advanced age; the mean age was 75.42 ± 10.58 years. This finding is similar to the data published recently in series of patients with IC in our country[16,19,20]. It is noteworthy that, despite the advanced age of the patients, the prognosis was fairly good: only 5.9% required surgery and 4.7% died during hospitalization[17].

Our study has a number of limitations, the first of which is that it was a retrospective study. Although the collection of clinical history data was based on a protocol drawn up at our hospital, conditions related to development of IC, such as constipation and irritable bowel syndrome, could not be evaluated[11,13,14,20]. The control group might suffer from selection bias as a result of it being a group of patients who required admission. However, the selection criterion - performance of a colonoscopy - was not related to the variables analyzed. At all events, in view of the fact that these were patients who required admission, the control group might have had a frequency of cardiovascular risk factors, cardiovascular diseases and drug use higher than that of the general population. In this respect, the conclusions of this study must be validated in the context of a population-based cohort study. Since incidence of IC in the general population is low, ranging from 4.5 to 44 cases per 100 000 population[11], designing a cohort study to assess the effect of these variables may prove complicated.

Another of this study's limitations resides in the lack of some clearly defined criteria for diagnosis of IC. At our health center, diagnosis of IC, after excluding other etiologies, was based on typical endoscopic findings of IC associated with a definitive or compatible histology. Endoscopic findings are known to have a high degree of accuracy for diagnosis of IC[28]. We used widely accepted histological criteria for diagnosis of IC[1,29,30], with definitive histology being defined as detection of mucosal infarction, clots or fibrin in the capillaries or submucosal hemorrhage. In addition, compatible histology was defined as detection of a loss of mucin and superficial epithelial cells, mild or moderate inflammatory infiltrate, edema or vascular congestion. Currently, there are no diagnostic criteria that enable NSAIDs-related colitis to be specifically diagnosed. The doubt remains, therefore, as to whether two different entities are involved or whether NSAIDs in general, and ASA in particular, have a role in the pathogeny of IC. In a previously published study, we reported a statistically significant relationship between recurrent IC and continued treatment with ASA[17].

In conclusion, we detected a statistically independent relationship between some cardiovascular risk factors and cardiovascular diseases, and development of IC. This relationship must, however, be confirmed in cohort studies. Similarly, the role of ASA in the development of IC must be evaluated.

COMMENTS

Background

Ischemic colitis (IC) is the most frequent form of intestinal ischemia (70%) and arises in cases where the colon is transitorily deprived of vascular flow. Development of IC has been associated with a series of diseases and risk factors. In general, any condition or factor that reduces the blood flow to the colon could generate IC. In recent years, different population-based studies have been published which have sought to detect risk factors associated with the development of IC, especially irritable bowel syndrome.

Research frontiers

Although IC has been related to may conditions, the relationship with cardiovascular diseases and associated treatments is based on low evidence studies.

Innovations and breakthroughs

In this case-control study based on clinical data recordings we have performed a logistic regression analysis. We found an independent association between cardiovascular diseases (presence of heart failure, peripheral arterial disease) and cardiovascular risk factors (diabetes, dyslipidemia) and IC. Furthermore, we have detected that acetylsalicylic acid is independently associated with the development of IC. As long as there are no definite criteria that differentiate nonsteroidal anti-inflammatory drugs-related colitis from IC, the doubt remains as to whether two different entities are involved or whether acetylsalicylic acid has a role in the pathogeny of IC.

Applications

IC should be considered as a cardiovascular disease. This relationship must, however, be confirmed in cohort studies. Finally, the role of acetylsalicylic acid in the pathogeny of IC should be prospectively evaluated.

Peer review

This paper is well-organized and well-investigated about the risk factors associated with the development of IC.

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BRIEF ARTICLE

Recurrent abscess after primary successful endo-sponge treatment of anastomotic leakage following rectal surgery

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Abstract

AIM: To assess long-term efficacy of initially successful endo-sponge assisted therapy.

METHODS: Between 2006 and 2009, consecutive patients who had undergone primary successful endo-sponge treatment of anastomotic leakage following rectal cancer surgery were enrolled in the study. Patients were recruited from 6 surgical departments in Vienna. Clinical and oncologic outcomes were assessed through routine endoscopic and radiologic follow-up examination.

RESULTS: Twenty patients (7 female, 13 male) were included. The indications for endo-sponge treatment were anastomotic leakage (n = 17) and insufficiency of a rectal stump after Hartmann's procedure (n = 3). All patients were primarily operated for rectal cancer. The overall mortality rate was 25%. The median follow-up duration was 17 mo (range 1.5-29.8 mo). Five patients (25%) developed a recurrent abscess. Median time between last day of endo-sponge therapy and occurrence of recurrent abscess was 255 d (range 21-733 d). One of these patients was treated by computed tomography-guided drainage and in 3 patients Hartmann's procedure had to be performed. Two patients (10%) developed a local tumor recurrence and subsequently died.

CONCLUSION: Despite successful primary outcome, patients who receive endo-sponge therapy should be closely monitored in the first 2 years, since recurrence might occur.

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Key words: Rectal surgery; Anastomotic leakage; Endo-sponge; Endo-vacuum treatment

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INTRODUCTION

Anastomotic leakage following rectal cancer surgery is

regarded as one of the most feared postoperative complications[1]. Together with a significant increase in both morbidity and mortality rate, several studies found anastomotic leaks after curative colorectal resections for malignancy associated with poor survival and a higher tumor recurrence rate[2,3].

Once leakage occurs, treatment strategies are limited and depend on the septic condition of affected patients. The anastomosis can be renewed or taken down; simultaneously a protective stoma is usually created. In cases of mild sepsis and localised abscesses, computed tomography (CT)-guided drainage can be attempted.

The use of an endoluminal vacuum therapy represents a novel method to treat patients with anastomotic leakage after rectal resection[4]. An open-pored sponge is inserted into the abscess cavity and connected *via* draining tube to a vacuum drainage system. Through continuous negative pressure a shrinking and cleaning of the wound can be achieved.

To date, only few studies are available focusing on endo-sponge treatment[5-7]. Taking into account the limited number of included patients, promising short-term results have been reported. Weidenhagen *et al*[4] described a success rate of nearly 96% in their series. Another study observed complete healing in only 75% of patients[8].

Long-term results after primary successful endo-sponge therapy have not been published to date. Therefore, it is still under debate as to whether vacuum therapy-derived granulation tissue is stable and not prone to develop recurrent abscesses. This problem might occur especially in those patients who require further chemotherapy after endo-sponge therapy has been completed. Another question arises as to whether endo-sponge therapy negatively influences oncologic results. It is tempting to speculate that through stimulation of granulation tissue angiogenesis, growth of residual local tumor cells becomes reactivated.

The present multicenter study was designed to evaluate long-term efficacy of initially successful endo-sponge assisted therapy after anastomotic leakage following rectal resection for malignancy. Additionally, oncologic outcome was assessed.

MATERIALS AND METHODS

Between 2006 and 2009, consecutive patients who had undergone initially successful endo-sponge assisted treatment of anastomotic leakage following rectal cancer surgery were included in the study. Patients were recruited from 6 surgical departments in Vienna. The study was approved by the institutional Ethics Committee.

All patients were initially operated for rectal cancer. Rectal tumor was located in the upper third of the rectum in 2 patients, in the middle third in 8 patients and in the lower third in 10 patients. In 15 patients a low anterior resection, in 3 patients a rectal resection with a coloanal anastomosis, and in 2 patients a Hartmann's procedure with resection of the tumor were performed. The type of anastomosis was end-end in 14 patients, side-end in 1 patient and a J-Pouch in 3 patients. A protective stoma

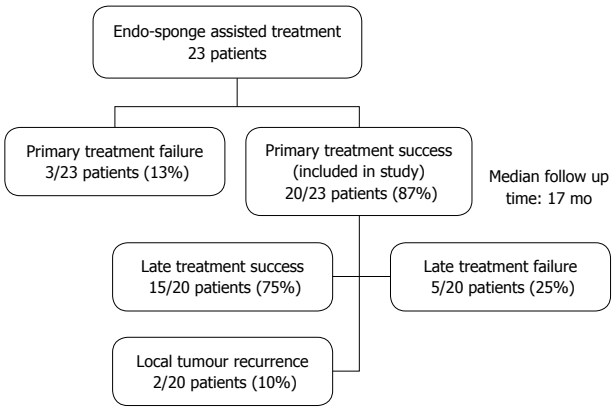

Figure 1 Study outline.

was created in 14 patients [ileostomy: n = 10 patients, colostomy: n = 4 patients, none (including Hartmann's procedure): n = 6 patients]. One patient had neoadjuvant short-term radiotherapy and 5 patients required long-term radio/chemotherapy before primary rectal resection. The remaining 14 patients did not receive any additional treatment prior to surgery. According to the International Union Against Cancer (UICC) classification, the rectal cancers were grouped as stage Ⅰ (n = 9), stage Ⅱ (n = 5), stage Ⅲ (n = 4) and stage Ⅳ (n = 2).

The indication for endo-sponge assisted therapy was an anastomotic leakage in 17 patients and insufficiency of a rectal stump after Hartmann's procedure in 3 patients. The Hartmann's procedures were conducted after anastomotic leakage following anterior rectal resection, after rectal perforation following palliative endoscopic stent implantation, and as primary treatment of bleeding rectal cancer in a patient who was in poor condition.

Time from primary operation to anastomotic leakage showed a median duration of 12.5 d (range 3-668 d).

In 2 patients (10%), a surgical approach (abscess drainage and stoma formation) and in 3 patients (15%) CT-guided drainage were attempted prior to endo-sponge treatment.

Those patients with an unsuccessful primary endo-sponge treatment were excluded. The study design is outlined in Figure 1.

Patients were investigated during annual routine follow-up examination. A colonoscopy was performed to assess the anastomotic site with regard to local recurrence and reappearance of any leakage. Whenever possible a CT or magnetic resonance tomography scan was performed to obtain additional information.

Patient data were obtained from the institutional database of each participating hospital and individual chart reviews retrospectively. Additional information was gathered through a face to face questioning.

The technique of inserting an endo-sponge (B. Braun, Austria, GmbH) and installing an endoluminal vacuum system has been described previously[9].

Statistical analysis

Continuous data are shown with median and minimum-

maximum values. Associations between late treatment failure and variables were analyzed by nonparametric Wilcoxon rank sum test. A *P*-value ≤ 0.05 was considered significant. All data were collected and statistically analyzed using SPSS (14.0.1. Chicago, USA).

RESULTS

Twenty patients (7 female, 13 male) who had primary successful endo-sponge therapy were included in the study. The median body mass index (BMI) was 26.5 kg/m^2 (range 17.3-31.3 kg/m^2) and the median age was 66.3 years (range 54.8-91.2 years). The median follow-up duration was 17.1 mo (range 1.5-29.8 mo). Five patients (25%) died during the follow-up period but were included in the analysis: four patients died due to tumor progression (2 patients had liver metastases at the time of endo-sponge therapy) and one patient died because of acute liver failure due to known liver cirrhosis.

One patient developed a benign anal stenosis which was improved by anal dilatation.

Interestingly, we identified five patients (25%) who developed a recurrent symptomatic abscess. The following interventions were performed for recurrent abscess: in 3 patients anastomosis was taken down by Hartmann's procedure, which classified abscesses as stage C (definition by the International Study Group of Rectal Cancer)[10]; one abscess was classified as stage B, requiring CT-guided drainage; one patient had only minimal clinical signs, therefore abscess was classified as stage A and further therapy (e.g. Hartmann's procedure) is still under discussion.

Additionally, we analyzed whether we could identify predictive factors for recurrent abscess formation (Table 1). None of the demographic factors, such as age or BMI were significantly different between successful and refractory groups. The same factors related to standard tumor care were similar in both groups. Specifically, neoadjuvant radiotherapy or radiochemotherapy and adjuvant chemotherapy administration was evenly distributed in both groups. With respect to anti-angiogenic treatment with Bevacizumab (Avastin®, Roche), there was 1 patient in the successful group and 1 patient in the treatment failure group.

One patient received Bevacizumab before endo-sponge therapy was started. This patient also developed a recurrent abscess.

Similarly, there was no difference in the indication for endo-sponge therapy (anastomotic leakage *vs* rectal stump insufficiency). At the time of endo-sponge treatment 9 patients (45%) had a diverting ileostomy and 8 patients (40%) a colostomy. Three patients (15%) were treated without protective stoma. There was no difference regarding recurrence between stoma groups during therapy. In 13 patients (76.5%) the stoma was closed after successful endo-sponge treatment.

We then analyzed endo-sponge therapy-related factors. The procedure for endo-sponge was the same in both groups with a sponge change at 2-3 d intervals. One patient had a fibrin glue injection after successful treatment to improve local healing. In one patient a stent was placed through the anal canal for 7 d until sufficient healing was achieved. Both patients had no recurrence during the follow-up period. The median duration of endo-sponge therapy was 21 d in both groups. Median interval between primary operation and onset of anastomotic leakage was longer in the non-successful group. This difference was statistically significant ($P < 0.05$).

Table 1 Characteristics of patients with and without successful long-term outcome after endo-sponge treatment[1]

	Successful outcome	No successful outcome
No. of patients	15	5
Age (yr)	67 (55-91.2)	63.4 (54.8-69.8)
BMI	24.9 (17.3-31.2)	24.5 (19.7-28.9)
Male:female ratio	10:5	3:2
Neoadjuvant treatment		
None	10	4
Radiotherapy	1	0
Chemo/radiotherapy	4	1
Indication		
Anastomotic leakage	12	5
Rectal stump insufficiency	3	0
Interval between primary operation and anastomotic leakage (d)	12.5 (3-668)	42 (21-668)
Duration of endo-sponge therapy (d)	21 (7-56)	21 (19-106)
Chemotherapy after endo-sponge therapy	3	1
Follow up time (mo)	16.1 (1.5-29.8)	17.1(8.38-26.2)
Interval between final endo-sponge therapy and recurrent abscess (d)		240 (21-730)
Local tumor recurrence	1	1

[1]Values are given as absolute numbers and frequencies (%) or as medians (min-max). BMI: Body mass index.

With regard to the oncological outcome, two patients (10%) developed a local tumor recurrence during the follow-up period and subsequently died. The UICC stage was Ⅱ and Ⅲ, respectively. Neither patient received any additional chemo- or radiotherapy prior to or after primary rectal resection. Notably, this was declined by one of them. Two patients (10%) developed distant liver metastasis.

DISCUSSION

This multicenter study was conducted to assess long-term outcome after primary successful endo-sponge treatment in patients with rectal cancer. The major finding of this analysis is the fact that 25% of patients developed recurrent abscess formation.

The endo-sponge system has been shown to be a suitable instrument for the treatment of anastomotic leaks following rectal cancer surgery. Glitsch *et al*[11] reported primary successful healing in 16 of 17 included patients. Each patient had a follow-up colonoscopy 2 mo after complete closure of the abscess cavity. Other studies with slightly longer follow-up periods found initial success rates ranging from 75% to nearly 100%[4-6].

Thus, this is the first study that indicates that late treatment failure might also occur. The consequence of our

finding is a recommendation of ongoing surveillance (at least 2 years) for recurrent abscess, since this might be easier to treat if diagnosed early.

In the present study, for 2 of 5 patients who developed a new abscess, recurrence occurred 30 d after endo-sponge therapy had been finished. The reason for reappearance, which obviously occurred soon after the therapy was considered a success, remains unclear. It is tempting to speculate that the wound healing was superficial, whereas in deeper areas of the cavity a new abscess formation was established. Notably, both patients had a late anastomotic leak (668 and 427 d after initial surgery), which could have further contributed to the worse outcome.

In one patient endo-sponge treatment was continued for 106 d. This extended duration was due to the firm desire of the patient not to receive a stoma formation. Although after that time period the abscess cavity was healed, such prolonged treatment might be predictive for a less successful outcome. This observation was comparable to previous published results[9].

We have also attempted to identify predictive parameters for reoccurrence of abscess. Taking into account the limited number of patients available for statistical analysis, we found that late onset of anastomotic leakage is associated with a tendency for a high probability of recurrent abscess formation. This correlated with the fact that late onset of endo-sponge therapy correlates with a worse outcome of primary therapy[8].

Interestingly, concomitant chemo- or radiotherapy was not associated with an increased risk of recurrent abscess. This is in contrast to the finding of von Bernstorff *et al*[12], who did observe a negative influence of radio-chemotherapy on the primary success rate. Therefore, the present finding is important as it relieves fear associated with application of chemotherapy in this setting.

The humanized monoclonal antibody Bevacizumab represents another therapy associated with potential colorectal anastomotic complications[13]. Of the 20 patients analyzed herein, 10% received Bevacizumab after initial endo-sponge therapy. At present, we do not see antiangiogenic therapy as an obstacle for endo-sponge therapy.

In addition, we observed 2 local recurrences of tumor growth. We cannot differentiate whether this high number of patients is an effect of anastomotic leakage or correlates with endo-sponge treatment. There are controversies regarding the impact of anastomotic leakage on oncologic outcome. Several studies found anastomotic leaks significantly associated with a worse cancer specific mortality and increased local recurrence[3,14,15]. However, others could not detect any correlation[16,17].

Since endo-sponge therapy is believed to rely on induction of angiogenesis, induction of tumor growth is a potential theoretical concern[18]. The question remains whether this has a biological consequence since angiogenesis formation is also part of chronic inflammation. Furthermore, tumor recurrence might also be induced by chronic inflammation. There is growing evidence that systemic inflammatory response, characterized by raised levels of circulating C-reactive protein, predicts poor survival in patients undergoing curative resection for colorectal malignancies[19,20]. Thus, it is possible that an ongoing septic condition caused by anastomotic leakage might negatively influence oncologic outcome. On the other hand, a more rapid healing as induced by endo-sponge therapy might even be beneficial.

Before the era of endo-sponge treatment anastomotic leakage was treated by defunctioning stoma. This procedure could save anastomosis in around 60% to 75% of cases[21,22]. Thus, the question arises, whether despite obvious macroscopic early improvements, endo-sponge treatment is of great benefit. Based on late treatment failures requiring Hartmann's procedures, we believe that a randomized trial with 2 years of follow-up will be necessary to solve this question. In the meantime, endo-sponge treatment can still be recommended as a therapeutic option for anastomotic leakage. The great advantages of this novel method are still rapid leak control and the avoidance of a defunctioning stoma in selected cases.

In conclusion, in the current multicenter study we observed that 25% of patients developed recurrent abscesses after primary successful endo-sponge assisted treatment for anastomotic leakage following rectal resection. Notably, 2 patients showed a local tumor recurrence during the follow-up period. This has to be investigated in larger series of patients.

COMMENTS

Background

Anastomotic leakage following rectal cancer surgery is regarded as one of the most feared postoperative complications with a considerable morbidity and mortality rate. The incidence varies from 2% to 19%. Strategies to treat anastomotic leaks are limited and depend on the septic condition of affected patients. The use of an endoluminal vacuum therapy (endo-sponge) represents a novel method to treat patients with anastomotic leakage after rectal resection.

Research frontiers

An open-pored endo-sponge is inserted into the abscess cavity and connected *via* draining tube to a vacuum drainage system. Through continuous negative pressure a shrinking and cleaning of the wound can be achieved. The research hotspot is to assess the long-term effect of endo-sponge therapy. It is still under debate, as to whether vacuum therapy-derived granulation tissue is stable and not prone to develop recurrent abscesses.

Innovations and breakthroughs

It has been recently published that endo-sponge assisted closure can be used as an alternative in the treatment of colorectal anastomotic leakage. The few available studies focusing on endo-sponge treatment found short-term healing rates of nearly 96%. Others concluded that extended leakages should be treated by different approaches having little probability of successful healing but these can lead to discomfort for the patient.

Applications

In the present study it was observed that 25% of patients developed recurrent abscesses after primary successful endo-sponge assisted treatment for anastomotic leakage following rectal resection. Consequently, patients need to be monitored closely as abscess recurrence might occur. Notably, 2 patients showed a local tumor recurrence during the follow-up period. This has to be investigated in larger series of patients.

Terminology

Endo-sponge treatment means the insertion of an open-pored sponge into the abscess cavity. The endo-sponge is connected *via* draining tube to a vacuum drainage system. Through continuous negative pressure a shrinking and cleaning of the wound can be achieved.

Peer review

This is a well-written hypothesis-generating paper. Although there might be many contributing factors, the authors present data that suggest endo-sponge may promote abscess formation a long time after treatment of a failed low anastomosis. This is worth knowing.

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BRIEF ARTICLE

A predictive factor for the response to S-1 plus cisplatin in gastric cancer

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Abstract

AIM: To prove that the protein expression level of thymidylate synthase is a predictive factor for the response to S-1/cisplatin (CDDP) chemotherapy in gastric cancer.

METHODS: We measured the protein expression levels of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and orotate phosphoribosyltransferase (OPRT) in advanced gastric cancer. Before S-1/CDDP chemotherapy, tumor specimens from primary sites were obtained by endoscopic biopsy and analyzed by enzyme-linked immunosorbent assay. The chemotherapeutic effects on the primary sites were evaluated by endoscopic biopsy performed more than once after S-1/CDDP chemotherapy. The effects are a predictive factor for the response to S-1/CDDP chemotherapy in patients with advanced gastric cancer, as evaluated by endoscopic biopsy over time.

RESULTS: The protein expression level of TS was significantly higher ($P < 0.05$) in the tumor than in the normal tissue, and significantly lower ($P < 0.05$) in the responders than in the non-responders. We were able to evaluate the correlation between changes in the protein expression levels of TS, DPD and OPRT and chemotherapeutic responses in 7 patients by assessing tumor tissues more than twice. In the responders, the protein expression level of TS was < 40 ng/mg protein. However, there were significant increases in the protein expression levels of TS ($P < 0.01$) and DPD ($P < 0.05$) after chemotherapy in 3 patients. In these cases, the patient assessment changed from "responder" to "non-responder". In the non-responders, the protein expression level of TS was > 40 ng/mg protein.

CONCLUSION: We have confirmed that the protein expression level of TS is a predictive factor for the response to S-1/CDDP chemotherapy in patients with advanced gastric cancer.

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Key words: Gastric cancer; Thymidylate synthase; Dihydropyrimidine dehydrogenase; Orotate phosphoribosyltransferase; Biopsy

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INTRODUCTION

In Japan, S-1 plus cisplatin (S-1/CDDP) chemotherapy is currently the most commonly used first-line chemotherapeutic regimen in patients with advanced gastric cancer. This is attributed to the high response rate (76%)[1] and significantly longer survival of patients administered with S-1/CDDP chemotherapy than with S-1 alone, as demonstrated in a randomized phase Ⅲ study[2]. However, it has also been reported that about 25% of patients treated with S-1/CDDP chemotherapy failed to show a significant response. Therefore, accurate prediction of the response to chemotherapy is essential for identifying the most effective drug and form of chemotherapy.

For predicting the response to chemotherapy, metabolic enzymes have recently received considerable attention as possible predictors. While the mechanism of metabolism of 5-fluorouracil (5-FU), a principal fluoropyrimidine used against colorectal cancer, has been clarified by many researchers, there have also been reports on the relationship between various metabolic enzymes and drug sensitivity, as well as between enzymes and clinical response. Recent studies have focused on the relationship between thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) or orotate phosphoribosyltransferase (OPRT) and prediction of response to fluoropyrimidines[3-7] (Figure 1).

The aim of this study was to confirm whether TS, DPD and OPRT can be used as predictors of the response of patients with advanced gastric cancer to S-1/CDDP chemotherapy by measuring their expression level from biopsy specimens over time. Measurements over time were carried out in biopsy specimens sampled using an endoscope. Endoscopic biopsy specimens have conventionally been considered to pose difficulties in the measurement of enzyme expression level owing to their small size. Nevertheless, we succeeded in measuring enzyme expression level in small specimens by enzyme-linked immunosorbent assay (ELISA)[8]. Effect of treatment on advanced gastric cancer was then examined by endoscopy. Endoscopic examination made it possible to confirm precisely whether TS, DPD and OPRT can be used as effective predictors of the response to S-1/CDDP chemotherapy using gastric cancer specimens. In addition, we also assessed whether the commonly used tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) can also be used as effective predictors of the response to S-1/CDDP chemotherapy.

MATERIALS AND METHODS

Patient eligibility

Patients with locally advanced or metastatic gastric cancer with primary sites were considered eligible. Further eligibility criteria were as follows: (1) histologically confirmed gastric adenocarcinoma; (2) an Eastern Cooperative Oncology Group performance status of 0-2; (3) over 20 years of age; (4) no prior chemotherapy or radiotherapy; (5) sufficient hematological, renal and hepatic functions; and (6) life expectancy over 12 wk. Written informed consent was obtained from all patients. This study was approved by the institutional ethical review board of Tokyo Medical University Hospital.

Chemotherapy and endoscopic assessment of chemotherapeutic effects

Patients received S-1 orally twice daily at least 1 h after breakfast and supper on days 1 to 21, followed by a 14-d recovery period. S-1 dosage according to the body surface area (BSA) of a patient was as follows: BSA < 1.25 m^2, 40 mg twice daily (80 mg day 1); BSA $\geqslant 1.25$ m^2 but < 1.5 m^2, 50 mg twice daily (100 mg day 1); BSA $\geqslant 1.5$ m^2, 60 mg twice daily (120 mg day 1). CDDP was administered intravenously over 2 h at 60 mg/m^2 on day 8. Chemotherapy cycles were repeated every 35 d.

Chemotherapeutic effects on primary sites were evaluated endoscopically by response assessment of chemotherapy and radiotherapy for gastric carcinoma: clinical criteria (Japanese Classification of Gastric Carcinoma-2nd English Edition)[9]. Patients were classified into 2 groups: "responders" and "non-responders". "Responders" was defined as patients with complete response (CR: disappearance of all tumoral lesions and no diagnosis of any cancers) or partial response (PR: dramatic regression, flattening on endoscopic examination roughly corresponding to at least a 50% decrease in tumor size). In this study, many metastatic lesions were presented in the form of lymph node or peritoneal dissemination. The shrinkage or growth of these lesions was difficult to evaluate only through the imaging procedure, and therefore only the primary lesions were evaluated. We accordingly added tumor markers for evaluation.

Specimens and measurement of protein expression level

Biopsy specimens were obtained from 2 sites of the tumor and 2 sites of the normal stomach area before the first chemotherapy and after every 2 cycles of chemotherapy. The forceps used were FB-25K-1 (Olympus Corp., Tokyo, Japan) and Radial Jaw3 1534 (Boston Scientific Corp., MA, USA). All specimens were immediately frozen and stored at -80℃. The specimens were assigned anonymous marks and enzyme expression level was measured by the Pharmacokinetic Research Laboratory of Taiho Pharmaceutical Co., Ltd. Specifically, the protein expression levels of TS, DPD and OPRT were determined by ELISA[10].

Statistical analysis

To evaluate the correlation between the protein expression levels of TS, DPD and OPRT and the anti-tumor effects of S-1/CDDP chemotherapy, the protein expression level before chemotherapy and the endoscopic assessment of chemotherapeutic effects after S-1/CDDP chemotherapy were ascertained. The Student's *t*-test was used to compare protein expression level and various factors. The paired *t*-test was used to compare tumor and normal areas, responders and non-responders, and the first and last

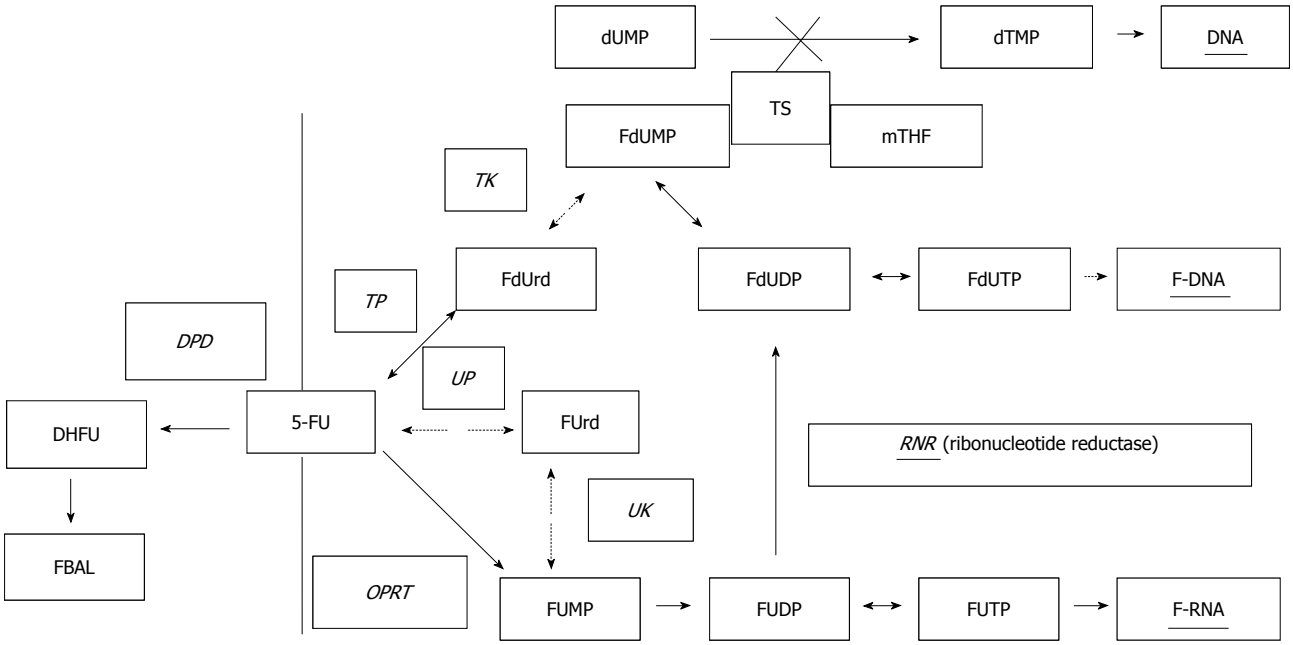

Figure 1 Metabolic pathway of 5-fluorouracil. DPD: Dihydropyrimidine dehydrogenase; TS: Thymidylate synthase; TK: Thymidine kinase; TP: Thymidine phosphorylase; UP: Uridine phosphorylase; OPRT: Orotate phosphoribosyl transferase; UK: Uridine kinase.

Table 1 Patient characteristics

Sex	Age (yr)	Performance status	Site	Size (cm)	Pathology	Metastasis
Male	62	1	Antrum	5	Diffuse	Peritoneum
Female	52	0	Fundus	4	Diffuse	Ovary
Female	67	2	Corpus	10	Diffuse	Lymph node
Male	68	2	Corpus	8	Intestinal	Lymph node
Male	78	2	Corpus	15	Intestinal	Lymph node
Male	64	2	Corpus	8	Intestinal	Liver
Male	67	2	Fundus	5	Intestinal	Liver
Female	70	2	Corpus	5	Diffuse	Peritoneum
Male	65	1	Corpus	4	Diffuse	Peritoneum
Female	59	2	Corpus	15	Diffuse	Peritoneum
Male	67	2	Corpus	5	Diffuse	Liver
Male	39	2	Corpus	20	Diffuse	Peritoneum
Female	62	0	Antrum	7	Intestinal	Liver
Male	77	1	Antrum	3	Intestinal	Liver

measurements in each patient. The JMP software program (version 7.0) was used in all analyses, and $P < 0.05$ was considered significant.

RESULTS

Patient characteristics

Fourteen patients were enrolled in this study between December 2005 and July 2007 conducted at the Department of Gastroenterology and Hepatology, Tokyo Medical University Hospital, Japan. Patient characteristics are shown in Table 1. All of the patients (9 males and 5 females; median age, 64 years) had primary sites of advanced gastric cancer. The primary site was the gastric body in 9 patients, gastric fundus in 2, and vestibular area in 3. The size of the primary tumor was < 5 cm in 3 patients, < 10 cm in 7, and at least 10 cm in 4. The histological type was highly differentiated adenocarcinoma in 6 patients and poorly differentiated adenocarcinoma in 8. The metastatic site was the lymph node in 3 patients, peritoneum in 5, liver in 5, and ovary in 1.

Protein expression levels of TS, DPD and OPRT in tumors and normal areas

The protein expression levels of TS, DPD and OPRT were measured in all 14 patients by ELISA before the first S-1/CDDP chemotherapy. The tumors and normal areas could also be measured in all 14 patients for DPD but in only 12 patients for TS and 12 patients for OPRT. The protein expression levels of TS, DPD and OPRT were 42.9 ± 19.1 ng/mg protein, 156.6 ± 63.3 ng/mg protein, and 9.3 ± 5.8 ng/mg protein in the tumors, and 21.5 ± 13.7 ng/mg protein, 168.1 ± 36.5 ng/mg protein, and 10.1 ± 6.1 ng/mg protein in the normal areas, respectively.

Table 2 Protein expression of thymidylate synthase, dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase in tumour tissues and matched normal tissues (ng/mg protein)

	No. of cases	Tumour tissue	Normal tissue	Paired *t*-test
TS	12	42.9 ± 19.9 (median 40.5)	21.5 ± 13.7 (median 19.9)	$P < 0.001$
DPD	14	156.6 ± 63.3 (median 143.3)	168.1 ± 36.5 (median 170.3)	NS
OPRT	12	9.3 ± 5.8 (median 8.7)	10.1 ± 6.1 (median 8.2)	NS

TS: Thymidylate synthase; DPD: Dihydropyrimidine dehydrogenase; OPRT: Orotate phosphoribosyl transferase; NS: Not significant.

Table 3 Protein expression of thymidylate synthase, dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase in relation to responder or non-responder on the first S-1/CDDP therapy (ng/mg protein)

	Responder (n = 6)	Non-reponder (n = 4)	*t*-test
TS	27.4 ± 9.4 (median 29.2)	56.9 ± 19.9 (median 53.6)	$P < 0.05$
DPD	170.9 ± 91.4 (median 135.7)	148.9 ± 33.5 (median 155.6)	NS
OPRT	6.1 ± 3.7 (median 6.5)	9.6 ± 9.7 (median 5.8)	NS

TS: Thymidylate synthase; DPD: Dihydropyrimidine dehydrogenase; OPRT: Orotate phosphoribosyl transferase; NS: Not significant.

The protein expression level of TS, but not of DPD and OPRT, in the tumors was significantly higher than that in the normal areas ($P < 0.001$) (Table 2). There was no difference in the protein expression levels of TS, DPD and OPRT in the tumors with regard to patient characteristics, gender, tumor size and pathological type.

Protein expression levels of TS, DPD and OPRT before chemotherapy and in response to the first chemotherapy

The relationship between the protein expression levels of TS, DPD and OPRT in the tumors and the endoscopic assessment of the first chemotherapeutic response was investigated. Measurement of expression levels from biopsy specimens, administration of S-1/CDDP chemotherapy, and endoscopic assessment of the primary tumor could be carried out in 10 of the 14 patients. The protein expression level of TS in responders was significantly lower (27.4 ± 9.4 ng/mg protein) than that in non-responders (56.9 ± 19.9 ng/mg protein) ($P < 0.05$, Table 3). There was no marked difference in the protein expression levels of DPD and OPRT between responders and non-responders.

Correlation between the changes in the protein expression levels of TS, DPD and OPRT and chemotherapeutic responses

The correlation between the changes in the protein expression levels of TS, DPD and OPRT and the chemotherapeutic responses could be evaluated more than twice in each of 7 patients. After the first S-1/CDDP chemotherapy, 5 of the 7 patients showed a response and the remaining 2 showed no response. Of the 5 responders, 2 showed a continuous response and the remaining 3 started to show a worse response after several courses. Two non-responders in the first chemotherapy underwent a second S-1/CDDP chemotherapy but showed no response.

The protein expression levels of TS, DPD and OPRT before the first chemotherapy were compared with those after tumor progression or with the latest measurement in each patient. In 2 patients, a continuous but nonsignificant response was observed from the initial response to the last response with regard to the protein expression levels of TS, DPD and OPRT. On the other hand, in 3 patients who showed tumor progression after the initial response, a significant increase in the protein expression levels of TS ($P < 0.01$) and DPD ($P < 0.05$) was observed at the time of non-response compared with the time of the initial response. In addition, in the 2 non-responders after the second chemotherapy, a significant increase in the protein expression level of DPD ($P < 0.05$) was observed. These results clearly show that the changes in the protein expression level of TS in the primary tumors correlated with the changes in the response to S-1/CDDP chemotherapy in these patients (Table 4). The changes in the individual protein expression levels of TS, DPD and OPRT in the 7 patients are shown in Figure 2. In these 7 patients, the protein expression level of TS was 27.6 ± 6.5 ng/mg protein in the responders and 66.1 ± 22.4 ng/mg protein in the non-responders ($P < 0.05$). The protein expression levels of DPD and OPRT were 181.1 ± 75.7 ng/mg protein and 6.2 ± 3.2 ng/mg protein, respectively, in the responders and 231.0 ± 100.6 ng/mg protein and 6.1 ± 2.0 ng/mg protein, respectively, in the non-responders.

Correlation between CEA or CA19-9 level and endoscopy-assessed chemotherapeutic response

Correlations between CEA or CA19-9 level and the endoscopy-assessed chemotherapeutic response of the primary tumor were also studied. We could evaluate the correlation between CEA or CA19-9 level and the endoscopy-assessed response of the primary tumor after the first S-1/CDDP chemotherapy in 10 of the 14 patients. The CEA and CA19-9 levels in 6 responders were 6.0 ± 4.2 mg/dL and 110.4 ± 116.8 mg/dL, and 51.4 ± 86.7 mg/dL and 7168.9 ± 14268.8 mg/dL in 4 non-responders, respectively. In 7 patients, although the correlation between the changes in the CEA and CA19-9 levels and the responses could be evaluated more than twice in each patient, no correlations were found.

DISCUSSION

This study examined whether the protein expression level of TS is a predictive factor for the response to S-1/

Table 4 Correlation between changes of protein expression of thymidylate synthase, dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase and responses (ng/mg protein)

	1st evaluation	2nd evaluation	Paired *t*-test
Responder(1st evaluation) → responder (2nd evaluation) (n = 2)			
TS	30.0 ± 2.8	19.2 ± 2.3	NS
DPD	161.7 ± 53.3	172.6 ± 22.8	NS
OPRT	9.8 ± 0.6	4.7 ± 1.7	NS
Responder(1st evaluation) → non-responder(2nd evaluation) (n = 3)			
TS	31.7 ± 4.6	53.9 ± 3.9	$P < 0.01$
DPD	204.4 ± 119.5	279.1 ± 124.7	$P < 0.05$
OPRT	4.3 ± 3.1	5.3 ± 1.3	NS
Non-responder(1st evaluation) → non-responder (2nd evaluation) (n = 2)			
TS	73.4 ± 10.7	77.3 ± 45.4	NS
DPD	142.3 ± 52.6	247.5 ± 60.5	$P < 0.05$
OPRT	5.9 ± 1.3	8.4 ± 1.9	NS

TS: Thymidylate synthase; DPD: Dihydropyrimidine dehydrogenase; OPRT: Orotate phosphoribosyl transferase; NS: Not significant.

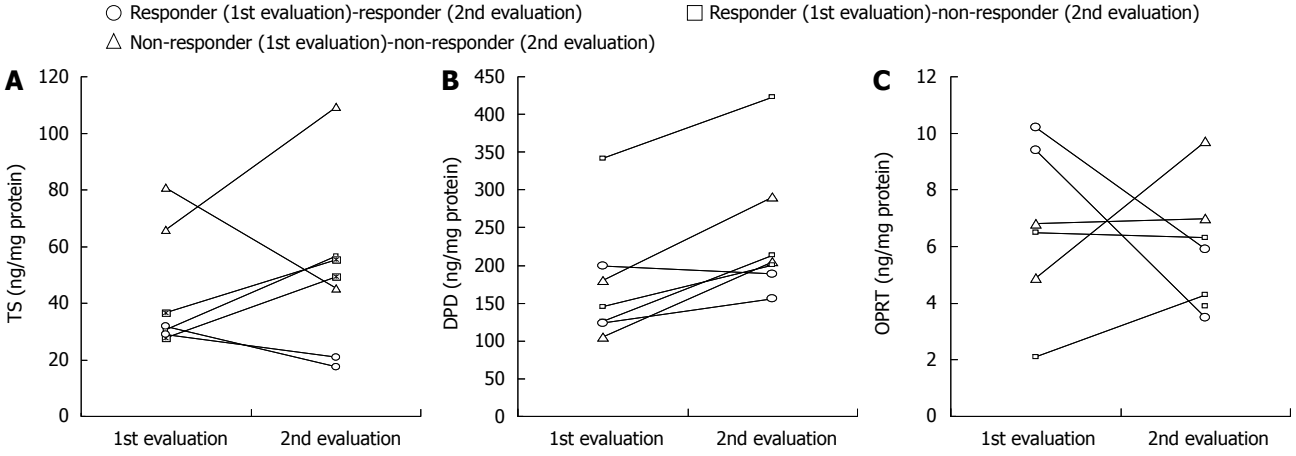

Figure 2 Changes in the protein expression level of thymidylate synthase, dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase. 1st evaluation: Protein expression and response at the first S-1/CDDP chemotherapy; 2nd evaluation: Protein expression and response at the first progression after response or the latest S-1/CDDP chemotherapy. TS: Thymidylate synthase; DPD: Dihydropyrimidine dehydrogenase; OPRT: Orotate phosphoribosyl transferase.

CDDP chemotherapy in patients with advanced gastric cancer. Patients with a low protein expression level of TS showed a response to S-1/CDDP chemotherapy, whereas those with a high protein expression level of TS showed no response to S-1/CDDP chemotherapy. Furthermore, in 3 patients who showed tumor progression after the initial response, a significant increase in the protein expression level of TS ($P < 0.01$) was observed at the time of tumor progression compared with the time of the initial response. On the other hand, although the protein expression level of DPD had not been expected to be correlated with the response to S-1/CDDP chemotherapy, the results indicate a possible association.

S-1 is a dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine, which has been shown to produce a high response rate against advanced gastrointestinal cancer in phase Ⅱ studies[11,12]. S-1 is an oral anticancer agent consisting of tegafur, the prodrug of 5-FU, 5-chloro-2,4-dihydroxypyridine, a strong DPD inhibitor, and potassium oxonate, which inhibits OPRT in the gastrointestinal tract, resulting in the suppression of gastrointestinal toxicity caused by the phosphoribosylation of 5-FU[13]. Considering that cancer has a high proliferative ability, nucleic acid metabolism is more active and the nucleic acid metabolic enzyme normally shows increased levels in cancer[14].

Amatori *et al*[15] reported that high sensitivity to 5-FU was associated with a low expression level of TS *in vitro*. Salonga *et al*[16] described that the intratumoral gene expression level of DPD was associated with tumor response to 5-FU. Meropol *et al*[17] conducted a biomarker analysis and provided preliminary evidence that the expression level of TP (thymidine phosphorylase) may be a predictive marker for treatment response in patients with metastatic colorectal cancer. In addition, Honda *et al*[18] reported that TP and DPD are predictive factors for the therapeutic efficacy of capecitabine monotherapy for breast cancer. On the other hand, Harada *et al*[19] reported that the expression of TS in biopsy samples before S-1 chemotherapy was significantly lower in responders than in non-responders with oral squamous cell carcinoma ($P = 0.0001$). Ichikawa *et al*[20] investigated simple combinations of 2 genes, namely, OPRT and TS, which

may allow the identification of gastric cancer patients who will benefit from S-1 chemotherapy. Shimizu *et al*[21] reported that S-1 may be effective even in gastric scirrhous carcinoma with a high level of DPD activity.

These previous results are consistent with ours. However, the specimens used for assessment in most of these past studies had been resected during surgery. In contrast, our assessment is based on endoscopic biopsy specimens. Since the amount of specimens that were endoscopically collected by biopsy in our study was very small, we used ELISA for all measurements to confirm whether the protein expression levels of TS, DPD and OPRT may be a predictive factor for the response to S-1/CDDP chemotherapy. Fukui *et al*[22] investigated the differences in the protein expression levels of TS, DPD and OPRT by ELISA in various tumor tissue specimens. They reported that comparison of the protein expression levels of these enzymes among matched tumor and non-tumor tissue specimens revealed significantly higher expression levels of TS (25.3 ng/mg protein) and DPD (150.3 ng/mg protein) in gastric cancer. This suggests that measurements of even small amounts of endoscopic biopsy materials would not be different from those of large-scale studies. Further, Koga *et al*[23] collected biopsy materials from the oral cavity of a great number of patients and tried to determine TS and DPD with ELISA. Their published report also showed that a small amount of specimens could produce correct measurements.

The present study indicated that the protein expression level of TS was significantly lower ($P < 0.05$) in the responders than in the non-responders. This result is consistent with the findings of previous reports which showed fluorinated pyrimidine to be effective in the case of a low expression level of TS. In 2 patients who continued to show low protein expression levels of TS, the responses to S-1/CDDP chemotherapy continued for more than 5 and 8 mo during the study period. In addition, 2 non-responders after the first course of S-1/CDDP chemotherapy demonstrated continuously high protein expression levels of TS. As expected, the second course of S-1/CDDP chemotherapy was also not effective. Because 2 responders continued to show both low protein expression level of TS and a high chemotherapeutic response, unfortunately we could not identify 5 patients whose chemotherapeutic evaluations changed from "responder" to "non-responder" during the study period. However, these results clearly proved that the protein expression level of TS in gastric cancer is a predictive factor for the response to S-1/CDDP chemotherapy. In all responders, the protein expression level of TS was $\leqslant$ 36.7 ng/mg protein, whereas it was $\geqslant$ 45.2 ng/mg protein in all non-responders. It was considered that 40 ng/mg protein was therefore the approximate cutoff value between responders and non-responders.

We suspected that the protein expression level of DPD may not be related to response to S-1/CDDP chemotherapy, because S-1 is a strong DPD-inhibitory fluoropyrimidine drug. In the present study, there was no difference in the protein expression level of DPD between the responders and the non-responders after the first course of S-1/CDDP chemotherapy. Therefore, it is speculated that the protein expression level of DPD is not a predictive factor for response to S-1/CDDP chemotherapy in gastric cancer. However, in this study on the relationship between the changes in the protein expression level of DPD and the endoscopy-assessed response at the individual level, a significant increase in the protein expression level of DPD ($P < 0.05$) was observed in the patients with proceeding tumor progression.

The results indicate that a significant increase in the protein expression level of DPD can be considered a predictive factor for the progression of S-1/CDDP chemotherapy at the individual level. The protein expression level of DPD was increased in 6 of 7 patients (Figure 2); it will be necessary to investigate a large number of patients in the future before any definitive conclusion can be made.

Regarding the relationship between the changes in the protein expression level of OPRT and the response to S-1/CDDP chemotherapy in patients with advanced gastric cancer, no data indicating any type of relationship was obtained.

How to select an effective chemotherapeutic agent to avoid unnecessary treatment in patients with advanced gastric cancer, specifically in terms of how comfortably patients can spend their survival time, in addition to the problem of how to improve patient survival whenever possible, are highly important questions that must be taken into consideration. Presently in Japan, S-1/CDDP chemotherapy is the most popular regimen as first-line chemotherapy in patients with advanced gastric cancer. Therefore, one alternative method is to attempt a tailor-made treatment to predict the effectiveness of S-1/CDDP chemotherapy for advanced gastric cancer by monitoring the protein expression level of TS in endoscopic biopsy specimens.

Based on the results of this study, it has been confirmed that the response to S-1/CDDP chemotherapy in patients with a low protein expression level of TS should be determined, and that the same S-1/CDDP chemotherapy should be continuously administered as the first-line treatment. However, the first-line treatment should be changed to the second-line treatment in patients whose protein expression level of TS tends to increase, considering that S-1/CDDP chemotherapy will become ineffective. In gastric cancer, specimens cannot easily be collected from the oral cavity or other parts of the body surface. Unlike surgical samples, many specimens cannot be obtained from different sites. Therefore, the number of samples was small in our study. While this was a weakness, the advantage was that the specimens could repeatedly be collected. Further investigation of a larger number of patients is required.

There is as yet no report in which the correlation between the protein expression levels of TS, DPD and OPRT and the effects of chemotherapy was studied more than

twice in each patient. This study strongly suggested that the protein expression level of TS is a predictive factor for the response to S-1/CDDP chemotherapy in patients with advanced gastric cancer, because changes in the protein expression level of TS correlated with changes in the response to S-1/CDDP chemotherapy after evaluating biopsy specimens more than twice in all patients.

COMMENTS

Background

In Japan, S-1 plus cisplatin (S-1/CDDP) chemotherapy is currently the most commonly used first-line chemotherapeutic regimen in patients with advanced gastric cancer. Medical doctors usually rely on their experience in deciding the best timing for changing first-line chemotherapy to second-line chemotherapy. Determination of the optimum timing for changing the treatment modality from first-line to second-line chemotherapy, together with the precise prediction of response to chemotherapy, is therefore expected to improve clinical outcome.

Research frontiers

The specimens used for assessment in most past studies had been resected during surgery. In contrast, the authors' assessment is based on endoscopic biopsy specimens. They measured the protein expression levels of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and orotate phosphoribosyltransferase (OPRT). Before S-1/CDDP chemotherapy, tumor specimens from primary sites were obtained by endoscopic biopsy. Since the amount of specimens that were endoscopically collected by biopsy in their study was very small, they used enzyme-linked immunosorbent assay for all measurements to confirm whether the protein expression levels of TS, DPD and OPRT may be a predictive factor for the response to S-1/CDDP chemotherapy.

Innovations and breakthroughs

The protein expression level of TS was significantly higher in tumors than in normal tissue, and significantly lower in the responders than in the non-responders. There is as yet no report in which the correlation between the protein expression levels of TS, DPD and OPRT and the effects of chemotherapy was studied more than twice in each patient.

Applications

The authors have confirmed that the protein expression level of TS is a predictive factor for the response to S-1/CDDP chemotherapy in patients with advanced gastric cancer. This will assist medical doctors in avoiding chemotherapeutic regimens with strong side effects and thus prevent a decrease in the quality of life of patients.

Terminology

"S-1" is an oral anticancer agent consisting of tegafur, the prodrug of 5-fluorouracil (5-FU), 5-chloro-2,4-dihydroxypyridine, a strong DPD inhibitor, and potassium oxonate, which inhibits orotate phosphoribosyltransferase in the gastrointestinal tract which results in the suppression of gastrointestinal toxicity caused by the phosphoribosylation of 5-FU.

Peer review

This is an original article which discussed whether the quantity of the expression of TS proteins could be a predictive factor for the response of S-1/CDDP chemotherapy. This is a very significant and interesting topic, and also clinically very important and estimable.

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BRIEF ARTICLE

Clinicopathological evaluation of duodenal well-differentiated endocrine tumors

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Abstract

AIM: To assess the clinicopathological characteristics of duodenal well-differentiated endocrine tumors.

METHODS: We examined clinicopathological characteristics in 11 consecutive patients with duodenal well-differentiated endocrine tumors treated by endoscopic therapy or surgery in our hospital from 1992 through 2007. Patients with well-differentiated endocrine tumors of the papilla of Vater or with gastrinoma were excluded.

RESULTS: Three patients received endoscopic treatment, and 8 underwent surgery. In patients who received endoscopic treatment, the tumor diameter was less than 1.0 cm, with no histopathological evidence of lymphovascular invasion or invasion of the muscularis. There were no complications such as late bleeding or perforation after treatment. Among 8 patients with tumors less than 1.0 cm in diameter, 3 underwent partial resection, and 2 underwent radical surgery. Three patients had lymphovascular invasion, 1 had invasion of the muscularis, and 1 had proximal lymph node metastasis. Among 3 patients with tumors 1.0 cm or more in diameter, 1 underwent partial resection, and 2 underwent radical surgery. One patient had lymphovascular invasion, with no lymph node metastasis. After treatment, all patients are alive and have remained free of metastasis and recurrence.

CONCLUSION: Duodenal well-differentiated endocrine tumors less than 1.0 cm in diameter have a risk of lymphovascular invasion, invasion of the muscularis, and lymph node metastasis, irrespective of procedural problems.

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Key words: Duodenal well-differentiated endocrine tumors; Endoscopic resection; Surgical operation

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INTRODUCTION

Neuroendocrine tumor is defined as a tumor associated

with neuroendocrine differentiation. There has been confusion regarding the concept of neuroendocrine tumor. This has been especially complicated by the long standing concept of "Karzinoide Tumor" proposed by Oberndorfer in 1907[1], which develop more slowly than carcinomas arising at the same site clinically. Neuroendocrine tumor is currently classed into: (1) Well-differentiated endocrine tumor (WDET) (synonymous with carcinoid tumor); (2) Well-differentiated endocrine carcinoma (synonymous with malignant carcinoid tumor); (3) Poorly-differentiated endocrine carcinoma (synonymous with small cell carcinoma); (4) Mixed-endocrine tumor; and (5) Tumor-like lesion associated to its degree of differentiation, cell proliferation or other histological features[2].

About 70% of WDET arise from the gastrointestinal tract. In Japan the most common site is the rectum (41.5%), followed by the stomach (26.3%), duodenum (16.5%), and cecum (7.2%). In Europe and North America, the cecum is the most common site, followed by the ileum and rectum. Duodenal WDET account for only 2.6% of all neuroendocrine tumors[3,4]. Increased use of upper gastrointestinal endoscopy for health checkups has led to increased detection rates of WDET. However, duodenal WDET are a rare disease diagnosed in only a small number of patients. The natural history of duodenal WDET is therefore poorly understood, and standard treatment strategies have yet to be established.

Soga[5] reported that lymph node metastasis was associated with 9.8% of gastrointestinal neuroendocrine tumors with submucosal invasion, even when the tumor diameter was 1.0 cm or less, suggesting that the risk of metastasis does not differ appreciably from that of carcinomas. Burke *et al*[6] studied a series of 99 patients with duodenal WDET and reported that a tumor diameter of 2.0 cm or greater, invasion of the muscularis propria, and mitotic figures are risk factors for lymph node metastasis. On the basis of safety, effectiveness, and patients' quality of life, Dalenbäck *et al*[7] recommended endoscopic therapy for the management of duodenal WDET 1.0 cm or less in diameter with no evidence of distinct invasion of the muscularis on endoscopic ultrasonography.

Many studies have reported the usefulness of endoscopic treatment for WDET of the rectum[8] and stomach[9]. Duodenal WDET have also been treated endoscopically[10]. At present, the decision to perform endoscopic treatment for duodenal WDET is primarily made on the basis of tumor diameter (1.0 cm or less) and the depth of invasion (up to submucosal). However, even small lesions have a risk of lymph node metastasis[4,5,11]. The indications for endoscopic treatment and radical surgery with lymph node dissection remain controversial. We studied the clinicopathological characteristics in 11 patients with duodenal WDET treated in our hospital.

MATERIALS AND METHODS

The study group comprised 11 patients with duodenal WDET who received endoscopic treatment or surgery at the Department of Gastroenterology or the Department of Gastrointestinal Surgery, Kitasato University East Hospital from 1992 through 2007. Before treatment, all patients underwent upper gastrointestinal endoscopy. WDET were diagnosed by biopsy. Patients with WDET of the papilla of Vater and those with gastrinoma were excluded from the study. Abdominal computed tomography (CT) and upper gastrointestinal endoscopic ultrasonography (EUS) were performed to evaluate the depth of tumor invasion and the presence or absence of metastasis. Local resection (endoscopic treatment or partial resection) or radical surgery with extended (D2) lymph node dissection was performed.

From 1992 to 2005, all patients underwent open surgery. Local resection was performed if the tumor diameter was less than 1.0 cm on preoperative evaluation, and more radical resections with lymph node dissection were performed if the tumor diameter was 1.0 cm or greater. (Table 1, No. 1 to 7). However, curative resection was additionally performed in patients who were found to have a tumor diameter of 1.0 cm or greater or invasion of the muscularis, lymphovascular invasion, mitotic figures, or nuclear atypia on postoperative histopathological examinations. From 2005 through 2007, tumors less than 1.0 cm in diameter on preoperative evaluation were treated endoscopically. Curative resection was additionally performed on the basis of the results of histopathological examination (Table 1, No. 8 to 11).

For endoscopic treatment, endoscopic aspiration mucosectomy was performed as described by Tanabe *et al*[12]. The lesion margins were marked by argon plasma coagulation (APC), and a solution of 10% glycerin plus fructose (Glyseol, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was locally injected into the submucosa to cause the lesion to bulge. To perform endoscopic treatment safely, the endoscope (GIFXQ-230; Olympus Optical Co., Tokyo, Japan) was inserted through an overtube (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). An aspiration mucosector (Top Co., Ltd., Tokyo, Japan) was attached to the tip of the endoscope. The endoscope was then reinserted, and the lesion was aspirated into a hood. The tumor margin was confirmed, the snare was opened, and the lesion was strangled. Mucosectomy was then performed by applying high-frequency current.

The following clinicopathological findings were recorded: age, sex, the presence or absence of symptoms, the presence or absence of carcinoid syndrome, endoscopic findings (the presence or absence of a central depression, erosions, and ulcers), tumor diameter, depth of invasion, lymphovascular invasion, mitotic figures, grade of nuclear atypia, and the presence or absence of lymph node metastasis. Proliferative activity of tumor cells was assessed by immunostaining with a monoclonal mouse antihuman Ki-67 antibody (MIB-1, N1633, DAKO, ChemMate Envision kit) and a monoclonal mouse antihuman p53 antibody (DO-7, M7001, 1:500, DAKO, ChemMate Envision kit). Tumor diameter was measured postoperatively on histopathological specimens. WDET were diagnosed histopathologically according to the criteria of the World Health Organization International Histological Classification of Tumors[2,13].

Table 1 Clinicopathological features of 11 patients with duodenal neuroendocrine tumors

Patient No.	Location	Age (yr)	Sex	Size (cm)	EUS	Accuracy rate	Depth of invasion	Lymphatic invasion	Venous invasion	LN	Treat	L/D metastasis
1	Bulbs	42	M	0.2	sm		sm	0	0	0	LR	None
2	Bulbs	68	F	0.7	NE		sm	0	0	0	LR	None
3	Bulbs	56	F	1.1	m		sm	0	0	0	LR	None
4	Bulbs	57	F	0.9	sm		mp	0	2	0	SG	None
5	Bulbs	62	M	1.1	sm	7/9 (77%)	sm	0	2	0	SG	None
6	2nd portion	55	M	1.2	sm		sm	0	0	0	PD	None
7	Bulbs	71	M	0.7	sm		sm	0	1	0	LR	None
8	2nd portion	59	M	0.9	sm		sm	0	0	0	EMR	None
9	Bulbs	56	M	0.9	sm		sm	0	1	1 (No. 4 d)	SG	None
10	Bulbs	60	M	0.7	sm		sm	0	0	0	EMR	None
11	Bulbs	54	M	0.7	NE		sm	0	0	0	EMR	None

EUS: Endoscopic ultrasonography; NE: Not evaluated; LN: Lymph node metastasis; No. 4 d LN: Lymph node metastasis along the right gastroepiploic vessels; sm: Submucosa; mp: Muscularis propria; EMR: Endoscopic mucosal resection; LR: Local resection; SG: Subtotal gastrectomy; PD: Pancreaticoduodenectomy; L/D metastasis: Local/distant metastasis.

Figure 1 Upper gastrointestinal endoscopy showed a submucosal-tumor-like, protruding lesion 0.7 cm in diameter, arising in the anterior wall of the duodenal bulb. The top of the tumor was yellowish white, with dilated blood vessels.

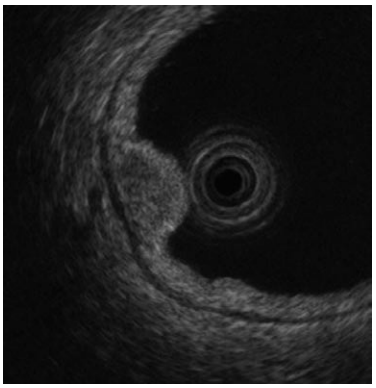

Figure 2 Upper gastrointestinal endoscopy showed a homogenous, oval hypoechoic mass, mainly located in the third layer.

Follow-up

The site that underwent endoscopic treatment was confirmed by the presence of a scar. To check for local recurrence around the scar formed at the site of endoscopic therapy, upper gastrointestinal endoscopy was performed 2, 6 and 12 mo after treatment and at 6 mo intervals thereafter. To confirm the presence or absence of distant metastasis, CT was performed at 6 mo intervals. In patients who underwent surgical resection of their tumors, upper gastrointestinal endoscopy and CT were performed at 6 mo intervals to confirm the presence or absence of recurrence.

RESULTS

Eleven consecutive patients with duodenal WDET (8 men and 3 women) were studied. Their median age was 57 years (range 42 to 71 years). The median follow-up period was 54 mo (range 6 to 201 mo) (Table 1). The tumors were located in the duodenal bulb in 9 patients and in the descending duodenum in 2 (Figure 1). All patients had only 1 lesion. No patient had carcinoid syndrome. No WDET was associated with von Recklinghausen disease, multiple endocrine neoplasm type I or asynchronous or synchronous malignant tumors. As for symptoms, 1 patient had dysphagia, and 1 had melena. All other patients were asymptomatic. Most WDET were diagnosed coincidentally on follow-up evaluation of gastric ulcers, follow-up after endoscopic mucosal resection (EMR) of early gastric cancer, follow-up for duodenal ulcers, or routine health screening. Among 9 tumors in the duodenal bulb and 2 in the descending duodenum, 7 had a central depression, including 1 with a deep depression. No patient had erosions or ulcers.

Upper gastrointestinal EUS was performed in 9 patients. All lesions had round or oval, homogenous, low-level internal echoes (Figure 2). Invasion of the muscularis was misdiagnosed as submucosal invasion in only 1 patient. As compared with the results of histopathological examination of the resected specimens, the depth of invasion was correctly diagnosed on EUS in 7 (77%) of 9 patients, indicating good results. On preoperative abdominal CT, no patient had evidence of lymph node metastasis, liver metastasis, or distant metastasis to other organs. Three patients were treated endoscopically, and 8 underwent surgery. The median tumor diameter was 0.9 cm (range 0.2-1.2 cm). All 3 patients who received endoscopic treatment had tumors less than 1.0 cm in diameter that were confined to the submucosa, with no distinct evidence

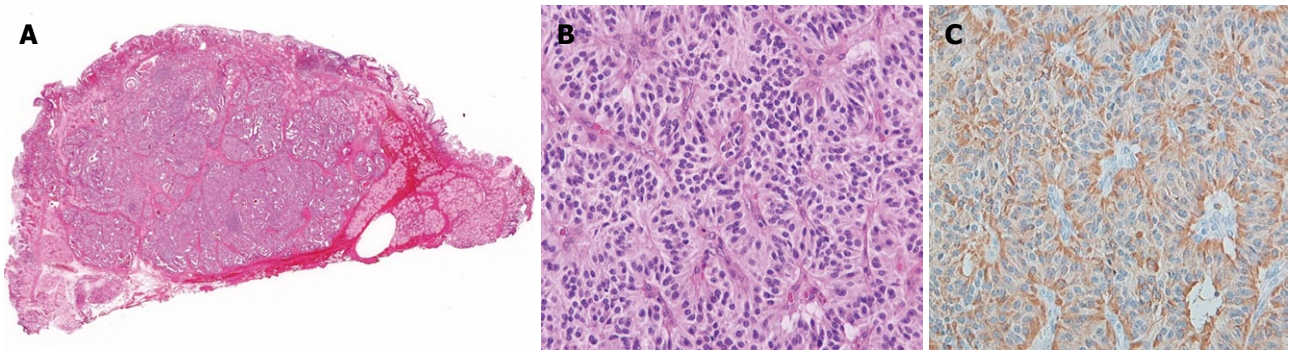

Figure 3 Histopathological examination. A: Macroscopic view of resected specimens obtained by endoscopic mucosal resection (hematoxylin and eosin staining). The longest diameter was 0.7 cm; B: Histopathological examination of specimens (hematoxylin and eosin staining, × 10) showed that cubic, atypical cells forming follicular or glandular patterns, with rounded nuclei and eosinophilic syncytia; C: Histopathological examination of specimens (chromogranin A staining, × 10) showed that tumors stained positively for chromogranin A.

Table 2 Pathological findings of 11 patients with well-differentiated endocrine tumor

Patient No.	Location	Size (cm)	Histological type	Depth of invasion	Lymphatic invasion	Venous invasion	Mitotic count (HPF)	Ki67/MIB1 Index (%)	LN	Direct invasion	Treatment
1	Bulbs	0.2	WD	sm	0	0	< 2	< 1	0	0	LR
2	Bulbs	0.7	WD	sm	0	0	< 2	< 1	0	0	LR
3	Bulbs	1.1	WD	sm	0	0	< 2	< 1	0	0	LR
4	Bulbs	0.9	WD	mp	0	2	< 2	< 1	0	0	SG
5	Bulbs	1.1	WD	sm	0	2	< 2	< 1	0	0	SG
6	2nd portion	1.2	WD	sm	0	0	< 2	< 1	0	0	PD
7	Bulbs	0.7	WD	sm	0	1	< 2	< 1	0	0	LR
8	2nd portion	0.9	WD	sm	0	0	< 2	< 1	0	0	EMR
9	Bulbs	0.9	WD	sm	0	1	< 2	< 1	1 (No. 4 d)	0	SG
10	Bulbs	0.7	WD	sm	0	0	< 2	< 1	0	0	EMR
11	Bulbs	0.7	WD	sm	0	0	< 2	< 1	0	0	EMR

WD: Well-differentiated; LN: Lymph node metastasis; No. 4 d LN: Lymph node metastasis along the right gastroepiploic vessels; sm: Submucosa; mp: Muscularis propria; EMR: Endoscopic mucosal resection; LR: Local resection; SG: Subtotal gastrectomy; PD: Pancreaticoduodenectomy; 10HPF (high power field): At least 10 fields (at 40 × magnification) evaluated in area of highest mitotic density.

of lymphovascular invasion or invasion of the muscularis on histopathological examination (Figure 3). There were no treatment-related complications, such as bleeding or perforation. Among 8 patients with tumors less than 1.0 cm in diameter, 3 received partial resection and 2 curative resection (distal gastrectomy in both). Three patients had lymphovascular invasion, 1 had invasion of the muscularis, and 1 had proximal lymph node metastasis (Table 1, No. 9). Among 3 patients with tumors 1.0 cm or greater in diameter, 1 received partial resection and 2 curative resection (distal gastrectomy in 1 and pancreaticoduodenectomy in 1). One patient had lymphovascular invasion, with no evidence of lymph node metastasis. No tumor showed distinct nuclear atypia or mitotic figures. On immunostaining, all tumors had a Ki-67 labeling index of 1% or less and tested negative for p53. In the patient with proximal lymph node metastasis (Table 1, No. 9), the tumor diameter was 0.9 cm, with no invasion of the muscularis, nuclear atypia, or mitotic figures. The Ki-67 labeling index was less than 1%, but lymphovascular invasion was positive. In 1 patient with a tumor less than 1.0 cm in diameter, lymphovascular invasion was found on local resection (Table 2). Because of advanced age, the patient was followed up without performing additional resection (Table 1, No. 7). At the time of this writing, all patients are alive, with no distinct evidence of metastasis or recurrence.

DISCUSSION

Our retrospective study showed even duodenal WDET 1.0 cm or less in diameter can be associated with invasion of the muscularis or lymphovascular invasion, considered high-risk factors for metastasis. One patient in our series had lymphovascular invasion with proximal lymph node metastasis. Whether endoscopic treatment is indicated for duodenal WDET has not been fully examined because of the rarity of these tumors. As for biologic malignancy, duodenal WDET are characterized by lower grades of atypia and malignancy than carcinomas. Similar to rectal WDET[5,14-16], endoscopic therapy has been used to treat duodenal WDET up to 1.0 cm in diameter that are limited to the submucosa. Such lesions are considered to have a relatively low risk of lymph node metastasis. Duodenal WDET arise from endocrine cells in the gastrointestinal mucosa and penetrate beyond the muscularis mucosae and invade the submucosa at an early stage. Because of these features, duodenal WDET appear to be submucosal tumors, although they arise from the mucosal endothelium[17]. EUS is

Table 3 Risk factor without metastasis of duodenal well-differentiated endocrine tumors

Author	*n*	Risk factor without metastasis of duodenal WDET
Burke *et al*[6], 1990	99	2.0 cm or less in diameter, no mitotic figures, no invasion of the muscularis propria
Zyromski *et al*[21], 2001	27	2.0 cm or less in diameter
Mullen *et al*[11], 2005	24	1.0 cm or less in diameter, submucosal lesions

WDET: Well-differentiated endocrine tumors.

very useful for evaluating the depth of invasion of duodenal WDET. If the tumor is confined to the submucosa, the lesion is mainly present in the third layer, depicted as a well demarcated, hypoechoic mass with homogenous, low-level internal echoes[18,19]. In our series, a correct diagnosis was made on EUS in 7 (77%) of 9 patients. Preoperative EUS is thus considered useful for diagnosis.

In patients with gastrointestinal neuroendocrine tumors, tumor diameter and depth of invasion are related to the risk of metastasis. The depth of invasion is mucosal in 1.7% of tumors, submucosal in 10.5%, the muscularis propria in 29.6%, and subserosal or serosal in 42.8%[20]. The incidence of metastasis in patients with gastrointestinal neuroendocrine tumors invading the submucosa increases in parallel to tumor diameter: 0.5 cm or less, 6.0%; 1.0 cm or less, 13.3%; 2.0 cm or less, 23.9%; and more than 2.0 cm, 38.4%[5]. Soga[5] retrospectively studied 1914 cases of gastrointestinal neuroendocrine tumors limited to the submucosa and found that tumor diameter was 0.5 cm or less in 8.3% of lesions, 1.0 cm or less in 10.5%, 2.0 cm or less in 13.8%, and greater than 2.0 cm in 25.8%. In Western countries, Burke *et al*[6] studied 99 patients with duodenal WDET and found that lesions that were 2.0 cm or less in diameter or had no mitotic figures or invasion of the muscularis propria had a low risk of lymph-node metastasis (Table 3). Zyromski *et al*[21] studied 27 patients with duodenal WDET and reported that tumors 2.0 cm or less in diameter could be safely and effectively treated by local resection alone, without recurrence. On the basis of the safety, effectiveness, and patients' quality of life, Dalenbäck *et al*[7] recommended endoscopic therapy for the management of duodenal WDET 1.0 cm or less in diameter that have no evidence of muscular invasion on EUS. Among 24 patients with duodenal WDET, however, Mullen *et al*[11] found that 2 of 7 patients with lymph node metastasis had submucosal lesions that were 1.0 cm or less in diameter, indicating that lymph node metastasis could not be accurately predicted solely on the basis of tumor diameter or depth of invasion. Biologic markers of cell proliferative activity, such as Ki-67 and p53, have sporadically been reported to be related to metastasis from gastrointestinal neuroendocrine tumors[13,14,22], but these markers were negative in all of our patients.

At present, endoscopic treatment is mainly indicated for duodenal WDET 1.0 cm or less in diameter that are confined to the submucosa, with no distinct invasion of the muscularis. In our series, radical surgery with lymph node dissection was performed in all patients with tumors 1.0 cm or more in diameter or with suspected invasion of the muscularis on preoperative examinations, including EUS. Tumors that were less than 1.0 cm in diameter and confined to the submucosa underwent local resection or endoscopic treatment. However, patients with duodenal WDET should be carefully followed up, including histopathological examination after endoscopic treatment, because postoperative examination of histopathological specimens showed that even duodenal WDET less than 1.0 cm in diameter can be associated with lymphovascular invasion, muscular invasion, or proximal lymph node metastasis. The incidence of metastasis associated with duodenal WDET is estimated to be about 10% even when the tumor diameter is 1.0 cm or less, similar to that of carcinomas[23]. The biologic malignancy of duodenal WDET may thus differ from that of carcinoid tumors arising in the rectum[5,8,14-16] and stomach[9].

As for the endoscopic treatment of duodenal WDET, EMR is more difficult to perform in the duodenum than in the stomach because of its very thin wall and narrow lumen[24]. Moreover, EMR can cause complications such as late bleeding and perforation. In particular, the incidence of late bleeding is very high (25.5% to 33.0%) after EMR for duodenal tumor[25,26], as compared with early gastric cancer (1.4%)[27], early esophageal cancer (3.6%)[28], and early colorectal cancer (0.3% to 2.7%)[29,30]. Lépilliez *et al*[26] reported that therapeutic or prophylactic hemostasis by clipping or APC decreased the rate of late bleeding from 21.7% to 0% in patients who underwent EMR for sporadic duodenal adenomas. In our study, EMR was done in 3 patients with tumors arising in the duodenal bulb or descending duodenum. After the procedure, the exposed vessels at the ulcer floor were treated with a hemostatic forceps. Complications such as late bleeding were prevented by performing second-look endoscopy on the day after treatment. Future studies examining the correlation between tumor diameter in millimeters and the presence or absence of lymph node metastasis in large numbers of patients may help to more clearly define the indication range for endoscopic treatment. The discovery of new biomarkers may also assist physicians in deciding whether additional surgery is needed.

In conclusion, we clinically and histopathologically studied 11 patients with duodenal WDET treated in our hospital. Duodenal WDET less than 1.0 cm in diameter have a risk of lymphovascular invasion, invasion of the muscularis, and lymph node metastasis, irrespective of procedural problems. Fully informed consent should be obtained, and patients should be closely followed up, including histopathological evaluation, after endoscopic therapy.

COMMENTS

Background

The diameter and depth of invasion of well-differentiated endocrine tumors (so-called carcinoid tumors) have been shown to correlate with lymph node metastasis. The treatment strategy of choice remains controversial.

Research frontiers

Many studies described tumor diameter of 1.0 cm or greater, invasion of the

muscularis propria, and mitotic figures as risk factors for lymph node metastasis of well-differentiated endocrine tumors.

Innovations and breakthroughs

Duodenal well-differentiated endocrine tumors less than 1.0 cm in diameter have a risk of lymphovascular invasion, invasion of the muscularis, and lymph-node metastasis.

Applications

Patients with duodenal well-differentiated endocrine tumors should be closely followed up, including histopathological evaluation, if endoscopic treatment has been performed.

Peer review

Ishido *et al* reported their institutional experience on the clinicopathological evaluation of carcinoid tumors of the duodenum.

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BRIEF ARTICLE

Endoscopic removal of gastric ectopic pancreas: An initial experience with endoscopic submucosal dissection

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Abstract

AIM: To evaluate the therapeutic usefulness and safety of endoscopic resection in patients with gastric ectopic pancreas.

METHODS: A total of eight patients with ectopic pancreas were included. All of them underwent endoscopic ultrasonography before endoscopic resection. Endoscopic resection was performed by two methods: endoscopic mucosal resection (EMR) by the injection-and-cut technique or endoscopic mucosal dissection (ESD).

RESULTS: We planned to perform EMR in all eight cases but EMR was successful in only four cases. In the other four cases, saline spread into surrounding normal tissues and the lesions became flattened, which made it impossible to remove them by EMR. In those four cases, we performed ESD and removed the lesions without any complications.

CONCLUSION: If conventional EMR is difficult to remove gastric ectopic pancreas, ESD is a feasible alternative method for successful removal.

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Key words: Ectopic pancreas; Endoscopic resection; Endoscopic ultrasonography

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INTRODUCTION

Ectopic pancreas, also called heterotopic or aberrant pancreas, is defined as pancreatic tissue lying outside its normal location and lacking anatomic or vascular connections with the pancreas. It has been found in 0.6% to 13% of autopsies and has also been noted in approximately one of every 500 surgical operations involving the upper abdomen[1]. Ectopic pancreas is mostly found in the upper gastrointestinal tract adjacent to the pancreas; in 90% of patients with ectopic pancreas, it was found in the stomach, duodenum, or proximal part of the jejunum[2]. Histologic diagnosis of ectopic pancreas is usually difficult when tissue specimens are obtained using a standard endoscopic biopsy forceps[3].

Pathological diagnosis of ectopic pancreas is usually unachievable for two reasons: because adequate tissue samples cannot usually be taken during endoscopic biopsy using standard forceps[3] and because surgery is usually unnecessary for most asymptomatic patients. Recently, endoscopic ultrasonography (EUS) was reported to be helpful for diagnosing ectopic pancreas[4,5]. However, the accuracy of EUS for the diagnosis of subepithelial tumors is limited[6].

Most patients with ectopic pancreas are asymptomatic although a minority may present with a variety of symptoms, the most common being epigastric pain[7]. Options for treatment for gastric ectopic pancreas include observation, surgery[7,8], or endoscopic resection[6,9-11]. However, previous reports describing various methods of endoscopic resection were based on a limited number of cases. Therefore, we evaluated the therapeutic usefulness and safety of endoscopic resection in eight cases of gastric ectopic pancreas.

MATERIALS AND METHODS

We retrospectively analyzed our database of all patients who underwent endoscopic resection at Pusan National University Hospital from July 2006 to December 2009. We identified a total of eight patients who were diagnosed as ectopic pancreas after endoscopic resection. All of them underwent EUS before endoscopic resection. This study was reviewed and approved by the Institutional Review Board at Pusan National University Hospital.

EUS was performed with a radial scanning 20 MHz catheter probe (UM3D-DP20-25R, Olympus, Tokyo, Japan). The probe was passed through the instrument channel of a one-channel endoscope (GIF-H260, Olympus) or a two-channel endoscope (GIF-2T240, Olympus). All examinations were performed under intravenous conscious sedation (midazolam with or without meperidine). Scanning of the lesion was performed after filling the stomach with 400-800 mL of deaerated water. EUS features of the lesions such as size, sonographic layer of origin, border appearance, echogenicity, and homogeneity were evaluated.

Endoscopic resection was performed by two methods. If the lesion was properly elevated after saline injection, endoscopic mucosal resection (EMR) was performed by the injection-and-cut technique (Figure 1). If the lesion was not properly elevated after saline injection, endoscopic mucosal dissection (ESD) was performed (Figure 2). First, the margins of the lesion were marked by needle knife and submucosal saline injection with a small amount of epinephrine (0.025 mg/mL) and indigo carmine was used to lift the lesion. Then, a circumferential incision into the submucosa and submucosal dissection was performed around the lesion with an insulation-tipped (IT) knife. After removal, the *en bloc* pathologic specimen was mounted and oriented to facilitate histologic examination.

RESULTS

The eight patients included one man and seven women and ranged in age from 18 to 57 years (mean, 36 years). Four patients presented with dyspepsia or epigastric pain. The subepithelial lesions were incidentally diagnosed in the other four patients without preceding symptoms. Five lesions were located at the antrum and three lesions were located at the lower body. None of the lesions showed endoscopic findings such as umbilication or central dimpling. Conventional biopsies were performed on five lesions, but none were diagnosed as ectopic pancreas as based on pathology.

According to EUS, the lesions were mainly located in the second (deep mucosal) or third (submucosal) layer and ranged from 6 to 12 mm (mean 8 mm) in size (Table 1). All lesions were hypoechoic; five lesions were homogenous and three lesions were heterogeneous. The border was distinct in five lesions (5/8, 62.5%) and indistinct in three lesions (3/8, 37.5%). An undulated margin was observed in six lesions (6/8, 75%) and anechoic cystic or tubular structures appeared in three lesions (3/8, 37.5%).

For accurate diagnosis of the subepithelial lesions, EMR was performed. To decrease the risk of perforation or bleeding, we first planned to remove the lesions by EMR. In all cases, we injected saline solution including a small amount of epinephrine and indigo carmine beneath the lesions. In four cases, the lesions were properly elevated and then were resected by the injection-and-cut technique. However, in the other four cases, the saline spread into surrounding normal tissue and the lesions became flattened, which made it impossible to remove the lesions *via* the injection-and-cut technique. Therefore, we decided to perform ESD on these four lesions and removed them successfully without any complications. There were no recurrences during the median follow-up period of 30 mo (range 24 to 40 mo).

DISCUSSION

Most patients with ectopic pancreas are asymptomatic, but symptoms may rarely occur due to the irritating effect of hormones and enzymes secreted by the ectopic pancreas[8]. Rare complications resulting from ectopic pancreas have been reported, including gastric outlet obstruction, obstructive jaundice, intestinal obstruction, and intussusceptions[8]. Asymptomatic patients with ectopic pancreas can generally be monitored with treatment reserved for patients who are symptomatic, have enlarging lesions or require diagnostic certainty.

Ectopic pancreas is most often detected as an incidental finding during routine upper endoscopy. The typical endoscopic finding is a firm round or oval subepithelial lesion with a central depression, which corresponds to the opening of a duct. The gross appearance of central dimpling or umbilication implies a presumptive diagnosis of ectopic pancreas during preoperative endoscopy[12]. The characteristic EUS features of ectopic pancreas, including indistinct margins, heterogeneous echogenicity (mainly hypoechoic accompanied by scattered small hyperechoic areas), presence of an anechoic area and fourth-layer thickening, and location within the second, third, and/or fourth layers are very useful for establishing a preoperative diagnosis of

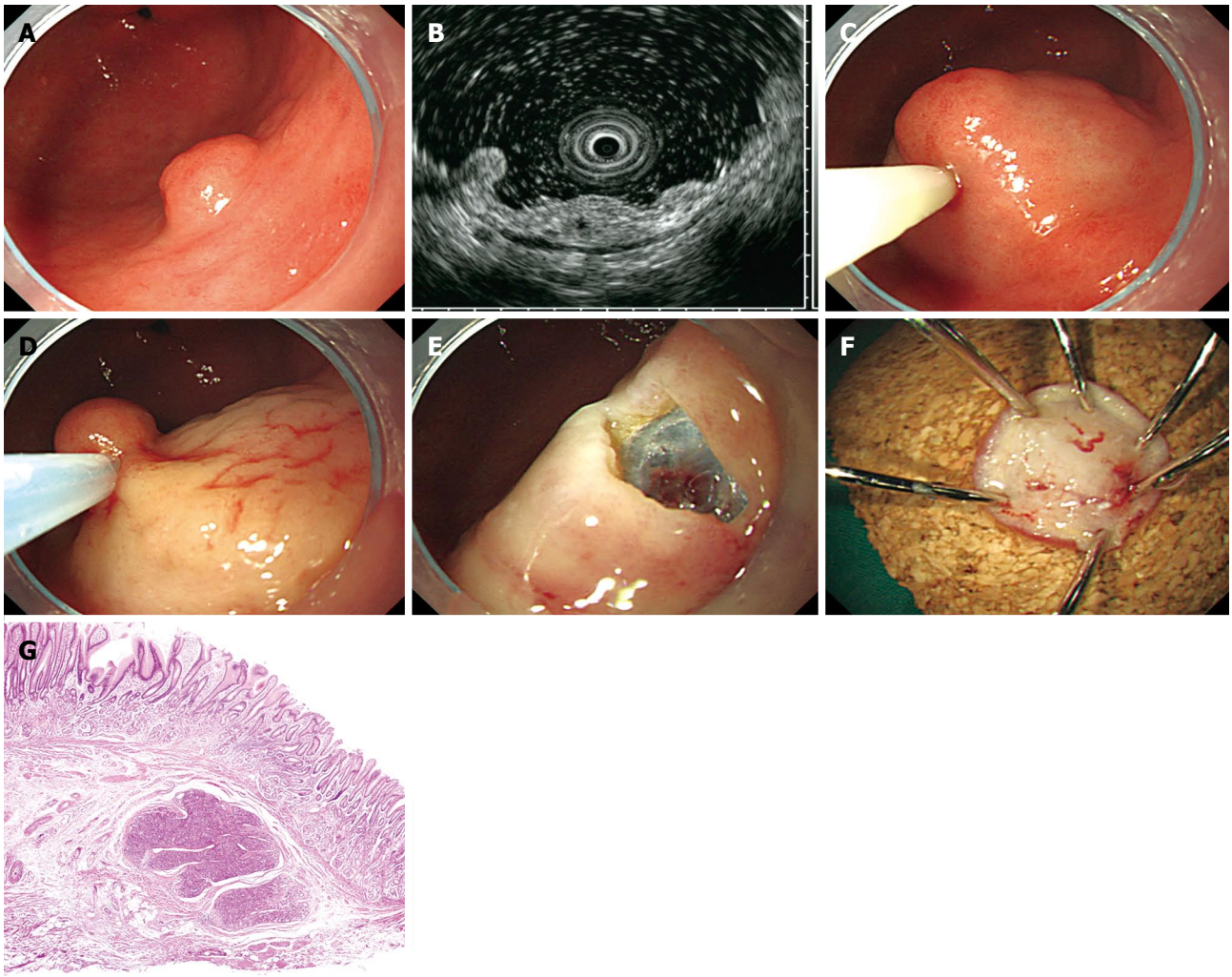

Figure 1 Endoscopic mucosal resection of ectopic pancreas by injection-and-cut technique (case 3). A: A subepithelial lesion is observed on the greater curvature of the antrum; B: Endoscopic ultrasonography image obtained with a 20 MHz catheter probe. An indistinct, heterogeneous, and hypoechoic lesion with a small anechoic space is located within the submucosal layer; C: Saline with indigo carmine is injected into the submucosa beneath the lesion; D: The lesion is resected using an electrocautery snare; E: The lesion is completely removed; F: The inner surface of the resected specimen; G: Histologically, the ectopic pancreas is located in the submucosa (HE, × 40).

Table 1 Summary of clinicopathologic and endoscopic ultrasonography features in eight patients with ectopic pancreas

Case	Sex	Age (yr)	Symptoms	Location	EUS features						Treatment	Follow-up period (mo)
					Layer	Size (cm)	Echogenicity	Homogenicity	Border	Anechoic area		
1	F	18	Dyspepsia	Antrum	2, 3	0.9	Hypoechoic	Homogenous	Distinct	Absent	EMR	28
2	F	44	None	Antrum	2, 3	0.6	Hypoechoic	Homogenous	Distinct	Absent	EMR	40
3	F	37	None	Antrum	3	0.7	Hypoechoic	Heterogeneous	Indistinct	Absent	EMR	35
4	M	45	None	Lower body	3	0.9	Hypoechoic	Homogenous	Distinct	Present	EMR	21
5	F	43	None	Lower body	3	0.8	Hypoechoic	Homogenous	Distinct	Absent	ESD	27
6	F	22	Epigastric pain	Antrum	3	0.6	Hypoechoic	Heterogeneous	Indistinct	Present	ESD	38
7	F	57	Epigastric pain	Antrum	2, 3	1.2	Hypoechoic	Heterogeneous	Indistinct	Present	ESD	26
8	F	24	Dyspepsia	Lower body	2, 3	0.9	Hypoechoic	Homogenous	Distinct	Absent	ESD	24

EUS: Endoscopic ultrasonography; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

ectopic pancreas[4,12]. Heterogeneous hypoechoic or mixed echogenicity, resembling that of the normal pancreatic parenchyma, corresponds to the presence of acinous tissue with scattered adipose tissue within the lesion[4]. Anechoic areas indicate duct dilatation, and fourth-layer thickening is considered a consequence of the hypertrophy of the muscularis propria[4].

Although these endoscopic and EUS findings are suggestive of ectopic pancreas, the accuracy for the diagnosis of subepithelial tumors is limited[6,13]. In fact, none

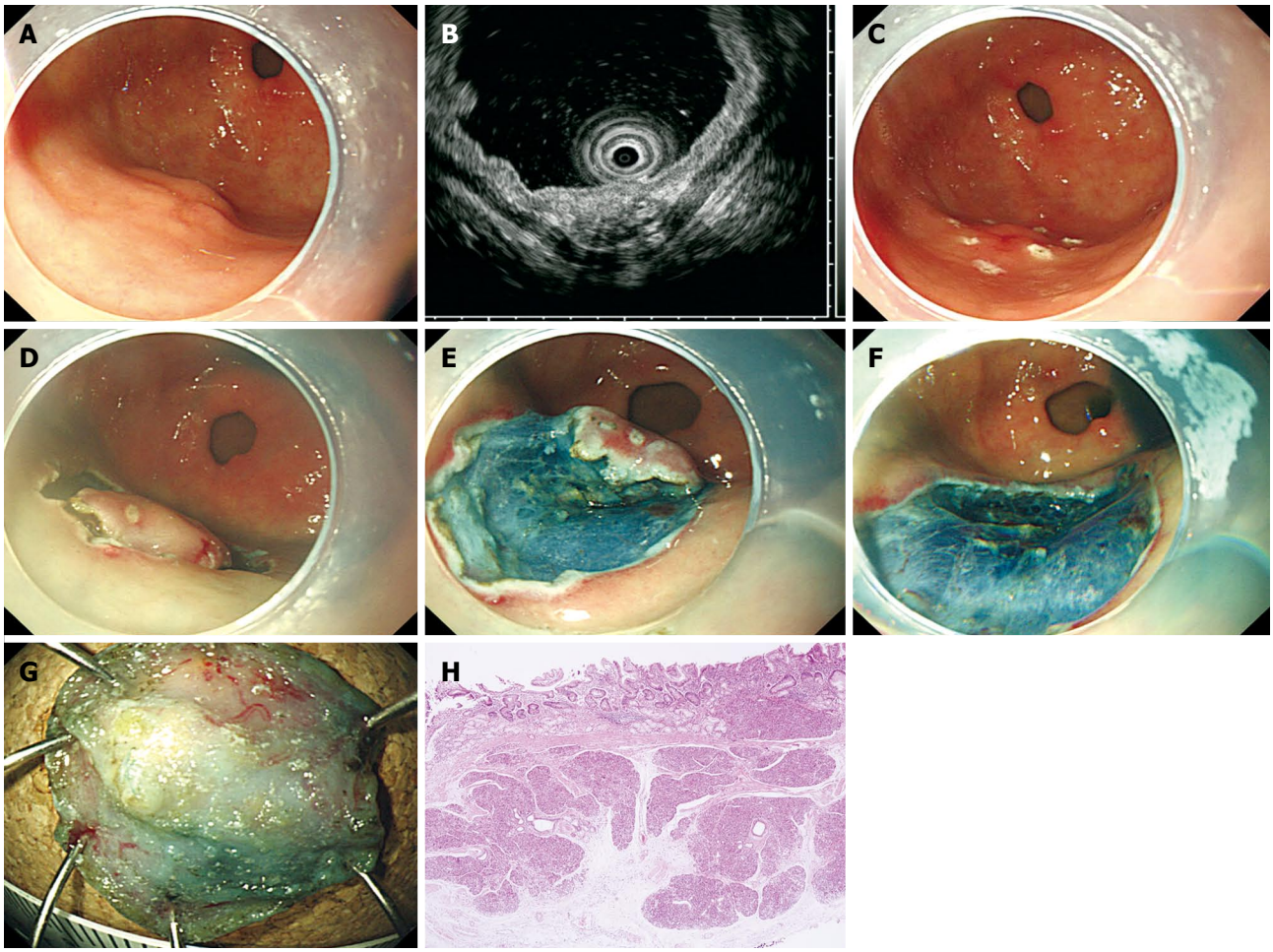

Figure 2 Endoscopic submucosal dissection of ectopic pancreas (case 7). A: A subepithelial lesion with a tiny erosion due to a previous biopsy is observed on the greater curvature of the antrum; B: Endoscopic ultrasonography image obtained with a 20 MHz catheter probe. An indistinct, heterogeneous, and hypoechoic lesion with small anechoic spaces is located within the deep mucosal and submucosal layer; C: By using a standard needle knife, a mark is made on the margin of the lesion; D: Saline injection with epinephrine and indigo carmine is injected into the submucosa beneath the lesion, and then a complete circumference incision is made using an IT knife; E: The IT knife is used to dissect the submucosa; F: The lesion is completely removed; G: The inner surface of the resected specimen; H: Histologically, the ectopic pancreas tissue is located in the deep mucosa and submucosa (HE, × 40).

of our cases showed typical endoscopic findings such as central dimpling or umbilication and four of them did not exhibit a characteristic anechoic duct structure by EUS. Therefore, three lesions with anechoic duct structure were diagnosed as ectopic pancreas but the other five lesions, without anechoic duct structure, were suspicious as ectopic pancreas or were diagnosed as other diseases such as inflammatory fibrinoid polyp.

Histological diagnosis of ectopic pancreas is usually difficult when tissue specimens are obtained using conventional endoscopic biopsy forceps. For precise histological diagnosis, endoscopic techniques for obtaining deeper specimens are necessary, such as EUS-guided biopsy or combined strip biopsy and bite-on-bite biopsy[14-16]. Endoscopic removal of gastric ectopic pancreas is also useful for accurate diagnosis and treatment[11]. The diagnosis of ectopic pancreas was not made based on the pathological appearance of specimens taken with standard endoscopic biopsy forceps in any our cases.

EUS provides the most useful information regarding tumor location within the gastric wall, helps to distinguish subepithelial lesions, and assists in establishing indications for endoscopic removal[4]. Endoscopic removal of submucosal lesions, especially ESD, is considered dangerous because of the risk of perforation or bleeding[17]. There have only been a few reports describing EMR methods for gastric ectopic pancreas, such as strip biopsy[6,10], cap-assisted EMR[9,18], or ligation-assisted EMR[11]. In the present study, we first planned to remove the lesions by EMR and we therefore injected saline beneath the lesions. However, in four cases, we were forced to switch to ESD and removed the lesions without any complications. The current series, to our knowledge, is the first to describe the use of ESD for removal of gastric ectopic pancreas. Therefore, in cases for which conventional EMR is difficult or impossible, ESD may be used as an alternative method for successful removal of ectopic pancreas.

COMMENTS

Background

Ectopic pancreas is mostly found in the upper gastrointestinal tract adjacent

to the pancreas; in 90% of patients with ectopic pancreas, it was found in the stomach, duodenum, or proximal part of the jejunum. Histologic diagnosis of ectopic pancreas is usually difficult when tissue specimens are obtained using a standard endoscopic biopsy forceps. Recently, endoscopic ultrasonography (EUS) was reported to be helpful for diagnosing ectopic pancreas. However, the accuracy of EUS for the diagnosis of subepithelial tumors is limited.

Research frontiers

Options for treatment for gastric ectopic pancreas include observation, surgery, or endoscopic resection. There have only been a few reports describing endoscopic mucosal resection (EMR) for gastric ectopic pancreas, such as strip biopsy, cap-assisted EMR, or ligation-assisted EMR. In the present study, the authors first planned to remove the lesions by EMR and we therefore injected saline beneath the lesions. However, in some cases, they were forced to switch to endoscopic submucosal dissection (ESD) and removed the lesions without any complications. The current series is the first to describe the use of ESD for removal of gastric ectopic pancreas

Innovations and breakthroughs

To decrease the risk of perforation or bleeding, EMR is usually used to remove submucosal lesions. Usually saline solution including a small amount of epinephrine and indigo carmine is injected beneath the lesions. Then, the lesions are properly elevated and are resected by the injection-and-cut technique. However, in some cases, the saline spreads into surrounding normal tissue and the lesions become flattened, which makes it impossible to remove the lesions *via* the injection-and-cut technique. In these cases, ESD may be used as an alternative method for successful removal of subepithelial lesions.

Applications

When conventional EMR is difficult or impossible, ESD may be used as an alternative method for successful removal of subepithelial lesions such as ectopic pancreas.

Terminology

Ectopic pancreas, also called heterotopic or aberrant pancreas, is defined as pancreatic tissue lying outside its normal location and lacking anatomic or vascular connections with the pancreas. It has been found in 0.6% to 13% of autopsies and has also been noted in approximately one of every 500 surgical operations involving the upper abdomen.

Peer review

The paper is well written and easy to read. It is also well supported by excellent endoscopic images and pathology slides.

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BRIEF ARTICLE

Double balloon endoscopy increases the ERCP success rate in patients with a history of Billroth Ⅱ gastrectomy

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Abstract

AIM: To evaluate the effect of double balloon endoscope (DBE) on the endoscopic retrograde cholangiopancreatography (ERCP) success rate in patients with a history of Billroth Ⅱ (B Ⅱ) gastrectomy.

METHODS: From April 2006 to March 2007, 32 patients with a B Ⅱ gastrectomy underwent 34 ERCP attempts. In all cases, the ERCP procedures were started using a duodenoscope. If intubation of the afferent loop or reaching the papilla failed, we changed to DBE for the ERCP procedure (DBE-ERCP). We assessed the success rate of afferent loop intubation, reaching the major papilla, selective cannulation, possibility of therapeutic approaches, procedure-related complications, and the overall success rate.

RESULTS: Among the 32 patients with a history of B Ⅱ gastrectomy, the duodenoscope was successfully passed up to the papilla in 22 patients (69%), and cannulation was successfully performed in 20 patients (63%). Six patients (2 with failure in afferent loop intubation and 4 with failure in reaching the papilla) underwent DBE-ERCP. The DBE reached the papilla in all the 6 patients (100%) and selective cannulation was successful in 5 patients (83%). Four patients (67%) who had common bile duct stones were successfully treated. One patient underwent diagnostic ERCP only and the other one, in whom selective cannulation failed, was diagnosed with papilla cancer proven by biopsy. There were no complications related to the DBE. The overall ERCP success rate increased to 88% (28/32).

CONCLUSION: The overall ERCP success rate increases with DBE in patients with a previous B Ⅱ gastrectomy.

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Key words: Double balloon endoscopy; Endoscopic retrograde cholangiopancreatography; Billroth Ⅱ gastrectomy

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is an important procedure for the diagnosis and treatment of hepatobiliary and pancreatic diseases. The ERCP suc-

cess rate exceeds 95% in patients with a normal gastrointestinal anatomy[1,2]. ERCP is also increasingly carried out in patients with a history of Billroth Ⅱ (B Ⅱ) gastrectomy, but its success rate in this group of patients is low (60%-92%)[3-7]. This procedure is more difficult because of problems encountered in entering the afferent loop, maneuvering the endoscope through the afferent loop to reach the major papilla, retrograde cannulation of the common bile duct, and performing an endoscopic sphincterotomy (EST) in a reverse direction[3,4]. The traditional examination method, using a side-viewing duodenoscope, is not always successful in some of these patients. If percutaneous transhepatic cholangiography and drainage (PTCD) or repeat surgery is not possible when the traditional method fails, double balloon endoscope (DBE) may be an excellent alternative treatment for these patients.

DBE was first introduced by Yamamoto *et al*[8] in 2001 as a novel endoscopic technique that allows examination of the entire small bowel. Presently, DBE is not only applied in diagnosis but also in endoscopic therapeutic interventions such as argon plasma coagulation for hemostasis, polypectomy, balloon dilation of small bowel strictures, and placement of enteral stents[9-12]. ERCP using DBE has been performed in patients with Roux-en-Y anastomosis[13-16], demonstrating that DBE system can be used to perform ERCP in patients with a surgically altered anatomy. In this paper, we describe the use of DBE in the ERCP procedure (DBE-ERCP) to increase the overall ERCP success rate in patients with a history of B Ⅱ gastrectomy. In these patients, the ERCP procedure using a traditional duodenoscope was unsuccessful after afferent loop intubation or reaching the major papilla with a long afferent loop failed.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Chang Gung Memorial Hospital. From April 2006 to March 2007, ERCP procedure was performed for 968 patients in our therapeutic endoscopy center. Of the 968 patients, 32 (21 men and 11 women) who had a previous B Ⅱ gastrectomy underwent 34 ERCP attempts (3.5%). The mean age of these 32 patients was 75.8 years (range 45-91 years). The procedure was always started with a side-viewing duodenoscope. If afferent loop intubation or reaching the papilla using the traditional duodenoscope was not possible, we changed to DBE for the ERCP procedure.

The DBE-ERCP procedure was performed using a 200-cm-long Fujinon double balloon endoscopy system which is 9.4 mm in diameter (EN-450 T5/W, Fuji Photo Optical Co., Ltd., Omiya, Japan). This double balloon endoscope has a 2.8-mm accessory channel through which therapeutic interventions may be carried out. Biliary cannulation was achieved using a long catheter (Glo-tip ERCP catheter, GT-1-TE, 320-cm, Cook Endoscopy, Winston-Salem, NC) and a long Axcess 21 guide wire (AX-21-650E, 650-cm, Cook Endoscopy, Winston-Salem, NC). After successful biliary cannulation, a controlled radial expansion (CRE) balloon dilation catheter (Boston Scientific Corporation, Natick, MA) was used for endoscopic papillary balloon dilation (EPBD). A long extraction balloon (ESCORT Ⅱ double lumen extraction balloon, EBL-18-320E, 320-cm, Cook Endoscopy) was used for retrieval of biliary stones. Fluoroscopic pictures of the DBE-ERCP procedure are shown in Figure 1, and endoscopic pictures are shown in Figure 2.

The endoscopic procedure was performed under conscious sedation with midazolam (median dose 3.5 mg, range 2-6 mg), and pethidine (median dose 40 mg, range 30-60 mg). The DBE-ERCP was performed using a standard push-and-pull technique. The patients were placed in the prone position with their blood pressure, heart rate, and pulse oximetry monitored. Oxygen was administered if its saturation level dropped below 90%. The DBE-ERCP technique was explained to the patients and their family members. Written informed consent was obtained from all patients.

Table 1 Success rate and failure of endoscopic retrograde cholangiopancreatography using a side-viewing duodenoscope in 32 patients with a history of Billroth Ⅱ gastrectomy

Characteristic	*n* (%)
Endoscope successfully reached the papilla	22 (69)
Successful cannulation	20 (63)
Cannulation by the rendezvous technique	2 (6)
Failed attempt	10 (31)
Failure in afferent loop intubation	2 (6)
Failure in reaching to the papilla	6 (19)
Endoscope-related perforation	2 (6)

RESULTS

Among the 32 patients with a history of B Ⅱ gastrectomy, the side-viewing duodenoscope was successfully passed up to the papilla in 22 patients (69%), and a successful cannulation was performed in 20 patients (63%). Two patients in whom selective biliary cannulation (n = 2, 6%) failed were successfully treated with a rendezvous technique. ERCP using a duodenoscope was unsuccessful in 10 patients (31%) when afferent loop intubation (n = 2, 6%), or in reaching the papilla failed because of a long afferent loop (n = 6, 19%), or endoscope-related small bowel perforation (n = 2, 6%) (Table 1).

Of the 8 patients (2 with failure in afferent loop intubation and 6 with failure in reaching the papilla, excluding those with perforation) who underwent ERCP using DBE, 2 were excluded because they refused to undergo DBE-ERCP since it was not covered by our national health insurance. The main indications for ERCP using DBE were biliary stones (n = 3, 50%), painless jaundice (n = 1, 17%), biliary pancreatitis (n = 1, 17%), and a dilated bile duct (n = 1, 17%). The DBE reached the papilla in all the 6 patients (100%). Selective cannulation and endoscopic treatment were successful in 5 patients (83%, 4

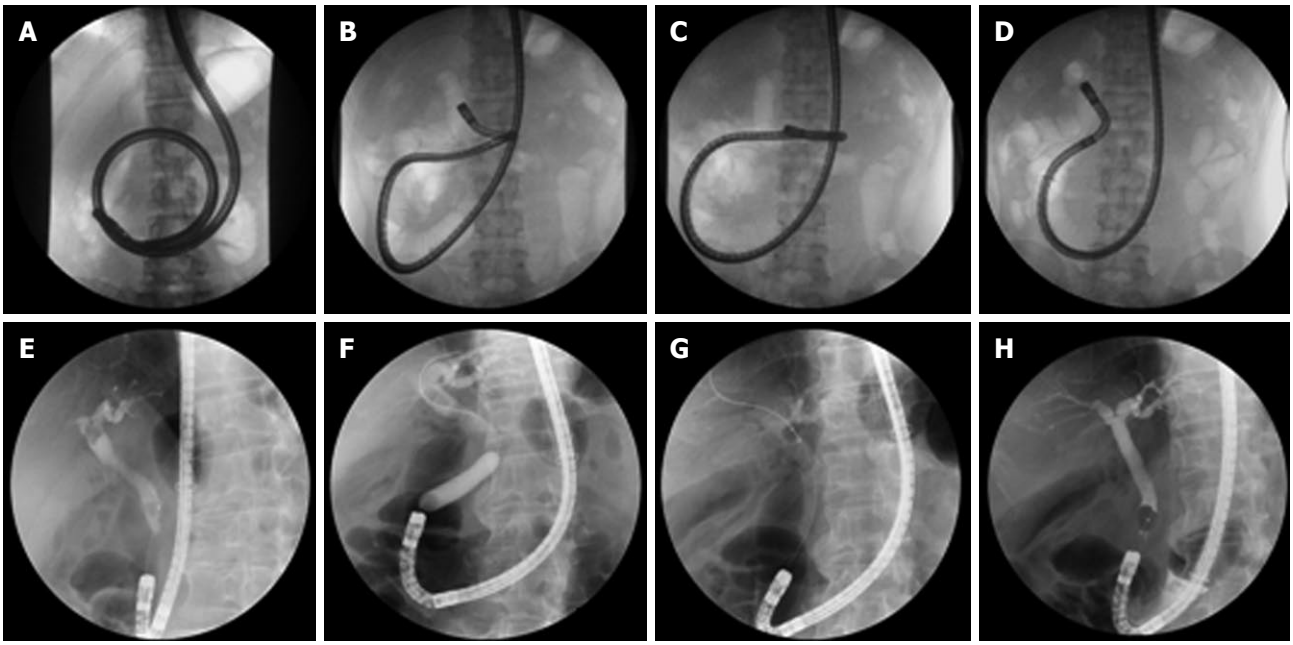

Figure 1 Fluoroscopy showing the long afferent loop and the major papilla that could not be reached by duodenoscope (A), the long afferent loop that could be shortened by double balloon endoscope and the papilla that could be reached (B-D), stones found after biliary cannulation (E) and endoscopic papillary balloon dilation (F), and stones removed using the balloon (G, H).

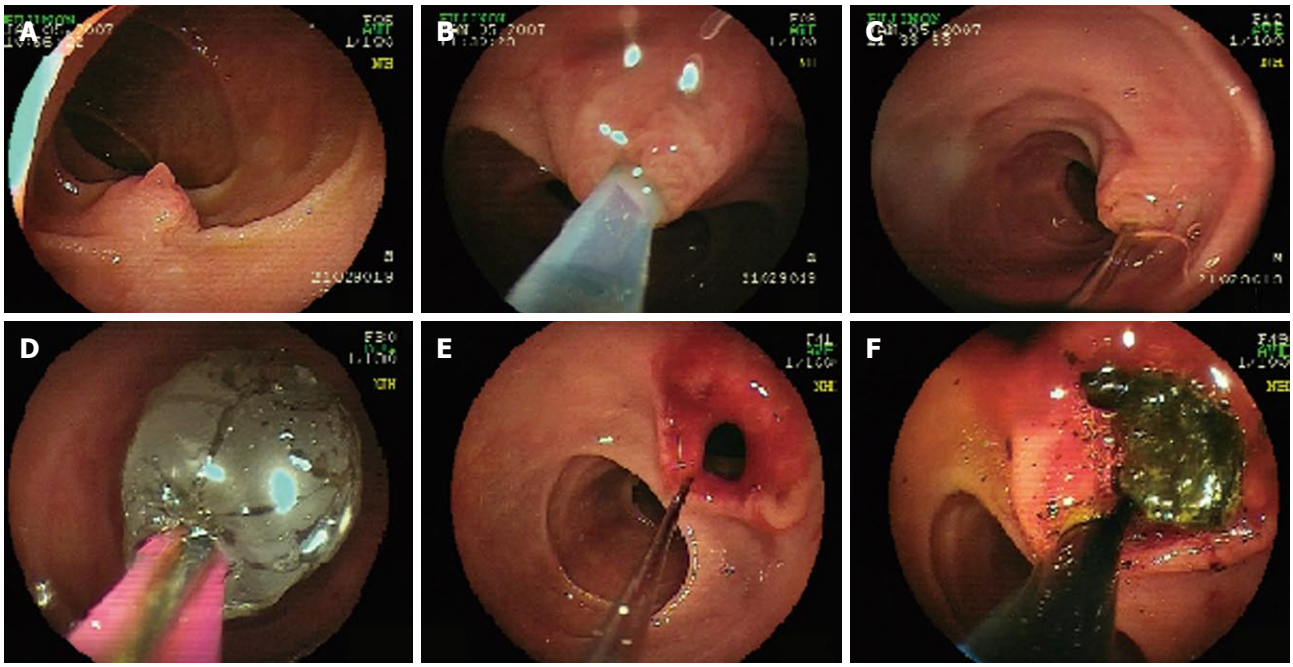

Figure 2 Endoscopy showing the papilla reached (A) and successfully cannulated (B) by double balloon endoscope, the guide wire left in the bile duct (C), endoscopic papillary balloon dilation performed (D, E), and stones found using the balloon (F).

with bile duct stones and 1 underwent diagnostic ERCP). A biopsy for the diagnosis of papilla of Vater tumor was needed in the remaining patients. There were no immediate or short-term complications related to the DBE-ERCP. Therapeutic interventions using DBE were EBPD with stone extraction in 4 patients (66%), papilla of Vater tumor biopsy in 1 patient (17%), and diagnostic ERCP in 1 patient (17%). The overall success rate of traditional side-viewing duodenoscopic ERCP and DBE-ERCP was 69% (22/32) and 88% (28/32), respectively, in the patients with a history of B Ⅱ gastrectomy.

DISCUSSION

Because of anatomical changes, ERCP is more challenging in patients who have undergone B Ⅱ gastrectomy.

The success rate of ERCP is lower in such patients than in those with a normal gastrointestinal anatomy[3-7]. ERCP is difficult because endoscopists encounter problems in afferent loop intubation, reaching the papilla, selective cannulation, and performing an EST in a reverse direction[3,4]. ERCP for patients with a history of B Ⅱ gastrectomy is associated with a higher rate of perforation and other complications due to the above mentioned technical difficulties. It was reported that the overall complication, perforation, and mortality rates are 8%-13%, 0.6%-11%, and 1%, respectively[3-5,17-19].

In our series, the side-viewing duodenoscope was successfully passed up to the papilla in 69% of the patients with a history of B Ⅱ gastrectomy, and the cannulation was successful in 63% of the patients. Six percent of the patients who failed in selective biliary cannulation were successfully treated with the rendezvous technique. ERCP using the duodenoscope was unsuccessful in 31% of the patients when afferent loop intubation and reaching the papilla failed, or endoscope-related small bowel perforation occurred in 6%, 19%, and 6% of the patients, respectively. The perforations, detected immediately after the procedure, usually occurred due to endoscope looping and over-manipulation, which caused tearing of the jejunal wall rather than direct perforation by the scope tip. Kim *et al*[7] have published a comparative study on the use of forward-viewing endoscope and side-viewing duodenoscope for ERCP in patients with a history of B Ⅱ gastrectomy, and found that side-viewing duodenoscope leads to considerably more bowel perforations, indicating that it may be safer to use a forward-viewing endoscope when a ERCP is performed for patients with a history of B Ⅱ gastrectomy. However, we prefer to use a side-viewing duodenoscope first, which allows the endoscopist to view the papilla en face. During therapeutic interventions such as EST, stenting, EPBD, and stone extraction, the manipulation of accessories is much easier using the elevator.

PTCD or surgery may be too invasive for patients in whom the afferent loop cannot be entered because of a sharp gastrojejunal anastomotic curve or the papilla cannot be reached because of the long afferent loop using a duodenoscope. In these cases, DBE is an excellent alternative treatment modality. DBE is a novel endoscopic procedure that allows examination of the entire small bowel in non-surgical patients[8]. In addition, it has been used for diagnostic and therapeutic ERCP in patients with Roux-en-Y anastomosis[13-16], demonstrating that the DBE system can be used to perform ERCP in patients with a surgically altered anatomy. Most of the complications that occur in patients with a history of B Ⅱ gastrectomy during ERCP examination with a duodenoscopy are due to the tortuous afferent loop and over-manipulation of the duodenoscope. The advantage of DBE is that the push-and-pull method can overcome the sharp angulation of the anastomosis, and shorten the tortuous and long afferent loop. In addition, too much pressure on the small bowel wall can be avoided, and the endoscope can be inserted more smoothly and deeply. The DBE method can also avoid some complications of duodenoscopic ERCP, such as perforation.

In the present study, the overall success rate of traditional side-viewing duodenoscopic ERCP and DBE-ERCP was 69% and 88%, respectively, in the patients with a history of B Ⅱ gastrectomy. Our study has some limitations, such as a small number of patients, and the study conducted at a single center. Multicenter, controlled studies are needed to confirm our results.

In conclusion, DBE can be used in ERCP for patients with a history of B Ⅱ gastrectomy due to failure in afferent loop intubation or reaching the papilla using the traditional duodenoscope, and is a useful, safe, and effective procedure for diagnosis and therapeutic interventions. The procedure increases the overall success rate of ERCP in this group of patients.

COMMENTS

Background

Performing endoscopic retrograde cholangiopancreatography (ERCP) for patients with a history of Billroth Ⅱ (B Ⅱ) gastrectomy is more difficult because of problems encountered in afferent loop intubation, reaching the papilla, selective cannulation and sphincterotomy.

Research frontiers

This is the first study using the double balloon endoscopy (DBE) for ERCP (DBE-ERCP) to increase the overall ERCP success rate in patients with a history of B Ⅱ gastrectomy.

Innovations and breakthroughs

From the literature review, the success rate of ERCP is lower in patients with a history of B Ⅱ gastrectomy than in those with a normal gastrointestinal anatomy. The results of this study demonstrate that the overall success rate of traditional side-viewing duodenoscopic ERCP and DBE-ERCP was 69% and 88%, respectively, for the patients with a history of B Ⅱ gastrectomy.

Applications

The results of this study suggest that using DBE for ERCP in patients with a history of B Ⅱ gastrectomy, when afferent loop intubation or reaching the papilla using the traditional duodenoscope fails, is a useful, safe, and effective procedure for diagnosis and therapeutic interventions.

Peer review

This manuscript, describing experiences of the authors in performance of the "DBE-ERCP", corresponds to a series of patients and the analysis of their results. As a conclusion, this is an interesting manuscript that describes and comments the results obtained by the authors with DBE-ERCP.

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BRIEF ARTICLE

Plasma miR-216a as a potential marker of pancreatic injury in a rat model of acute pancreatitis

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Abstract

AIM: To study the potential value and specificity of plasma miR-216a as a marker for pancreatic injury.

METHODS: Two rat models were applied in this article: L-arginine-induced acute pancreatitis was used as one model to explore the potential value of plasma miR-216a for detection of pancreatic injury; nonlethal sepsis induced in rats by single puncture cecal ligation and puncture (CLP) was used as the other model to evaluate the specificity of plasma miR-216a compared with two commonly used markers (amylase and lipase) for acute pancreatitis. Plasmas were sampled from rats at indicated time points and total RNA was isolated. Real-Time Quantitative reverse transcriptase-polymerase chain reaction was used to quantify miR-216a in plasmas.

RESULTS: In the acute pancreatitis model, among five time points at which plasmas were sampled, miR-216a concentrations were significantly elevated 24 h after arginine administration and remained significantly increased until 48 h after operation (compared with 0 h time point, $P < 0.01$, Kruskal-Wallis Test). In the CLP model, plasma amylase and lipase, two commonly used biomarkers for acute pancreatitis, were significantly elevated 24 h after operation (compared with 0 h time point, $P < 0.01$ and 0.05 respectively, Pairwise Bonferroni corrected *t*-tests), while miR-216a remained undetectable among four tested time points.

CONCLUSION: Our article showed for the first time that plasma miR-216a might serve as a candidate marker of pancreatic injury with novel specificity.

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Key words: MiR-216a; Plasma miRNA; Pancreatic injury; Acute pancreatitis; Biomarker

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INTRODUCTION

MicroRNAs (miRNAs) are endogenous small (18-25 nt), non-coding RNAs that repress expression of mRNAs by either cleavage or translational repression through perfect or imperfect binding to the 3' untranslated regions of target mRNAs. Since the discovery of the founding member named *lin-4* in *Caenorhabditis elegans* (*C. elegans*), miRNAs have now been shown to be involved in multiple important biological processes, including development, differentiation, and cancer, *etc*. The most recent release of the miRBase Registry (version 14, released on September 2009) lists 721 different miRNAs identified in humans[1]. It is estimated that miRNAs may contribute to the regulation of more than one third of all human genes[2].

In 2008, Jeyaseelan *et al*[3] first identified the existence of miRNAs in the circulation and suggested their possible use as biomarkers for stroke and related pathologies. This principle was validated in a series of literature reports and analyzing miRNAs levels in circulation is forecast to be a promising field for identifying biomarkers of cancer. For instance, miR-92 has been established to be a potential noninvasive molecular marker for colorectal cancer screening[4]. Most recently, two articles reported that miR-208, 122, 133a, and 124 held promise as biomarkers for injury of heart, liver, muscle, and brain respectively, indicating that tissue-specific miRNAs can be exploited as circulating accessible biomarkers for tissue injury[5,6].

In this study, we evaluated the hypothesis that pancreas-specific miRNA (miR-216a) might leak into the circulation from the injured pancreatic cells and this miRNA might serve as a good biomarker for pancreatic injury. Hence we used an arginine-induced pancreatitis model to study whether pancreas-specific miRNA can be detected in the circulation in the setting of pancreatic injury. As miR-216a is strictly expressed in pancreas, we further used the cecal ligation and puncture (CLP) model, a model simulating perforation, intestinal strangulation, sepsis, and multiple organ dysfunction syndrome (MODS), to evaluate the specificity of miR-216a to pancreas injury compared with the two most commonly used biomarkers for acute pancreatitis.

MATERIALS AND METHODS

Study design

This study was divided into three phases: Phase Ⅰ (Identification of miR-216a's specificity to pancreas): In this phase, thirteen different tissues including heart, liver, spleen, lung, kidney, thyroid gland, pancreas, small intestine, large intestine, brain, skeletal muscle, testis, and blood vessel were collected from healthy Sprague Dawley (SD) rats. reverse transcriptase-polymerase chain reaction (RT-PCR) was used as the means to quantify relative concentrations of miR-216a in various tissues. Specific expression of miR-216a in pancreas was identified for further analysis in phase Ⅱ. Phase Ⅱ (Validation of miR-216a's eligibility as a biomarker for pancreatic injury): In this phase, plasma samples were collected from SD rats at 5 different time points (0, 12, 24, 48, 72 h after induction of acute pancreatitis model) before they were sacrificed. Plasma miR-216a was quantified using RT-PCR and its differential expression between different time points was compared to testify its potential as a biomarker for pancreatic injury. Phase Ⅲ (Validation of miR-216a's specificity for pancreatic injury): Nonlethal sepsis induced in rats by single puncture CLP[7], a model simulating perforation, intestinal strangulation, sepsis, and MODS, will lead to non-specific hyperamylasemia and hyperlipasemia. We quantified plasma miR-216a in this model to see if it is more specific compared with amylase and lipase, two common laboratory markers used to establish the diagnosis of acute pancreatitis[8,9].

Animals and setup of two models

Male SD rats (200 ± 10 g) were purchased from the Experiment Animal Center of the Second Military Medical University (Shanghai, China). The rats were maintained in a temperature-controlled room on a 12-h light/12-h dark cycle and fed standard rat chow and tap water ad libitum. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai.

Acute pancreatitis was induced in SD rats (n = 50, 10 at each time point) by injecting 2 × 250 mg/100 g body weight of L-Arginine (Sigma) intraperitoneally in a 1-h interval, as a 20% solution in 0.15 mol/L NaCl. After sampling caval vein blood, rats were sacrificed at 0, 12, 24, 48, and 72 h following arginine administration. Acute pancreatitis was confirmed by plasma amylase and lipase level elevations and typical inflammatory features observed microscopically.

The induction of nonfulminant sepsis was performed under chloral hydrate anesthesia (300 mg/kg of body weight) using cecal ligation with a single, 18-gauge puncture as previously described[7,10]. After surgery, animals were fluid resuscitated with 40 mL/kg of subcutaneously administered sterile saline and were given free access to water but not food. At 0, 6, 12, and 24 h following single-puncture CLP operation, animals were reanesthetized with a 300 mg/kg intraperitoneal injection of chloral hydrate. Vena caval blood was collected for further RNA, amylase and lipase analysis.

Plasma amylase concentrations were measured with an automatic analyzer (Hitachi 7600-120); Plasma lipase concentrations were measured by using a Cobas-mira (Roche, USA).

Blood processing and isolation of plasma

All peripheral blood samples were collected in 2 mL BDVacutainer spray-coated K2 EDTA tubes (BD Diagnostic Systems). Samples were allowed to sit at room temperature for a minimum of 30 min and a max of 2 h. Separation of the blood sample was accomplished by centrifugation at 1200 × g at 4℃ for 20 min. Each plasma sample (300 μL at least) was removed into a 1.5 mL Ep-

pendorf tube, leaving enough plasma in the original tube such that the lowest point of the meniscus did not touch the clot. Then the samples were stored at -80℃ waiting for further extraction for total RNA isolation.

RNA isolation

All plasma samples were thawed on ice and 100 μL of each sample was transferred to a tube containing 750 μL of TRI Reagent BD and 20 μL of acetic acid (5 mol/L). Five microliters of synthetic *C. elegans* miRNAs (cel-miR-39, 50 pmol/L, synthesized by Qiagen) was added to each denatured sample as the spiked-in control[11,12]. RNA was isolated using the TRI Reagent® BD (cat. No. TB 126) following the manufacturer's protocol for RNA isolation. Each obtained RNA pellet was resuspended in 40 μL nuclease-free water and stored at -80℃.

Real-time quantitative RT-PCR analysis

A TaqMan miRNA real-time RT-PCR kit (Applied Biosystems) was used to detect and quantify the mature miRNA existing in total RNA extracted from tissues or plasmas. Briefly, 100 ng of tissue-derived total RNA or 2 μL of plasma-derived total RNA (from 5 μL of plasma) was reverse transcribed by TaqMan® MiRNA RT Kit. Negative controls were included with every real-time RT-PCR assay, and no amplification of the signal was detected when nuclease-free water was added instead of RNA or cDNA sample. Data were analyzed with 7500 software v.2.0.1. (Applied Biosystems), with the automatic Ct setting for adapting baseline and threshold for Ct determination. RT-PCR assays were performed in triplicate on each cDNA sample. Tissue expression levels of miR-216a were normalized to RNU6B[4,5], whereas cel-miR-39 was used to normalize the expression levels of miRNAs in plasma as described previously[11,12].

To relatively quantify miR-216a's concentrations in different tissues, we conducted RT-PCR with a known amount of synthetic miR-216a (Shanghai GenePharma Co., Ltd., Shanghai, China). In the presence of 0.67 amol (Ct = 35) to 48 fmol (Ct = 17.8) of synthetic miR-216a, we observed an excellent linearity (r^2 = 0.997) between the logarithm of the amount of input miR-216a and Ct value, suggesting that Taqman PCR assay is capable of detecting miR-216a at a detection limit equivalent to a Ct value of 35. Of note is that no miR-216a signal was detected in the plasma of healthy rats at all; no signal was detected even after 45 cycles of real-time PCR. The amount of miRNA not detected after 45 cycles of a real-time PCR was regarded in the present study as a Ct equivalent to 45. We set 35 as the baseline because the limit for reliably detecting synthetic miR-216a was 0.67 amol (Ct = 35).

Statistical analysis

With SPSS 13.0 software, data was compared between groups using analysis of variance (ANOVA), Kruskal-Wallis Test and Pairwise Bonferroni corrected *t*-test methods. The *P* value of less than 0.05 was defined as statistically significant.

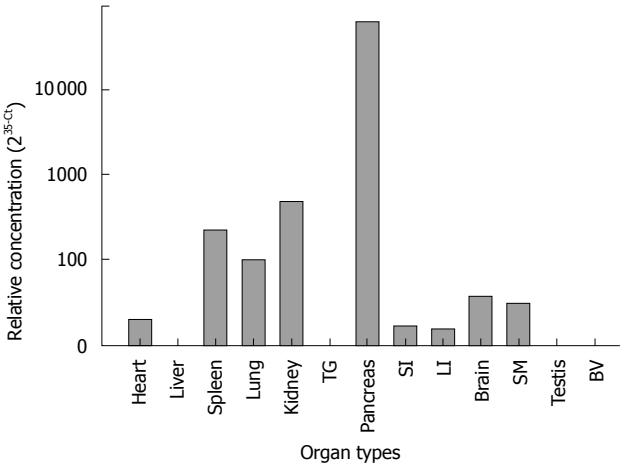

Figure 1 Expression of miR-216a in healthy rat tissues. Measurements of miR-216a in different samples were achieved by conducting real-time reverse transcriptase-polymerase chain reaction assay by means of 2^{35-Ct} as the relative expression level. Three rats were used in this experiment. Bars represent mean values of plasma miR-216a's quantification. TG: Thyroid gland; SI: Small intestine; LI: Large intestine; BV: Blood vessel.

RESULTS

Identification of miR-216a as a pancreas-specific miRNA

Various miRNA array analyses demonstrated that miR-216a was highly specific to the pancreas[13-15]. To verify that it is indeed produced specifically and abundantly to serve as a good biomarker candidate for pancreatic injury, we quantified the concentrations of miR-216a in 13 different tissues sampled from normal rats (Figure 1). As expected, pancreas tissue had the highest concentration of miR-216a among these samples, 128-fold higher than in kidney, which listed the next highest concentration.

Concentrations of plasma miRNAs in a pancreas-injury model

Arginine-induced pancreatitis was used as the pancreas injury model to investigate our hypothesis. We measured the plasma concentrations of amylase, lipase, and miR-216a. As miR-16 had been reported to be stably expressed across normal tissue types[16] and could be detectable at modest levels in normal plasmas[11], we tested its plasma concentrations to see if pancreas injury might lead to general elevation of plasma miRNAs.

As shown in Figure 2, plasma amylase and lipase concentrations were significantly elevated 24 h after intraperitoneal injection of arginine ($P < 0.01$, Kruskal-Wallis Test). Light micrographs of the pancreas showed interstitial edema, inflammatory infiltrate, acinar cell necrosis, and adipose tissue in interstitial spaces (Figure 3). Both laboratory tests and microscopic demonstrations supported our model of pancreatic injury. We further quantified plasma miR-216a using 35-Ct as its relative concentration. As shown in Figure 2A, though undetectable at baseline (Ct > 35), miR-216a concentrations in plasma were significantly elevated 24 h after arginine administration and remained significantly increased until 48 h after administration ($P < 0.01$, Kruskal-Wallis

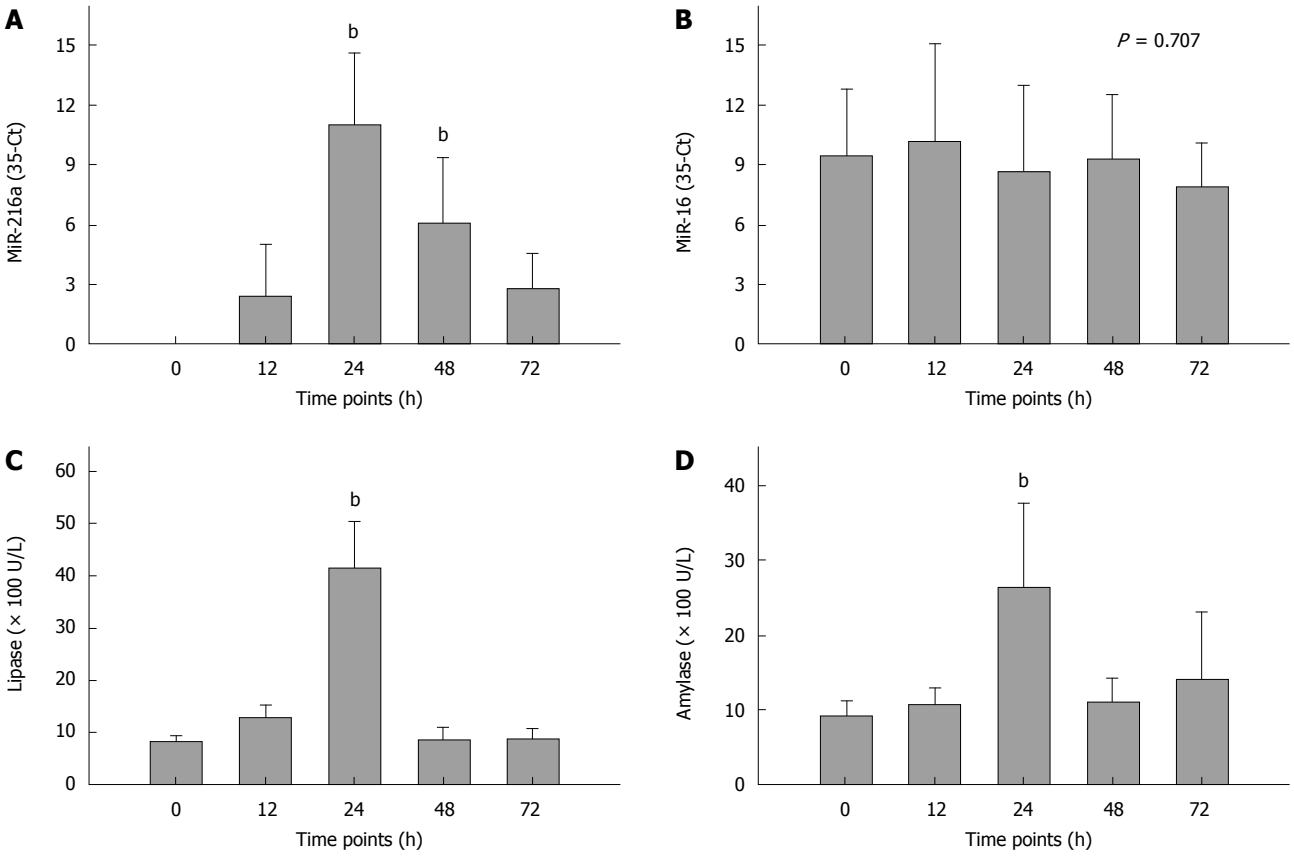

Figure 2 Plasma levels of miR-216a, miR-16, lipase, and amylase at different time points after induction of acute pancreatitis. A: Production of plasma miR-216a was significantly increased 24 h after L-arginine administration and remained significantly higher until 48 h after administration (Kruskal-Wallis Test); B: The amount of plasma miR-16 remained unchanged across all time points (One-way ANOVA); C and D: Plasma lipase and amylase levels were significantly elevated 24 h after administration (Kruskal-Wallis Test). Data are presented as the mean and SD. Ten rats were studied at each time point. $^{b}P < 0.01$ *vs* 0 h time point.

Test). Furthermore, we did not identify significant elevations of plasma miR-16 at any time points ($P = 0.707$, one-way ANOVA), indicating that pancreas injury did not lead to general increase of plasma miRNAs.

MiR-216a may be more specific than amylase and lipase as a biomarker for acute pancreatitis

As miR-216a is pancreas-specific and various pathologic conditions may lead to nonspecific hyperamylasemia and hyperlipasemia, we hypothesized that miR-216a might be more specific than amylase and lipase in diagnosing acute pancreatitis. In the CLP model of our experiment, plasma amylase and lipase were significantly elevated 24 h after operation ($P < 0.01$ and 0.05 respectively, Pairwise Bonferroni corrected *t*-tests, Figure 4B), while miR-216a remained undetectable. Microscopic examination showed no sign of pancreatic injury (Figure 4A), which further consolidated our hypothesis that miR-216a might be a reliable biomarker for pancreatic injury with novel specificity.

DISCUSSION

Accumulating evidence suggests that circulating miRNAs may be good biomarkers for specific tissue injury. For example, Jeyaseelan *et al*[3] provided evidence that some of the miRNAs that were highly expressed in the ischemic brain could be detected in blood samples; Kai Wang's exploration demonstrated that specific miRNA species, such as miR-122 and miR-192, exhibited dose- and exposure duration-dependent changes at a significantly early stage of drug-induced liver injury[17]. Most recently, two articles reported that miR-208, 122, 133a, and 124 held promise as biomarkers for injury of heart, liver, muscle and brain respectively, indicating that tissue-specific miRNAs could be exploited as circulating accessible biomarkers of tissue injury[5,6]. Furthermore, Ai *et al*'s[18] results, which revealed that circulating miR-1 might be a novel, independent biomarker for diagnosis of acute myocardial infarction, extended the principle of circulating miRNAs' eligibility as biomarkers into clinical settings.

Our data show for the first time that the plasma concentration of miR-216a, which is produced exclusively in pancreas, increases in the model of arginine-induced acute pancreatitis. This result provides clues that plasma miR-216a may be a good biomarker for pancreatic injury. Furthermore, the undetectable concentration of miR-216a in the control group compared with the extremely high concentrations seen in the acute pancreatitis model with histologically documented toxicity highlights the signal-to-noise ratios seen with miR-216a, suggesting that this miRNA may serve as a good biomarker to monitor the injury to pancreas.

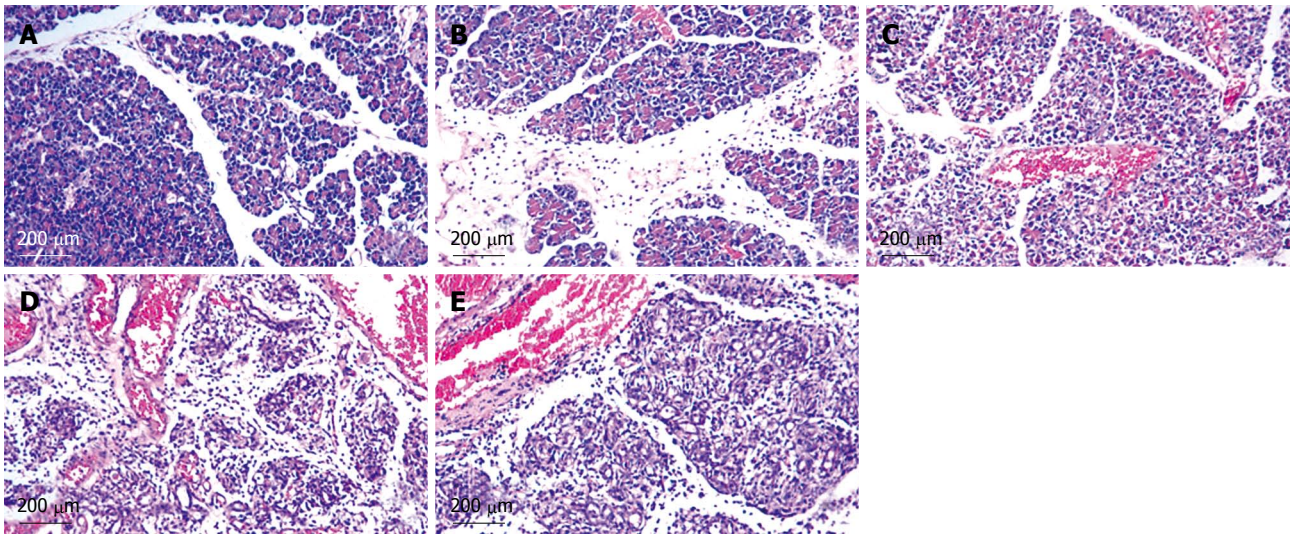

Figure 3 Light micrographs of the pancreas at 0 h (A), 12 h (B), 24 h (C), 48 h (D) and 72 h (E) after arginine injection. A: Neither interstitial edema nor acinar cell necrosis is seen; B: Interstitial edema and slight cellular infiltration in the interstitium can be seen; C: The acinar structures are partially destroyed. Interstitial edema and inflammatory infiltrate are greater in degree than at 12 h; D: The acinar architecture is markedly disrupted; E: Most pancreatic acinar cells show signs of degeneration or necrosis. Adipose tissue can be seen in the interstitial spaces.

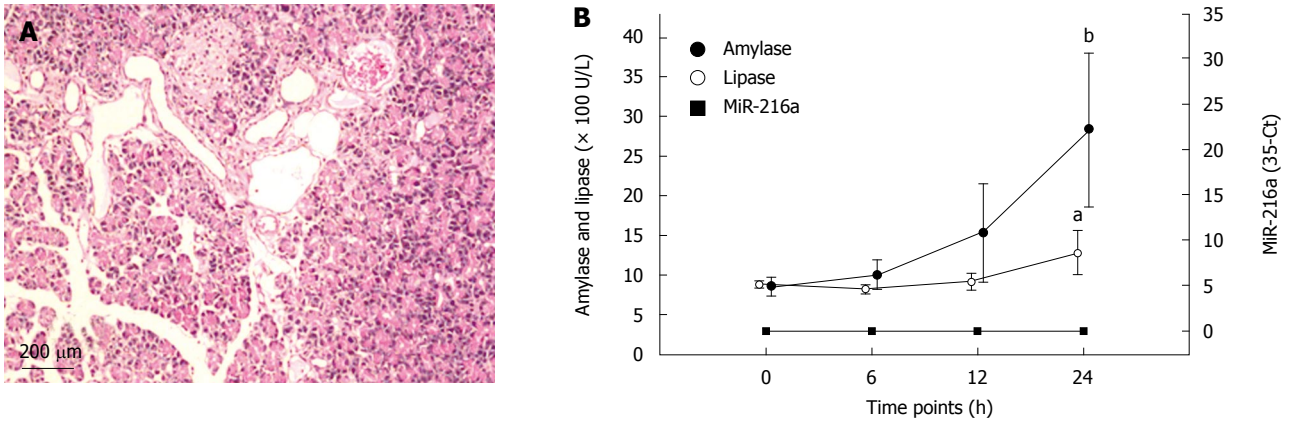

Figure 4 Amylase, lipase levels and light micrographs of pancreatic tissue in cecal ligation and puncture model. A: Pancreatic tissue was normal in the cecal ligation and puncture (CLP) model; B: Plasma amylase and lipase were significantly elevated 24 h after induction of this model, while plasma miR-216a remained undetectable. Data are presented as the mean ± SD. Eight rats were studied throughout the experiment with the CLP model. $^{a}P < 0.05$ *vs* 0 h time point; $^{b}P < 0.01$ *vs* 0 h time point. Pairwise Bonferroni corrected *t*-tests.

As miR-216a is specifically expressed in pancreas (Figure 1), we hypothesized that plasma miR-216a might be more specific than those commonly used in clinical settings. Although amylase and lipase are the two most common laboratory markers used to establish the diagnosis of acute pancreatitis[8,9], nonspecific hyperamylasemia and hyperlipasemia may occur under various conditions. For example, in diabetic ketoacidosis nonspecific elevations of amylase and lipase occur in 16%-25% of cases. Diagnosis of acute pancreatitis based solely on elevated amylase or lipase, even > 3 times normal, is not justifiable[19]. Furthermore, hyperamylasemia in the background of non-pancreatic diseases has been reported in a series of literature[20,21] whereas different groups have identified elevations of lipase in a number of conditions such as acute cholecystitis, intestinal infarction, duodenal ulcer, obstruction or inflammatory bowel disorders, liver diseases, and abdominal trauma[22-24]. Our article demonstrated that nonspecific elevations of amylase and lipase would occur in the CLP model while the plasma concentration of miR-216a remained undetectable, indicating that plasma miR-216a might be useful in justifying whether the elevated concentration of amylase or lipase was due to pancreatic injury in certain complex pathologic settings.

In this article, we validated for the first time the eligibility of a pancreas specific miRNA as a biomarker for pancreatic injury and its potential advantage of specificity over two previously confirmed markers of acute pancreatitis in certain pathology courses. The next question is whether elevation of plasma miR-216a has clinical significance and whether it offers advantages over measurement of amylase and lipase in human. As miR-216a is strictly conserved across species and its assessment (e.g. qRT-PCR) is simple and universally applicable, the discovery-validation pipeline for miRNA biomarkers will be more efficient than traditional proteomic biomarker discovery-

validation pipelines. Furthermore, highly sensitive PCR will possibly lower detection limits for plasma miR-216a compared to amylase and lipase. Future clinical assessments in humans are warranted to test its feasibility in patients.

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COMMENTS

Background

MicroRNA (miRNA) is a kind of small, noncoding RNA which can repress expression of target mRNAs. Various groups validated that miRNA could remain stable in circulation and served as a novel biomarker for different physiological or pathological conditions. More recently, several studies showed that certain miRNAs were strictly expressed in some tissues and these tissue-specific miRNA could leak into circulation, holding potential as non-invasive biomarkers with novel specificity. Nonetheless, there is no study concerning the diagnostic value of pancreas-specific miRNA in circulation for acute pancreas injury.

Research frontiers

The potential of pancreas-specific miRNA, miR-216a, as a biomarker for pancreatic injury has never been investigated previously.

Innovations and breakthroughs

This is the first report that pancreas-specific miRNA, miR-216a, could leak into the circulation to serve as a biomarker for pancreatic injury. Furthermore, this article showed that the specificity of circulating miR-216a is significantly higher than amylase and lipase, two most commonly used biomarkers in diagnosing acute pancreatitis.

Applications

Amylase and lipase are the two most commonly used biomarkers for pancreatitis detection. However, non-specific hyperamylasemia and hyperlipasemia will occur in plenty of clinical settings, which greatly lowers the specificity of these two biomarkers. MiR-216a is specifically expressed in pancreas and circulating miR-216a exhibited higher specificity than amylase and lipase. Furthermore, polymerase chain reaction is a highly sensitive technique which will possibly lower detection limits for plasma miR-216a than amylase and lipase.

Peer review

This paper reports, for the first time, that circulating miR-216a is a specific biomarker for pancreas injury. The study is nicely designed and the manuscript is pretty good.

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BRIEF ARTICLE

Laparoscopic low anterior resection for rectal carcinoma: Complications and management in 132 consecutive patients

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Abstract

AIM: To analyze the clinical manifestations and risk factors of complications in laparoscopic low anterior resection (LAR) for rectal cancer patients.

METHODS: A series of 132 consecutive patients who received laparoscopic LAR for rectal cancer in our center were included. The etiology, diagnosis, treatment and prevention of rectal cancer were studied among the patients with surgery-related complications using both univariate and multivariate regression analysis.

RESULTS: No conversion to open surgery was observed and 5 cases converted to hand-assisted laparoscopic operation. The overall morbidity rate was 20.5%. Complications occurred during the operation in 7 patients (5.3%), within 30 postoperative days in 24 patients (18.2%), and within 3 mo in 2 patients (1.5%). The most significant complications were anastomotic leakage (9.1%) and anastomotic hemorrhage (5.3%). Size and location of tumor, pathological staging and preoperative nutrition were significant factors associated with LAR complications, while gender, age and pathological type showed no relevance. Binary logistics regression showed that the size and location of tumor, and pathological staging were independent factors of laparoscopic LAR. All the complications were treated during their onset of clinical manifestations by interventional or conservative therapy.

CONCLUSION: Anastomotic leakage is a major complication in laparoscopic LAR. The complications may be associated with tumor size and site, and pathological stage. Interventional therapies are of value in the management of laparoscopic LAR complications.

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Key words: Laparoscopy; Low anterior resection; Complication; Rectal cancer; Logistic regression analysis

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INTRODUCTION

Despite important progress made in the past decade regarding surgical staplers, techniques and perioperative management, patients who receive low anterior resection (LAR) for rectal cancer may still inevitably experience surgical complications. With the lowering level of colo-anal anastomosis and increasing demands for anal-sphincter

preservation, risks such as anastomotic leakage are still the major concerns of the surgeons. It is crucial to understand their risk factors of complications for clinical applications and their impact on patient survival. The laparoscopic approach has been regarded as an attractive surgical alternative for low rectal cancer management because it offers better visualization and more delicate instrumentation and may reach an adequate dissection up to the pelvic floor with a better preservation of the hypogastric plexus and erigent nerves, thus resulting in an improved functional and oncological outcome, ensuring a relatively lower anastomosis and a reduced occurrence of complications.

Several recently published randomized studies have shown the better short-term benefits of the laparoscopic approach in colorectal cancer treatment compared with the open approach. However, such data are limited to the common complications related to laparoscopic LAR during or after surgery. Hence, we conducted this trial to study laparoscopic LAR in terms of perioperative and oncological outcomes in patients with rectal cancer. The aim of this study was to analyze the clinical manifestations and risk factors of the complications following LAR, and to summarize the management of the patients who suffered from these complications.

MATERIALS AND METHODS

Patients

Using a prospectively constructed database, we reviewed the outcomes of 132 consecutive patients who underwent laparoscopic LAR for rectal adenocarcinoma within 8 cm towards the dentate line at our Minimally Invasive Surgery Center. Preoperative localization of tumor was determined using the colonoscopy, double contrast barium enema, endoscopic ultrasonography and contrast-enhanced computed tomography of the abdomen and pelvis. Transcutaneous ultrasonography was not routinely performed. The excluding criteria were as follows: (1) the tumor was recurrent or metastatic according to the imaging test or perioperative biopsy; (2) those who had received any chemo- or radiotherapy preoperatively; (3) no total mesorectal excision (TME) technique was used during rectal resection; (4) emergent operation was performed for bowel obstruction, acute hemorrhage or perforation; and (5) patients who had other bowel diseases such as ulcerative colitis or Crohn's disease. The present study was performed in compliance with the guidelines issued by our institutional review board (IRB) and fulfilled the requirements for informed consent, and approved by our IRB. All patients provided informed consent for the laparoscopic LAR procedure. The identified clinical manifestations or imaging test presentations were demonstrated in all the cases for risk analysis.

Surgical procedure

Using a 5-trocar approach, the inferior mesenteric vessels were ligated after left ureter identification, followed by retromesenteric dissection using a medial to lateral route. The splenic flexure was then mobilized, followed by laparoscopic TME dissection with preservation of the hypogastric plexus and nerves.

For tumors located in the distal rectum (8 cm from anal verge), a complete TME was performed laparoscopically after splenic flexure mobilization. The rectum was transected with an endoscopic or conventional stapler through a low abdominal transverse incision at the level of the pelvic floor (with at least a 2-cm distal margin from the tumor). A colo-anal anastomosis was spared. Transanal anastomoses were performed at least 1 cm from the dentate line with an adequate oncological distal margin of 2 cm, using a double-stapling technique and end-to-end anastomosis (Tyco Healthcare Group LP, Norwalk, CT, USA, Proximate ILS, Ethicon-Endo Surgery, Inc., Cincinnati, OH, USA).

Protective loop ileostomy was not routinely performed on our patients. A conversion to hand-assisted laparoscopic LAR was carried out under conditions such as a thickening mesentery, enlarged tumor mass or a narrow pelvis for successful manipulation.

Study parameters included: (1) patient data: age, body mass index and preoperative morbidity; (2) perioperative data: operation time, blood loss and complications, including intraoperative complications of hemorrhage, bowel injury and anastomotic rupture, short-term complications such as anastomotic leakage, anastomotic hemorrhage, urinary retention, pulmonary or urinary infection and long-term complications such as anastomotic stricture, incisional hernia; (3) postoperative data: length of hospital stay (including preoperative night spent in hospital), time to first liquid intake, time to unrestricted food intake, time to first stool passage and postoperative complications; (4) pathological data: TNM staging, total number of lymph nodes harvested, length of the resected specimen, tumor diameter, tumor distance to distal resection margin and circumferential margin status; and (5) follow-up data: time to local recurrence, time to occurrence of distant metastases, overall survival and disease-free survival.

Statistical analysis

The SPSS 13.0 software package was used for statistical analysis. The results were presented as mean ± SD using Student *t* test for parametric analysis and within-group analysis of variance was used when appropriate. Comparisons for variables were performed using χ^2 test. All *P* values less than 0.05 were considered statistically significant. For univariate analysis, the binary logistic regression model was used to identify independent prognostic factors for overall complications related to operations. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Demographic data

A total of 132 patients (80 men and 52 women) underwent curative laparoscopic LAR in our center. Their average age was 64.40 (33-90) years and average operation time was 106.33 ± 42.45 (55-210) min, intraoperative blood loss was 49.02 ± 56.50 (5-200) mL. The average

Table 1 Category, management and prognosis of intraoperative complications

Intraoperative complications	*n* (%)	Management	Prognosis
Anastomotic rupture	1	Intermittent suturing with absorbable sutures under laparoscopy	Anastomotic hemorrhage and leakage
Hemorrhage	2	Hemostat with Hem O-lok clip intraoperatively; Hemostat with titan clips under colonoscopy after completion of operation	Recovered
Ureter injury	2	Intraoperative cannulation of double J catheter under cystoscopy, saturation of the ends with absorbable sutures, extubation no earlier than 2 mo after operation	Recovered
Deferent duct injury	1	Occlusion of the distal end under laparoscopy by titan clips	Partial sexual dysfunction
Bladder injury	1	Intermittent suturing with absorbable sutures under laparoscopy	Recovered
Total	7 (5.3)		

Table 2 Category, management and prognosis of postoperative complications

Postoperative complications		*n* (%)	Management	Prognosis
Short-term complications	Cardiopulmonary dysfunction	1	Cardiopulmonary resuscitation and tracheal intubation, the patient was transferred to SICU emergently	Dead
	Urinary retention and infection	2	Proper antibiotics and functional exercise	Recovered
	Incisional infection and colliquation	2	Frequent dressing	Recovered
	Anastomotic leakage	12	1 patient underwent proximal colostomy; others received abdominal lavage and intravenous fluid support	Recovered
	Anastomotic hemorrhage	7	Fluid expansion and proper hemostatics: 2 patients with detainment of anal tubes received irrigation of ice-cold saline dissolving noradrenaline, 2 patients with relative severe hemorrhage received a colonoscopy and finally the bleeding points were stopped using titanic clips	Recovered
Total (%)		24 (18.2)		
Long-term complication	Anastomotic stricture	2	Periodic distension under colonoscopy	Improved
Total (%)		2 (1.5)		

SICU: Intensive Care Unit of Surgery.

size of tumor was 12.4 ± 10.1 (2-49) cm^2, and the average distance of tumor was 7.86 ± 2.60 (3-15) cm from the anal verge. Postoperative pathological examinations revealed 9 cases of mucinous adenocarcinoma, 36 tubular adenocarcinoma, and 31 papillary adenocarcinoma. In TNM staging, 46 were stage Ⅰ, 51 stage Ⅱ, and 35 stage Ⅲ. The preoperative nutritional status was mainly evaluated by hemoglobin and albumin, 56 patients were found below the standard level, and all of those patients received preoperative intravenous nutritious support. Besides the 5 cases with hand-assisted LAR, all the operations were completed laparoscopically with no conversion to open surgery. Up till now within the follow-up period, we discovered no metastasis, no tumor-related mortality, and one fatality due to a cardiovascular accident.

Surgical complications and management

The overall incidence of surgical complications was 20.5% (27/132) and the incidence of intraoperative complications was 5.3% (7/132), including hemorrhage (2/132), ureter injury (2/132), bladder injury (2/132), and anastomotic rupture (1/132). The incidence rate for short-term complications (defined as occurring one month after the operation) was 18.2% (24/132), including one patient who was attacked with an severe cardiac dysfunction caused by acute myocardiac infarction and immediately sent to the Intensive Care Unit of Surgery, anastomotic leakage (9.1%, 12/132), anastomotic hemorrhage (5.3%, 7/132), incisional colliquation or infection (1.5%, 2/132), and urinary infection (1.5%, 2/132). The long-term complication is anastomotic stricture (1.5%, 2/132).

During operation, all hemorrhage was hemostated by titan clips or proper sutures. Injuries of ureter, bladder, or anastomosis were repaired immediately, and the double J catheters were detained after repair of ureters to avoid stricture. All the anastomotic hemorrhage occurring intra- or postoperatively were treated by conservative therapy such as fluid expansion to stabilize the hemodynamics and proper hemostatics. Two patients with detainment of anal tubes received irrigation of ice-cold saline dissolving noradrenaline, and two patients with relatively severe hemorrhage received a colonoscopy and the bleeding was stopped finally using titanic clips. Only one patient underwent a protective proximal colostomy because of diffused abdominal and pelvic infection, and all the patients with anastomotic leakage were healed by continuous lavage of pelvic cavity via drainage tubes, detaining anal tube for a better decompression of lumen, as well as proper antibiotics treatment. Two patients with anastomotic stricture were also relieved by periodic distension therapy under colonoscopy (Tables 1 and 2).

Risk factor analysis

The above general statistics implied that the major clinical types of complications after LAR were anastomotic leak-

Table 3 Univariate analysis of factors for low anterior resection complications

Variables	*n*	Complication *n* (%)	χ^2 value	*P* value
Gender				
Male	80	20 (15.15)	3.743	0.053
Female	52	7 (5.30)		
Age (yr)				
⩾ 55	104	21 (15.9)	0.021	0.886
< 55	28	6 (4.54)		
Tumor size (cm)				
Φ ⩾ 3	86	24 (18.2)	8.424	0.004
Φ < 3	46	3 (2.30)		
Pathological type				
Mucinous adenocarcinoma	9	3 (2.30)	2.440	0.486
Tubular adenocarcinoma	36	5 (3.80)		
Papillary adenocarcinoma	31	8 (6.06)		
Adenocarcinoma	56	11 (8.33)		
Tumor location (anal verge) (cm)				
> 6	88	10 (7.58)	6.615	0.010
⩽ 6	44	17 (12.88)		
TNM staging				
Stage Ⅰ	46	2 (1.52)	11.46	0.003
Stage Ⅱ	51	14 (10.61)		
Stage Ⅲ	35	11 (8.33)		
Preoperative nutritious status (g/L)				
HB > 100 and Ag > 32	76	11 (8.33)	3.938	0.047
HB ⩽ 100 or Ag ⩽ 32	56	16 (12.12)		

age and hemorrhage. The clinical parameters associated with surgical complications of LAR are listed in Table 3. The univariate analysis showed that the influencing factors for surgical complications were tumor size, location, pathological staging and preoperative nutrition while gender, age and pathological type were not significantly correlated with the occurrence of complications. To be specific, a neoplasm larger than 3 cm in diameter, 6 cm from anal verge, together with anemia or hypoproteinemia (HB ⩽ 100 g/L or Ag ⩽ 32 g/L) may significantly increase the risks of postoperative complications. Further multivariate analysis using binary logistic regression model demonstrated that tumor size, location and pathological staging were independent risk factors for surgical complications after LAR, their relative risk (RR) was 1.149, 0.552 and 2.816 (Table 4).

DISCUSSION

Laparoscopic LAR is minimally invasive with a rapid recovery and short length of hospital stay compared with laparotomic approach. Compared with the mortality rate (2%-3%) by the conservative surgery, the mortality rate remains about 1% and the main causes of death were systemic complications[1]. As for postoperative complications, a serial clinical trials including a COST study have demonstrated no significant difference between these two kinds of techniques, which indicated that both methods are safe and feasible[2-5]. The Randomized Controlled Trial-CLASICC, which includes 484 cases of laparoscopic colorectal surgery and 253 cases of conservative ones, has listed the commonly encountered types of complications and their incidence rates[2]: intraoperative complications (14%) were severe hemorrhage (7%), cardiopulmonary dysfunction (4%), vascular/bladder injury (2%), and bowel injury (1%); short-term (within 30 d after operation) postoperative complications of LAP group were incision infection (13%), pulmonary infection (10%), anastomotic leakage (10%), deep vein thrombosis (0.4%) for LAP group (total 40%); and the most common long-term complications were bowel obstruction and persistent incision infection. Our results revealed that anastomotic leakage and hemorrhage more frequently appeared than the intraoperative bleeding, ureter or other visceral injury, incision infection, and anastomotic stricture. We found no obvious difference from the results of the CLASICC study in complication types, but only a proportional variation.

After a statistical analysis of the factors which may influence the occurrence of surgical complications, we concluded that a tumor larger than 3 cm in diameter, less than 6 cm proximal to anal verge, and confirmed as stage Ⅲ by pathological diagnosis, i.e. tumor location, tumor size and pathological staging, were independent risk factors for LAR surgical complications. Anastomotic leakage has been regarded as one of the major types of LAR complications[5-7], a better understanding of the risk factors would certainly benefit the selection of appropriate treatment. In conservative therapy, the incidence of anastomotic leakage after LAR could rise up to 4%-25%[6], however the CLASICC study revealed that the incidence rate was around 10%, which seems to have no significant difference. Insufficient blood supply, over-tension, and difficult anastomosis are the causes of anastomotic leakage in the conservative method. Patients with a tumor larger in size or later in TNM staging usually endured a worsened systemic physical status, and sometimes their bowels were found relatively edematous, or there was a pelvic adhesion due to invasion of the large tumor mass. Besides, the whole procedure routinely accomplished by TME principle will probably run into an insufficient blood supply around the location of anastomosis for a too thorough resection of mesentery[8]. Our results also revealed that the tumor location influence the occurrence of leakage. To guarantee oncological safety, we may choose a more proximal anastomosis for a lower tumor mass, which would inevitably result in a higher tension or even anastomotic difficulty[9]. Lipska *et al*[10] performed a risk factor analysis for 98 cases of laparoscopic LAR and concluded that tumor located within 6 cm from the anal verge is an significant risk factor for surgical complications($P = 0.01$). This reminds us that some modified techniques which can help relieve the regional tension could be used as alternatives when performing some critical anastomosis during operation[11]. Our study indicated that tumor size, location and pathological staging are major independent risk factors for laparoscopic LAR. This conclusion is somewhat close to that in the previous literature.

With the extensive use of LAR, the prevention or management of surgical complications, especially some common types, has gained more attentions. Anastomotic leakage and hemorrhage are considered to be the two major complications which will directly influence the

Table 4 Multivariate analysis of factors for low anterior resection complications

Variables	Coefficient	Standard error	Wald statistics	Degree of freedom	*P* value	Exp (coefficient)
Location	-0.595	0.193	9.453	1	0.002	0.552
Size	0.139	0.039	12.989	1	< 0.001	1.149
Nutritious status	0.705	0.616	1.308	1	0.253	2.023
TNM staging	1.035	0.456	5.159	1	0.023	2.816

postoperative recovery of the patients[5]. First of all, a leakage should be discovered promptly, and a fasting should be ordered with an intimate observation of the patient's regional signs and physical status. If the overall status is stable, an abdominal or pelvic lavage through a drainage tube is recommended so as to speed up the regional healing progress. For the patients who present with an ineffective response to preservative treatment or a severe systemic symptom, an interventional therapy or operation should be performed without hesitation, reconstruction of the anastomosis or an ileostomy is both a favorable choice. A defunctioning stoma has always been regarded as a useful method in both preventing and controlling of leakage in conventional colorectal surgery, which could even significantly decrease the occurrence of peritonitis or sepsis. We used to follow this concept to create preventive stomas for those "high risk" cases, but the additional procedure for stoma closure and the potential mentally discomfort of patients have made us continuously explore for better management. We found that postoperative placement of silicon drainage tubes near anastomosis or presacral space could not only minimize the possibility of local adhesion and sinus tract caused by rubber tube, but also serve as a monitoring "instrument" for surgical trauma healing by detecting the color and characteristics of fluid. Moreover, it could be transformed into multifunctional drainage such as suction by inserting some pinheads. Placement of anal tubes in certain cases is also very useful, especially in those who underwent a low or ultra-LAR. Since postoperative recovery of anal sphincter function, even evacuation and defecation resulted in elevation of intrarectal pressure, insertion of anal tube over the location of anastomosis would help relieve this pressure nearby, thus simultaneously decreasing the infection caused by early excreta. In this study, most of the patients who were recovered by conservative therapy were treated by these two methods. For anastomotic hemorrhage, although most of the conditions could be healed by intensive monitoring, sufficient fluid apply and proper intravenous hemostatics, in some recurrent cases, icy saline injection with noradrenaline through an anal tube was suggested in our center, and at the same time, attention should be paid to avoid artificial anastomotic eruption. If this method does not work well, detection for hemorrhaging spots under colonoscopy is also a worthy option, which could somewhat reduce the necessities of re-laprotomy for hemostasis. In this study, all the diagnosed hemorrhage cases were recovered by alternative conservative therapies so that patients could rescue from extra distress brought by another operation.

Another complication that is more likely to occur during operation is injury of ureter and bladder. The former occasion could happen whether in colon surgery or rectal surgery at some anatomical points, so we prefer to expose them at both sides in abdominal cavity when dissociating the bowels so as to avoid any useless dissection or clipping (especially near lateral- or retro-peritoneum). If any injury of ureter occurred, surgeons should quickly evaluate the severity before choosing the right method to repair. Under most circumstances, a side-to-side anastomosis of the injured ureter with catheterization of a "pigtail" ureteral catheter is sufficient, this procedure should better be completed under laparoscopy, but an open approach should be adopted as long as it is too difficult[12]. The injury of bladder is commonly due to false dissection or electronic-coagulation, even the false insertion of Veress needle or Trocar. So surgeons should pay attention to those thickening mesentery, enlarged tumor mass or narrow pelvis when dissecting the anterior wall of the rectum, and tightly move close to the inferior border whatever manipulation (especially when using harmonic scalpel) he or she is performing. For small perforation of bladder (3-5 mm), a detainment of urethral catheter for 7-10 d is enough for wound healing; for relatively large or irregular lesions, saturation using absorbable sutures and detainment of urethral catheter for 4-10 d are necessary, however, this surely depends on the specific location and size of the lesion.

With the development of techniques, some intra-abdominal complications could be managed by laparoscopy. For the patients with anastomotic leakage who need a laparotomy, abdominal cavity lavage, or replacement of drainage tubes, we could also complete these procedures with minimal invasion; even the patient needs to create a temporary stoma, we only need to make a small incision for pulling out the bowel. Since the adhesion of intra-abdominal cavity is often much more improved after laparoscopic surgery compared with conventional approach, the re-establishment of insufflation space could be achieved without much difficulty. It is promising that laparoscopic re-operation could possibly become another trend in abdominal surgery.

With the improvement of laparoscopic technique, widespread application of anastomotic devices, and greater demands for quality of life, more patients would receive laparoscopic LAR. Up till now, surgery-related complications are major factors prohibiting the improvement of overall quality of the surgery, so studies on how to better handle these problems would be beneficial for surgeons in accumulating necessary experiences as well as expanding the extent of application.

COMMENTS

Background

Despite important progress in the past decade regarding surgical staplers, techniques, and perioperative management, patients who receive low anterior resection (LAR) for rectal cancer may still inevitably have surgical complications. It is crucial to understand the risk factors for clinical applications and its impact on patient survival.

Research frontiers

The advanced skills and modified methods of laparoscopic colorectal surgery are widely recognized by surgeons, and several recently published randomized studies have shown the better short-term benefits of the laparoscopic approach in colorectal cancer compared with the open approach. However, such data are only limited to the common complications related to laparoscopic LAR during or after surgery. The authors performed this trial to study laparoscopic LAR in terms of perioperative and oncological outcomes in patients with rectal cancer.

Applications

With the improvement of laparoscopic technique, widespread application of anastomotic devices, and greater demands for improving quality of life, more patients would receive laparoscopic LAR. Up till now, surgical related complications are major factors prohibiting the improvement of overall quality of the surgery, so studies on how to better handle these problems would be beneficial for surgeons in accumulating necessary experiences as well as expanding the extent of application.

Terminology

LAR: A common surgery for rectal cancer in the proximal (upper) two-thirds of the rectum. Protective loop ileostomy: a surgical opening constructed by bringing the end or loop of small intestine (the ileum) out onto the surface of the skin. Anastomotic leakage: An anastomosis is a surgical connection between the stomach and bowel, or between two parts of the bowel. The surgeon attempts to create a water-tight connection by connecting the two organs with either staples or sutures, either of which actually makes a hole in the bowel wall. If the seal fails to form, for any reason, fluid from within the gastrointestinal tract can leak into the sterile abdominal cavity and give rise to infection and abscess formation.

Peer review

This is a retrospective study on 132 patients with rectal cancer dealing with management and prevention of complications of low anterior rectal resection. It is important that surgical and gastroenterological communities have an idea of what is made in China concerning laparoscopic surgery for rectal cancer.

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BRIEF ARTICLE

High expression level of EDIL3 in HCC predicts poor prognosis of HCC patients

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Abstract

AIM: To determine the role of epidermal growth factor-like repeats and discoidin I-like domains 3 (EDIL3) in pathogenesis of hepatocellular carcinoma (HCC) by investigating the EDIL3 expression in HCC and its prognostic value for HCC.

METHODS: EDIL3 expression was detected in 101 HCC surgical tissue samples with immunohistochemistry method, and its relation with clinicopathologic features and prognosis of HCC patients was analyzed.

RESULTS: EDIL3 was highly expressed in 48.5% of the HCC patients. Although the EDIL3 expression level did not correlate with any clinicopathological parameters, Kaplan-Meier survival analysis showed that high expression level of EDIL3 resulted in a significantly poor prognosis of HCC patients (log-rank test, P = 0.010). Multivariate Cox's analysis showed that the EDIL3 expression level was a significant and independent prognostic parameter for the overall survival rate of HCC patients (hazard ratio = 1.978, 95% confidence interval = 1.139-3.435, P = 0.015).

CONCLUSION: High expression level of EDIL3 predicts poor prognosis of HCC patients. EDIL3 may be a potential target of antiangiogenic therapy for HCC.

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Key words: Epidermal growth factor-like repeats and discoidin I-like domains 3; Hepatocellular carcinoma; Prognosis; Angiogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide[1]. Due to its high recurrence rate after surgical resection and chemotherapy resistance, the total survival rate of HCC patients is only 3%-5%[2]. There is evidence that angiogenesis plays a critical role in the development and progression of HCC, a highly vascularized tumor. It has been shown that microvascular density is significantly correlated with HCC prognosis and postoperative recurrence[3-6]. Therefore, it is crucial to understand the molecular mechanism underlying angiogenesis of HCC.

Epidermal growth factor-like repeats and discoidin

I-like domains 3 (EDIL3), also known as endothelial cell locus 1 (DEL1), is a glycoprotein secreted by endothelial cells, which was isolated and identified from the embryonic mouse lung in 1998[7]. EDIL3, containing a signal peptide, 3 epidermal growth factor-like repeats, and 2 discoidin I-like repeats, initiates angiogenesis by binding to integrin αvβ5 on resting endothelium and may play an important role in vessel wall remodeling and development during angiogenesis[8]. It has been confirmed that EDIL3 can induce mesentery and cerebral angiogenesis in mice[9,10]. Both animal experiments and clinical studies have demonstrated that EDIL3 gene therapy is effective for ischemic disease[11-13]. There is evidence that EDIL3 is involved in tumor angiogenesis and plays an important role in interaction between HCC cells and endothelial cells[14-16]. It has been reported that the EDIL3 gene is over-expressed in HCC[17]. As far as we know, no report is available on the actual expression level of EDIL3 and the correlation between clinicopathologic features and prognosis of HCC patients. Therefore, in this study, we investigated the EDIL3 protein expression profile in 101 primary HCC patients, and found that EDIL3 was highly expressed in 48.5% of the HCC patients, suggesting that the high expression level of EDIL3 is a reliable indicator for the poor prognosis of HCC patients.

MATERIALS AND METHODS

Patients and tissue samples

A total of 101 patients with primary HCC, who underwent routine surgery at Sun Yat-Sen University Cancer Center in 1998-2002, were enrolled in this study. Their diagnosis was made by a pathological examination, and all patients did not receive any preoperative treatment before admission. Histological cell types were assigned following the WHO classification criteria. Paraffin-embedded HCC tissue samples (n = 101) were obtained from surgical pathology files at Sun Yat-Sen University Cancer Center. All tissue blocks were cut into consecutive 4-μm thick sections. The patients were followed up for 3-81 mo with a median follow-up time of 30 mo.

Immunohistochemistry

The sections were deparaffinized in xylene and rehydrated with graded ethanol. Following rehydration, endogenous peroxidase was inactivated with 0.3% hydrogen peroxide. Antigen was retrieved by putting the sections in a boiling ethylenediaminetetraacetic acid buffer (1 mmol/L, pH 8.0) for 15 min. After rinsed with phosphate buffered saline (PBS), the sections were incubated with rabbit anti-EDIL3 antibody (Sigma-Aldrich, Inc. USA) diluted in a working solution (1:200) at 37℃ for 1 h, and then with horseradish peroxidase (ChemMate™ EnVision™ detection kit) at 37℃ for 30 min. Finally, the visualization signal was developed with 3,3'-diaminobenzidine tetrahydrochloride and all sections were then counterstained with hematoxylin. For negative controls, the sections were incubated in a solution without anti-EDIL3 antibody under the same experimental conditions. The percent of positive cells was scored as '0' (< 5%, negative), '1' (5%-25%, sporadic), '2' (25%-50%, focal), and '3' (> 50%, diffuse), respectively. The staining intensity was scored as '0' (no staining), '1' (weakly stained), '2' (moderately stained) and '3' (strongly stained), respectively. Both the percent of positive cells and cell staining intensity were decided in a double-blinded manner. The final EDIL3 immunostaining score was calculated using the percent of positive cell score × staining intensity score ranging 0-9. High EDIL3 expression level was defined as a total score ⩾ 4, and low EDIL3 expression level as a total score < 4.

Table 1 Relation between epidermal growth factor-like repeats and discoidin I-like domains 3 expression in hepatocellular carcinoma and clinicopathological features of hepatocellular carcinoma patients

Clinicopathologic features (n)	Low EDIL3 (total score < 4) n = 52	High EDIL3 (total score ⩾ 4) n = 49	P value
Age (yr)			0.889
< 60 (57)	29	28	
⩾ 60 (44)	23	21	
Gender			0.648
Male (87)	44	43	
Female (14)	8	6	
Tumor size (cm)			0.491
< 5 (26)	16	10	
5-10 (58)	28	30	
> 10 (17)	8	9	
Histological differentiation			0.379
Well (17)	9	8	
Moderate (63)	35	28	
Poor (21)	8	13	
Liver cirrhosis			0.617
Yes (50)	27	23	
No (51)	25	26	
Metastasis			0.202
Yes (15)	10	5	
No (86)	42	44	
Recurrence			0.817
Yes (38)	19	19	
No (63)	33	30	
HBSAg status			0.527
Positive (90)	45	45	
Negative (11)	7	4	
Serum AFP			0.707
Positive (62)	31	31	
Negative (39)	21	18	

EDIL3: Epidermal growth factor-like repeats and discoidin I-like domains 3; HBSAg: Hepatitis B surface antigen; AFP: α-fetoprotein.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Correlation of EDIL3 expression with immunohistochemistry and clinicopathologic parameters was evaluated by chi-square test or Fisher's exact probability test. Overall survival rate was calculated with the Kaplan-Meier method and the difference in survival curves was analyzed by the log-rank test. The follow-up time was calculated from the date of surgery to the date of death, or the last known follow-up. Independent prognostic factors were analyzed by the Cox

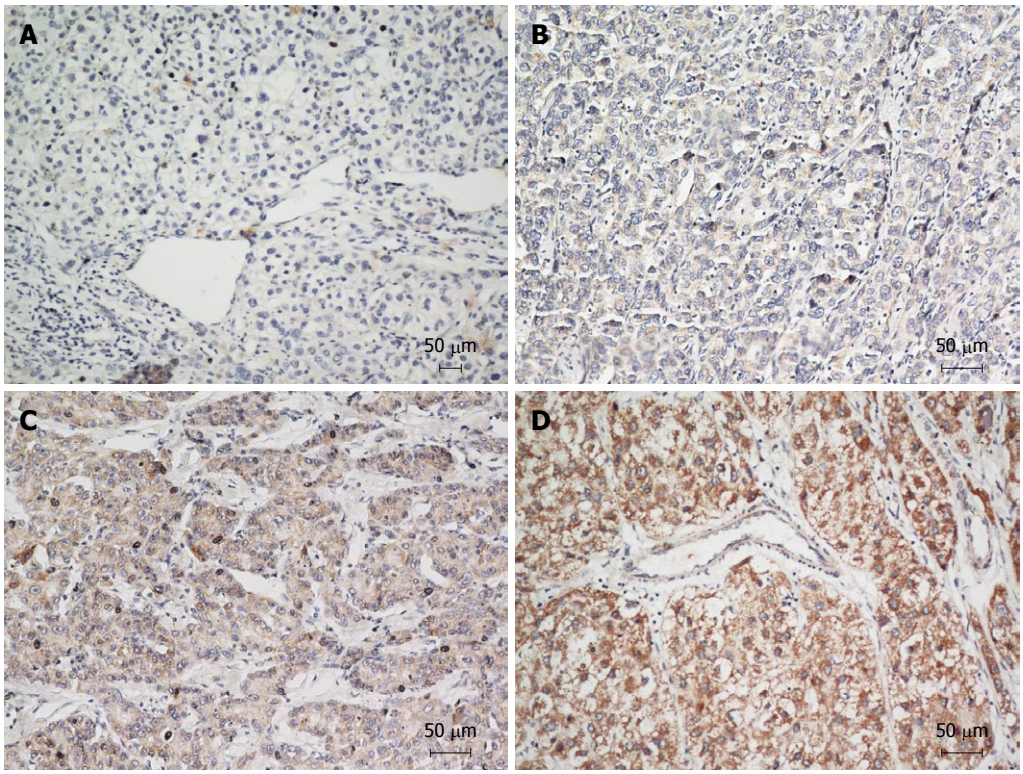

Figure 1 Immunohistochemical analysis of epidermal growth factor-like repeats and discoidin I-like domains 3 in hepatocellular carcinoma patients. A: Negative expression of epidermal growth factor-like repeats and discoidin I-like domains 3 protein; B: Low expression level of epidermal growth factor-like repeats and discoidin 1-like domains 3 protein; C, D: High expression level of epidermal growth factor-like repeats and discoidin 1-like domains 3 protein. A, B: Original magnification × 100; C, D: Original magnification × 200.

proportional hazards regression model. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of EDIL3 in HCC tissues

The expression level of EDIL3 protein in 101 HCC tissue samples was measured with immunohistochemical staining. The immunoreactivity of EDIL3 in cytoplasm was detected (Figure 1). Overall, EDIL3 was positively and negatively expressed in 95 (94.06%) and 6 (5.94%) of the 101 HCC patients, respectively (Figure 1B-D, total score ⩾ 1; Figure 1A, total score = 0). EDIL3 was highly and lowly expressed in 49 (48.5%) and 52 (51.5%) of the 101 HCC patients, respectively (Figure 1C and D, total score ⩾ 4; Figure 1A and B, total score < 4). No significant difference was found in EDIL3 expression level and clinicopathologic parameters including age, gender, tumor size, histological differentiation, liver cirrhosis, metastasis, recurrence, hepatitis B virus infection, and serum α-fetoprotein (Table 1).

Correlation between high EDIL3 protein expression level and low survival rate of HCC patients

The prognostic effect of EDIL3 on the overall survival rate of HCC patients with a high or low EDIL3 protein expression level was compared using Kaplan-Meier survival curves and the log-rank test respectively, showing that high expression level of EDIL3 protein was a significant prognostic factor for poor overall survival rate of HCC patients.

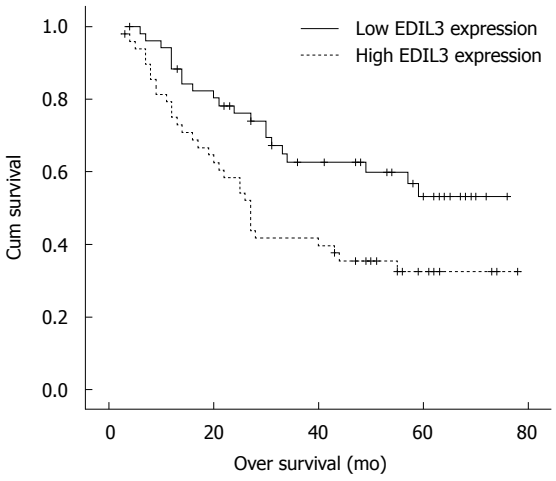

Figure 2 Overall survival rate of hepatocellular carcinoma patients estimated according to the epidermal growth factor-like repeats and discoidin I-like domains 3 expression level in hepatocellular carcinoma tissue samples (Kaplan-Meier method) with immunohistochemical staining.

The 5-year survival rate of HCC patients with a high or a low EDIL3 protein expression level was 32.4% and 53.2%, respectively. A significant difference was observed on the Kaplan-Meier survival curves for HCC patients with a high or a low expression level of EDIL3 ($P = 0.010$, log-rank test, Figure 2). Univariate Cox regression analysis showed that EDIL3 expression level and tumor size were the significant prognostic factors for HCC patients (Table 2).

Table 2 Univariate and multivariate analysis showing the overall survival rate for hepatocellular carcinoma patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
EDIL3	2.019	1.163-3.504	0.013	1.978	1.139-3.435	0.015
Gender	0.658	0.283-1.529	0.330			
Age	0.817	0.487-1.370	0.443			
Tumor size	1.662	1.133-2.437	0.009	1.492	0.980-2.270	0.062
Histologic grade	1.304	0.866-1.963	0.204			
Cirrhosis	0.683	0.410-1.139	0.144			
HBsAg status	1.244	0.565-2.738	0.588			
Serum AFP	1.592	0.923-2.744	0.094			
Metastasis	1.407	0.731-2.707	0.307			
Recurrence	1.349	0.804-2.263	0.257			

HR: Hazard ratio; CI: Confidence interval; EDIL3: Epidermal growth factor-like repeats and discoidin I-like domains 3; HBSAg: Hepatitis B surface antigen; AFP: α-fetoprotein.

The relative risk was 2.019 times higher for patients with a high EDIL3 expression level than for those with a low EDIL3 expression level. Multivariate Cox regression analysis showed that EDIL3 expression might play a role in prediction of the overall survival rate of HCC patients ($P = 0.015$, Table 2).

DISCUSSION

HCC is one of the most malignant cancers with no effective chemotherapy available for it at present. Antiangiogenic therapy is a novel systemic therapy for HCC by blocking the effect of angiogenic factors and inhibiting the proliferation of endothelial cells[18]. Further studies are needed to elucidate the mechanism underlying the angiogenesis of HCC in order to rationally use antiangiogenic agents in treatment of HCC. It has been shown that EDIL3 can enhance tumor angiogenesis by stimulating the proliferation of resting endothelial cells[14], indicating that EDIL3 may be a new target for antiangiogenic therapy and play an important role in the pathogenesis of HCC.

In the present study, EDIL3 was positively expressed in 94.06% of HCC patients and highly expressed in 48.5% of HCC patients, which is consistent with the reported data[14], indicating that human hepatocarcinoma cells can secrete EDIL3 involved in the angiogenesis of HCC.

In this study, the prognosis of HCC patients with a high expression level of EDIL3 was poor, and Cox regression analysis indicated that high expression level of EDIL3 was a significant prognostic factor for a poor overall survival rate of HCC patients, suggesting that EDIL3 may become a novel prognostic marker for HCC. However, the expression level of EDIL3 did not correlate with any clinicopathological parameters of HCC, which can be explained as follows. First, multivariate Cox regression analysis indicated that EDIL3 expression level was an independent predictor for the overall survival rate of HCC patients, and EDIL3 might have a direct effect on the prognosis of HCC patients rather than other factors. Second, some known risk factors for HCC, such as histological differentiation, presence of metastasis and disease recurrence, were not correlated with the prognosis of HCC.

Angiogenesis plays an important role in development and progression of HCC[19] that is the balanced result of the actions of multiple angiogenic and antiangiogenic factors from both tumor and host cells[18]. EDIL3 protein is involved in adhesion of HCC cells and HCC-derived endothelial cells, and initiates angiogenesis by binding to αvβ3 and αvβ5 in endothelial cells[8,16]. It has been confirmed that the expression level of αvβ3 and αvβ5 is higher in HCC-derived endothelial cells than in normal liver sinusoidal endothelial cells, suggesting that HCC cells with a high EDIL3 expression level can stimulate the growth of vascular endothelial cells and promote the angiogenesis in HCC. Furthermore, it has been shown that EDIL3 can prolong the survival time of endothelial cells by down-regulating their apoptosis-related gene expression[20], indicating that HCC cells with a high EDIL3 expression level can also promote angiogenesis by inhibiting the apoptosis of endothelial cells.

In conclusion, the prognosis of patients with a high expression level of the EDIL3 protein is poor, which may be attributed to the relation between EDIL3 and HCC angiogenesis. Current evidence identifies EDIL3 as a potential target of antiangiogenic therapy for HCC.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is a high malignancy with a poor prognosis. Angiogenesis plays a critical role in the development and progression of HCC. Epidermal growth factor (EGF)-like repeats and discoidin I-like domains 3 (EDIL3) can enhance tumor angiogenesis by stimulating the proliferation of resting endothelial cells and may be involved in HCC angiogenesis.

Research frontiers

EDIL3 is involved in tumor angiogenesis and plays an important role in interaction between HCC and endothelial cells. However, no report is available on

the actual expression level of EDIL3 and its correlation with clinicopathologic features and prognosis of HCC patients. In this study, the EDIL3 protein expression profile in primary HCC was studied.

Innovations and breakthroughs

In this study, EDIL3 was expressed in most HCC patients and highly expressed in 48.5% of the HCC patients. Furthermore, this is the first study to report that high expression level of EDIL3 in HCC tissues was a reliable indicator for the poor prognosis of HCC patients. The prognosis of HCC patients with a high expression level of EDIL3 protein was poor, which may be attributed to the relation between EDIL3 and HCC angiogenesis.

Applications

High expression level of EDIL3 in HCC was a significant prognostic factor for a poor overall survival rate of HCC patients, indicating that EDIL3 can become a novel prognostic marker and a target of antiangiogenic therapy for HCC.

Terminology

EDIL3, an acronym for "EGF-like repeats and discoidin I-like domains 3", contains a signal peptide, 3 epidermal growth factor-like repeats, and 2 discoidin I-like repeats. It is a glycoprotein secreted by endothelial cells.

Peer review

This is an interesting study. However, it is unclear why EDIL3 expression was associated with a poor prognosis, and factors known to have a negative impact on the outcome such as tumor size, histological differentiation, the presence of metastasis and disease recurrence. The authors should explain these findings in the DISCUSSION section.

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BRIEF ARTICLE

Clinicopathological features of gastric glomus tumor

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Abstract

AIM: To study the clinicopathological features of gastric glomus tumor and review the related Chinese literature published in 1990-2010.

METHODS: A case of gastric glomus tumor was reported. Clinicopathological findings in 56 cases of gastric glomus tumor were analyzed.

RESULTS: Gastric glomus tumor was far more common in women than in men with a female to male ratio of 1.6:1. The median age of the patients was 45 years (range 28-79 years). The patients often complained of epigastric pain and bloody stool. The tumor was located in antrum of the stomach. The greatest diameter of the tumor was 0.8-11cm. Histologically, the tumor was comprised of nests of glomus cells surrounding the capillaries. Glomus cells were small, uniform and round. Vimentin, smooth muscle actin and actin were expressed in the tumor. Other markers, including S-100 protein, CD34, CD117, desmin, CD56, synaptophysin, chromogranin A, neuron specific enolase and cytokeratin were all negative.

CONCLUSION: Gastric glomus tumor is a rare benign mesenchymal neoplasm. Its diagnosis depends on pathologic examination. Differential diagnosis includes gastrointestinal stromal tumor, paraganglioma and carcinoid tumor.

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Key words: Stomach; Glomus tumor; Clinicopathology; Immunohistochemistry

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Fang HQ, Yang J, Zhang FF, Cui Y, Han AJ. Clinicopathological features of gastric glomus tumor. *World J Gastroenterol* 2010; 16(36): 4616-4620 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i36/4616.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i36.4616

INTRODUCTION

Gastric glomus tumor is a rare benign mesenchymal neoplasm arising from the glomus body. The majority of glomus tumors occur in the distal extremities, particularly in the subungual region, hand, wrist, foot, bone and joints, skeletal muscle, soft tissue, mediastinum, trachea, kidney, lung, uterus and vagina. Since the first case of gastric glomus tumor was reported in 1951 by Kay *et al*[1], few cases have been reported[2-13]. The clinicopathological features of 56 cases of gastric glomus tumor were studied with a review of the related Chinese literature published in 1990-2010.

MATERIALS AND METHODS

A 60-year-old woman was admitted to our institution due to a gastric mass found at upper gastrointestinal endosco-

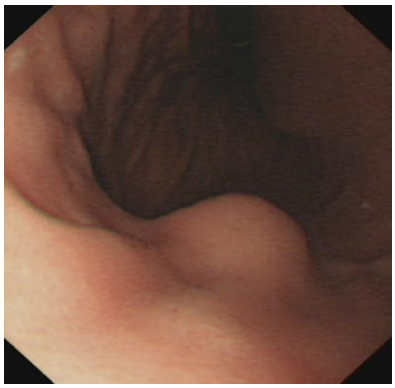

Figure 1 Upper gastrointestinal endoscopy showing a well-circumscribed elevated mass lesion measuring 1.5 cm × 1.5 cm with normal overlying mucosa in anterior wall of gastric corpus.

Figure 2 Endoscopic ultrasonography showing a 1.5 cm × 1.2 cm sharply demarcated homogeneous hypoechoic mass in the third and fourth sonographic layers of gastric wall.

py. Endoscopy demonstrated a well-circumscribed elevated mass measuring 1.5 cm × 1.5 cm with normal overlying mucosa in the anterior wall of gastric corpus (Figure 1). Endoscopic ultrasonography showed a sharply demarcated homogeneous hypoechoic mass measuring 1.5 cm × 1.2 cm in the third and fourth sonographic layers of gastric wall with rich blood supply (Figure 2). The patient underwent wedge resection of the tumor.

Resected specimens were fixed in 4% formaldehyde and embedded in paraffin. Tissue block was cut into 4-micrometer thick sections which were stained with routine staining methods.

Immunohistochemical staining of the formalin-fixed and paraffin-embedded tissue was carried out using an EnVison kit (DAKO). Primary antibodies including vimentin, smooth muscle actin (SMA), actin, CD117, CD34, S-100 protein, desmin, CD56, synaptophysin, chromogranin A, neurone specific enolase and cytokeratin were purchased from the DAKO Corporation.

Clinical, imaging and pathological features of 56 patients with gastric glomus tumor were analyzed according to a review of related Chinese literature published in 1990-2010.

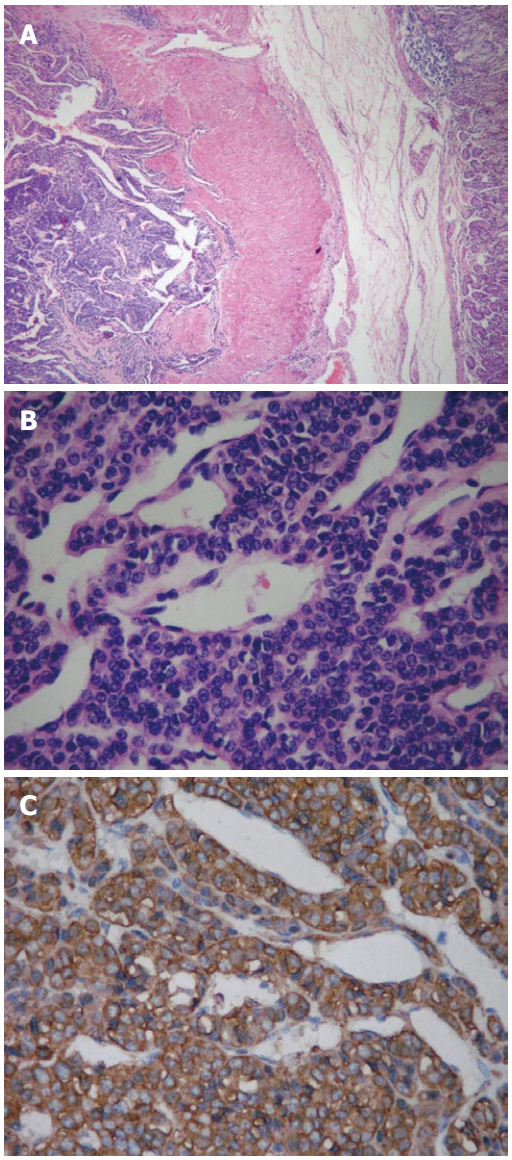

Figure 3 Gastric glomus tumor. A: Glomus tumor located in the muscularis of stomach (hematoxylin-eosin × 40); B: Clusters of uniform, round tumor cells around dilated blood vessels (hematoxylin-eosin × 200); C: Tumor cells positive for smooth muscle actin (immunohistochemistry staining × 200).

RESULTS

In our case, the cut surface of specimens demonstrated a grayish-white, circumscribed mass measuring 1 cm × 1 cm arising from the muscularis of stomach without involving the serosal surface. Histologically, the tumor was mainly located in the muscularis of stomach and comprised of small, uniform, rounded cells surrounding capillaries with diffuse sheet distributions but without nuclear pleomorphism and mitotic figures (Figure 3). Immunohistochemical staining was positive for vimentin, SMA and actin (Figure 3). Other markers, including S-100 protein, CD34, CD117, Desmin, CD56, synaptophysin, chromogranin A, neurone specific enolase and cytokeratin were all negative. A final diagnosis of gastric glomus tumor was made based

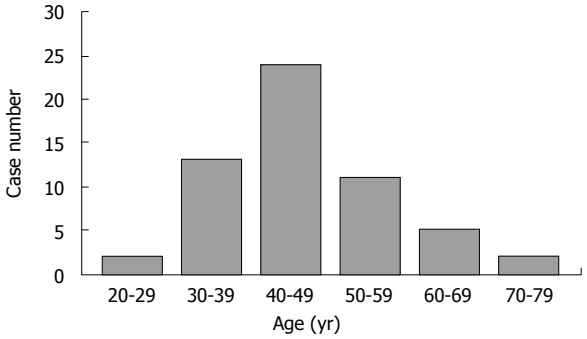

Figure 4 Distribution of age in 57 cases of gastric glomus tumor. The age distribution of 57 cases of gastric glomus tumors

Table 1 Clinical features of patients with gastric glomus tumor

	n (%)
Sex	
Male	22 (38.6)
Female	35 (61.4)
Symptoms	
Epigastric pain	35 (61.4)
Bloody stool	14 (24.6)
Asymptomatic	8 (14.0)
Location	
Antrum	53 (93.0)
Corpus	3 (5.3)
Junction of antrum-corpus	1 (1.7)
Histopathological subtype	
Solid glomus tumor	54 (94.7)
Glomangioma	3 (5.3)
Glomangiomyoma	0 (0)

on the typical histopathology and immunohistochemical staining.

Clinical features

Of the 57 patients with gastric glomus tumor including our case, 35 were females (64.1%), 22 were males (35.9%) with a female to male ratio of 1.6:1. The median age of the patients was 45 years (range 28-79 years) (Figure 4). Of the 57 patients, 35 complained of upper abdominal pain or fullness (61.4%), 14 had bloody stools (24.6%) and 8 were asymptomatic (14%). The course of the disease ranged 1 wk-over 10 years. Gastric glomus tumor was found in gastric antrum of 53 patients, in gastric corpus of 3 patients, and in junction of gastric antrum-corpus of 1 patient, respectively (Table 1).

Imaging features

Of the 57 patients with gastric glomus tumors, 8 had barium meal X-ray examination, which showed a circular or semi-circular filling defect with smooth surface and a broad base on the gastric angle. The abdominal sonogram revealed a sharply demarcated mass with a homogeneous hypoechoic pattern of gastric wall in 12 patients and a heterogeneous hypoechoic pattern with internal hyperechoic spots in a few patients. Endoscopic ultrasonography showed a hyperechoic mass in gastric antrum of 1 patient. However, endoscopic ultrasonography showed a sharply demarcated, homogeneous hypoechoic mass in the third and fourth sonographic layers of gastric wall with rich blood supply in our patient. Fifty patients underwent gastroscopy, showing round or oval elevated lesions with smooth surface on gastric mucosa of most patients and erosion or ulceration with blood clot attached in a few patients. Abdominal computed tomography scan revealed a demarcated soft tissue enhancing mass in the stomach of 16 patients, suggesting of a hypervascular benign gastric tumor.

Pathological features

Macroscopically, the greatest diameter of the tumor was 0.8-11 cm. On cut surface, the tumor was firm and solid or cystic, and gray or grayish-red or grayish-white, or dark brown in color.

Histologically, the tumor was well-circumscribed and located in gastric submucosa or muscularis and comprised of glomus cells surrounding capillaries. The glomus cells were small, uniform, and round without nuclear pleomorphism, mitotic figures or necrosis. The stroma showed hyalinization or myxoid change in some patients and sporadic mast cells could be seen in the stroma.

Immunohistochemical staining showed that gastric glomus tumor was strongly and diffusely positive for SMA (22/22), vimentin (18/18) and actin (10/10), calponin (5/7), type Ⅳ collagen (2/2) and laminin (1/1). Other markers, including desmin, CD31, CD34, CD99, CD117, cytokeratin, S-100 protein, synaptophysin, chromogranin A, and neuron specific enolase were negative.

According to the World Health Organization (WHO) Classification of Tumors of Soft Tissue and Bone (2002)[14], glomus tumour can be further divided into "solid glomus tumor", "glomangioma", and "glomangiomyoma" depending on the relative prominence of glomus cells, vascular structure and smooth muscle. According to the pathological features of 57 patients reported in Chinese literature including our patient, 54 patients had "solid glomus tumor" and 3 had "glomangioma".

Treatment and prognosis

Of the 57 patients with gastric glomus tumor, 18 underwent subtotal gastrectomy, 36 gastric wedge resection of the tumor and 3 tumor excision. Follow-up information was available from 15 patients who were alive without recurrence or metastasis 1-7 years after surgery.

DISCUSSION

Gastric glomus tumor is a rare benign mesenchymal tumor arising from the glomus body. Its clinical features are nonspecific. Patients often complain of epigastric pain and bloody stool. We reviewed 57 cases including our case reported in Chinese literature in 1990-2010. Gastric glomus tumor is far more common in women than in men with a female to male ratio of 1.6:1. The median age of the patients was 45 years (range 28-79 years). Most of the tumors were located in gastric antrum. The greatest diam-

eter of tumor was 0.8-11 cm, which is much larger than that in the distal extremities. The tumor is usually solitary but cases of multiple gastric glomus tumors have been reported[15-17].

The tumor can be further divided into "solid glomus tumor", "glomangioma", and "glomangiomyoma" depending on the relative prominence of glomus cells, vascular structure and smooth muscle[14]. According to the review of the pathological features of 57 patients including our case, 54 patients (94.7%) had "solid glomus tumor" and 3 (5.3%) had "glomangioma". Although gastric glomus tumor is usually benign, malignant behavior cannot be excluded. According to the WHO Classification of Tumors of Soft Tissue and Bone[14], "glomus tumor" should be defined as a malignant tumor when its size is > 2 cm and located at subfascia or viscera, with atypical mitotic figures or marked nuclear atypia and mitotic activity. However, no criteria are available for the diagnosis of gastric malignant glomus tumor. Several cases of malignant or metastatic gastric glomus tumor have been reported[11,18-20]. It seems that the biological behavior, especially metastasis, is more valuable for the diagnosis of malignant gastric glomus tumor.

Of the 57 patients with gastric glomus tumor, 18 underwent subtotal gastrectomy, 36 wedge resection of the tumor, and 3 tumor excision depending on the location and size of the tumor. Wedge resection with negative margins was the major treatment of choice. Follow-up information was available from 15 patients who were alive without recurrence or metastasis 1-7 years after surgery.

Gastric glomus tumor should be differentiated from other lesions, such as gastrointestinal stromal tumor (GIST), paraganglioma and carcinoid tumor, *etc*. GIST is the most common mesenchymal tumor in the stomach. Tumor often shows a variety of histological patterns, including the epithelioid pattern which may be confused with glomus tumor. However, GIST usually lacks of dilated capillaries and tumor cells are positive for CD117 and CD34, which is different from glomus tumor[21]. Paraganglioma cells are arranged in a characteristic alveolar or Zellballen pattern with rich thin-walled blood vessels in stroma which can easily be confused with glomus tumor. However, paraganglioma is comprised of large cells with nuclear enlargement, hyperchromatism and cytoplasm varying from pink to clear in color, or amphophilic and positive for S-100 protein, synaptophysin, chromogranin A, and neurone specific enolase. Carcinoid tumor is a neuroendocrine carcinoma and comprised of oval or spindle tumor cells arranged in cords or nests with thin-walled blood vessels. Carcinoid tumor cells are positive for cytokeratin, S-100 protein, synaptophysin, chromogranin A, and neurone specific enolase, but negative for SMA.

In conclusion, Gastric glomus tumor is rare benign mesenchymal neoplasm. Since patients have no specific clinical and imaging findings, it is difficult to diagnose before operation. Histopathological features of small, uniform and round tumor cells surrounding capillaries and strongly positive for SMA, are helpful to make its diagnosis. The differential diagnosis includes gastrointestinal stromal tumor, paraganglioma and carcinoid tumor.

COMMENTS

Background

Gastric glomus tumor is an extremely rare mesenchymal tumor. Few cases have been reported in the literature. Its clinicopathological features are unclear.

Research frontiers

In this study, the authors reported 1 case of gastric glomus tumor and reviewed 56 cases reported in Chinese literature in 1990-2010.

Innovations and breakthroughs

This is the first study to analyze the clinicopathological findings of gastric glomus tumor in large series.

Applications

This study provided important information for the diagnosis and treatment of gastric glomus tumor.

Terminology

Gastric glomus tumor is a rare benign mesenchymal neoplasm. Histopathological features of small, uniform and rounded tumor cells surrounding capillaries and strongly positive for smooth muscle actin, are helpful to make its diagnosis. Differential diagnosis includes gastrointestinal stromal tumor, paraganglioma and carcinoid tumor.

Peer review

This is an interesting paper and provides important information for the diagnosis and treatment of gastric glomus tumor.

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CASE REPORT

Complete resection of isolated pancreatic metastatic melanoma: A case report and review of the literature

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Abstract

Isolated metastatic melanoma of the pancreas is very rare. Currently, there is very limited experience with surgical resection of pancreatic metastasis. The potential benefit of metastasectomy can improve the quality of life and survival time of patients. We present a case of a 39-year-old Chinese male with a solitary pancreatic tumor which was considered a cystic benign lesion for years. Pathology and immunohistochemistry showed that the tumor in pancreatic tail was a metastasis from a malignant melanoma of the eyeball. No other metastastic foci were found in abdomen. The tumor was completely resected with combined distal pancreatectomy and splenectomy. The patient has survived 25 mo without any signs of local recurrence or other metastatic lesions after operation, indicating that complete surgical resection of a solitary metastatic melanoma of the pancreas can prolong the survival time of patients.

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Key words: Melanoma; Metastasis; Pancreas; Solitary tumor; Resection

Peer reviewer: Dr. Kai Bachmann, Department of Surgery, University Medical Center Hamburg, Martinistrasse 52, Hamburg 22529, Germany

He MX, Song B, Jiang H, Hu XG, Zhang YJ, Zheng JM. Complete resection of isolated pancreatic metastatic melanoma: A case report and review of the literature. *World J Gastroenterol* 2010; 16(36): 4621-4624 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i36/4621.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i36.4621

INTRODUCTION

Pancreatic metastasis from a non-pancreatic primary tumor is rare, accounting for approximately 2% of all pancreatic tumors[1,2]. The clinical occurrence of isolated metastasis to the pancreas is even less. There is currently very limited experience with surgical resection of isolated pancreatic metastasis[3]. In fact, pancreatic resection is associated with a high morbidity and mortality, and metastatic disease to the pancreas is considered a terminal-stage condition[4]. However, recent improvement in morbidity and mortality rates of such patients after pancreaticoduodenectomy has made the indication for this operation more acceptable[5]. The potential benefit of metastasectomy for such cases is documented because it can improve their quality of life and survival time[6,7]. Here, we present a rare male Chinese case of an isolated pancreatic metastatic melanoma, which was surgically treated at our hospital. The patient has survived 25 mo without any signs of local recurrence or other metastatic diseases after operation. The related literature was reviewed.

CASE REPORT

A 39-year-old male Chinese patient was admitted to the

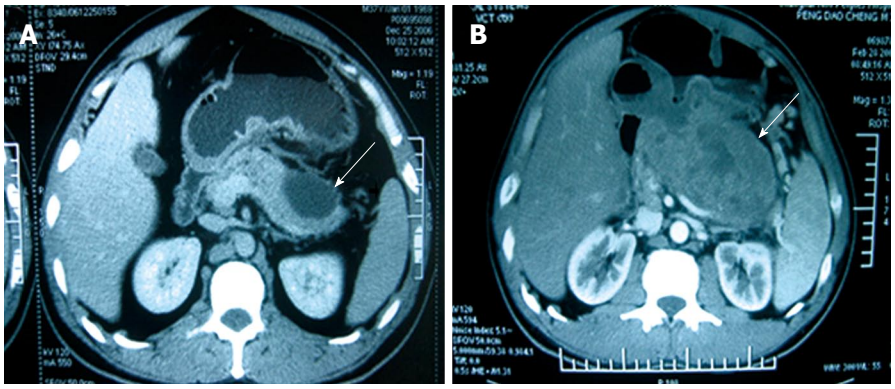

Figure 1 Computed tomography showing a pseudocystic tumor (arrows) as a benign cyst (A) and a bigger pseudocystic tumor poorly demarcated from the surrounding tissue (B).

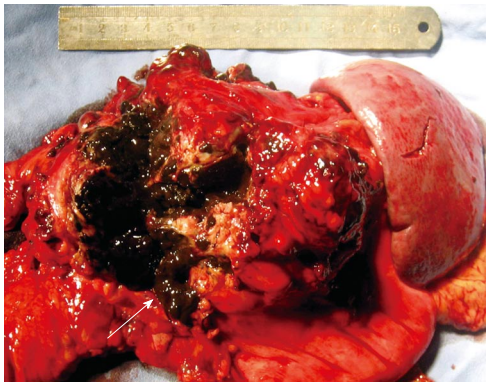

Figure 2 Specimen showing a metastatic melanoma as a black-brown mass (arrow) in pancreatic tail.

Department of General Surgery, Affiliated Changhai Hospital of Second Military Medical University in March 2008 because of a 3-year history of back pain and a cystic tumor in pancreatic tail. He underwent excision of a left eyeball melanoma in a local hospital 5 years ago (2003) with no regional and distant metastases observed. Pathological stage was pT4aN0M0, stage Ⅲ. The patient did not receive any chemotherapy or radiotherapy after surgery. Subsequently, the patient had back pain in 2006 with a tumor found in pancreatic tail. Computed tomography (CT) showed a cystic tumor in distal pancreas (Figure 1A). The clinical and CT manifestations suggested a benign disease for years. In 2008, because the tumor grew bigger and seemed invasive, the patient was surgically treated at our hospital. Physical examination upon admission in March 2008 revealed the excision of the left eyeball and no local recurrence. Laboratory tests showed normal CEA and CA199 levels, and blood cell count. CT showed a pseudocyst in pancreatic tail with a diameter of 7 cm and poor demarcation from surrounding tissue (Figure 1B). Magnetic resonance imaging (MRI) of the pancreas showed similar results. Endoscopic retrograde pancreatography (ERCP) revealed a deviated main pancreatic duct with no branching in pancreatic tail. Endoscopic examination did not reveal a tumor in any portion of duodenum. In March 2008, a partial pancreatic excision was performed. A tumorous mass measuring 18 cm × 13 cm × 8 cm was found in the pancreatic tail (Figure 2), causing adherence to duodenum and the back of stomach. Frozen section biopsy showed a malignant melanoma which was completely resected with combined distal pancreatectomy and splenectomy, during which no other metastatic foci were found in abdomen.

Histopathology revealed ill-defined epithelioid or polygon tumor cells infiltrating the pancreatic tail (Figure 3A) and dark brown granular intracytoplasmic pigmentation in most parts of the infiltration (Figure 3B). The histopathologic pattern of biopsy specimens was very similar to that of specimens examined postoperatively 5 years ago. Fontana-Masson histochemical staining confirmed a melanin pigment. Immunoreactivity was strongly positive for anti-HMB45 (Figure 3C) and anti-S100 protein (Figure 3D) and weakly positive for anti-melan A. Tumor cells were negative for CAM5.2, EMA, CK8/18, CA199 and CDX2. Ki-67 was over 80%. This malignant melanoma was proven to be a metastasis from the initial lesion of the eyeball. Solitary metastatic melanoma of the pancreas from the eyeball was thus diagnosed.

The patient underwent chemotherapy after surgery and was followed up for 2 years. He has survived 25 mo without any signs of local recurrence or other metastatic lesions after operation.

DISCUSSION

Only 5% of malignant melanomas have been found in the eye, and most ocular melanomas commonly metastasize to the viscera[8]. Ocular melanoma rarely metastasizes to lymph nodes due to lack of lymphatic vessels in the uveal tract[8,9]. Pancreatic metastases occur in less than 2% of patients with visceral melanoma metastases, but most pancreatic metastases disproportionately originate from primary ocular melanoma[9,10]. To date, few cases of pancreatic metastatic melanoma from ocular melanoma have been reported[9-14]. We present an unusual case of solitary pancreatic metastatic melanoma after a latency period of 5 years from melanoma of the eyeball. The patient had a 3-year history of back pain and a cystic mass in pancreatic tail. CT showed a pseudocyst in pancreatic tail with a diameter of 4-7 cm. The clinical and CT manifestations suggested a benign disease for years. Eventually, because the tumor grew bigger and seemed invasive, the patient was surgically treated at our hospital. The distal pancreas and spleen were carefully resected with no other metastatic foci observed in abdomen. Histopathology and immu-

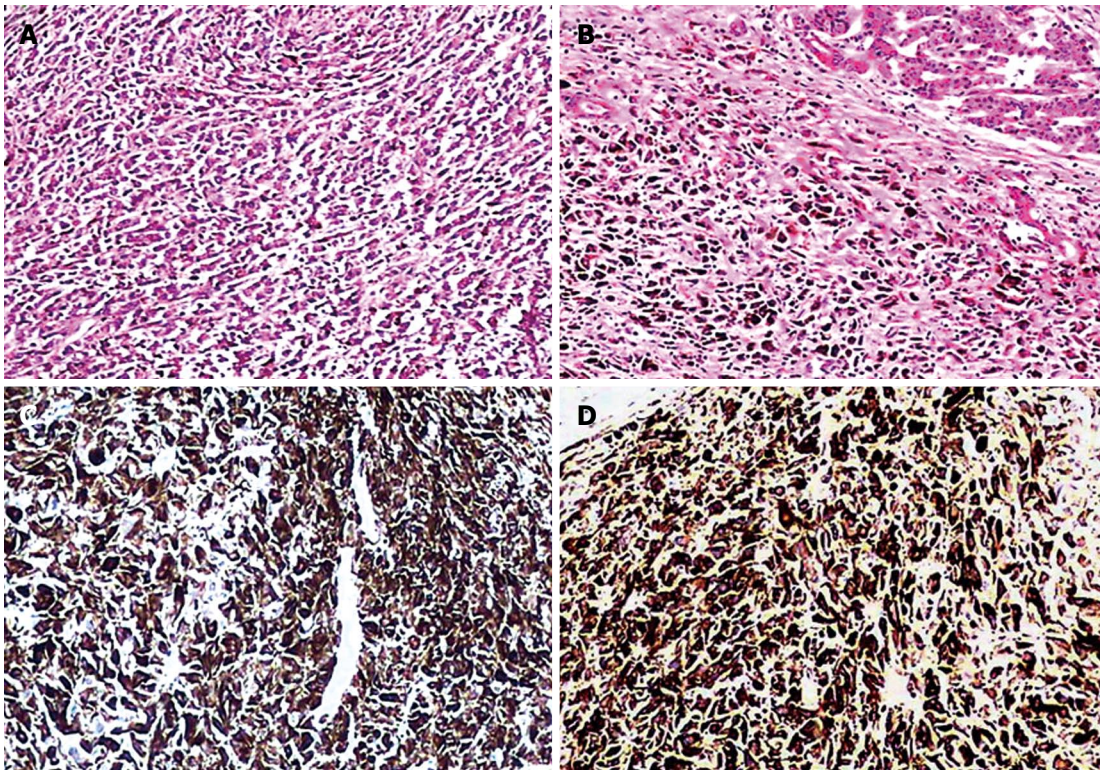

Figure 3 Microscopy showing epithelioid or polygon tumor cells infiltrating the pancreatic tail (A), dark brown granular intracytoplasmic pigmentation in most tumor cells (B), positive HMB-45 (C) and S-100 protein (D). Original magnification × 200. A and B: HE staining; C and D: Immunostaining.

nohistochemistry showed that the tumor was a melanoma, which was considered a metastasis from the initial lesion of eyeball, and chemotherapy was arranged. The patient has survived 25 mo without any signs of local recurrence or other metastatic lesions after operation.

Few reports are available on secondary pancreatic malignancies from a malignant melanoma, and most of them did not explain the features of the tumors. The clinical manifestations of most pancreatic metastases include disturbance of exocrine and/or endocrine function, various gastrointestinal problems, progressive back pain, with or without obstructive jaundice, and pancreatitis[8,9,11-13]. However, in some individuals, pancreatic metastasis is completely asymptomatic[9]. It has been reported that patients with previously diagnosed eye or occult melanoma presenting as a pancreatic metastasis have upper quadrant abdominal pain, nausea, back pain or are asymptomatic[10-14]. In our patient, the melanoma metastasized to the pancreatic tail to form a solitary lesion, and the tumor in pancreatic tail extended to the wall of duodenum and the back of stomach. However, the patient recently experienced back pain. CT showed that the tumor was a cystic lesion as a benign cystic disease for years. ERCP revealed a deviated main pancreatic duct with no branching in pancreatic tail but no obstructive jaundice in the patient. Such invasiveness and CT performance are unique[10-14]. Cystic pancreatic masses seen on imaging and a prolonged history of upper abdominal pain or back pain are clinically suggestive of benign diseases such as inflammatory pseudocyst, true cyst or cystadenoma. The patient was diagnosed as a benign pancreatic cystic lesion. Eventually, because the tumor grew bigger and seemed invasive, he was surgically treated. In fact, the exact preoperative diagnosis of the tumor could not be established, primarily due to the tumor location in the distal pancreas where biopsy remains difficult. The most reliable method for verifying the diagnosis remains histological biopsy of the pancreas. In this case, intraoperative frozen biopsy is necessary and helpful.

It has been reported that the 5-year survival rate of patients with metastatic disease from cutaneous melanoma and those with distant metastasis of ocular melanoma is 18% and 39%, respectively after complete resection[15], while their survival rate is 0% when they are managed non-operatively[16]. It has been shown that median survival time of patients with pancreatic metastatic melanoma after complete and incomplete resection is 24 and 8 mo with a 5-year survival rate of 37% and 0%, respectively[16,17], indicating that complete resection can improve the survival rate of such patients. We think that, if resection is not too dangerous to perform, as in the present case, it is beneficial for prolonging the survival time of such patients. If a concurrent metastatic disease is found elsewhere, it is prudent to adopt a less aggressive approach[2,12-14,16].

The prognosis of patients with pancreatic metastatic malignant melanoma is variable and unpredictable. The interval time from the diagnosis of a primary tumor to pancreatic metastasis is 2-28 years[17,18]. It was reported that the disease-free interval time is not related the survival time[19,20] and that a prolonged interval time free of malignant melanoma does not imply a better prognosis once

metastasis occurs[21,22]. Our patient with a solitary pancreatic metastatic melanoma after a latency period of 5 years from melanoma of the eyeball has survived 25 mo without any signs of local recurrence or other metastatic diseases after complete resection of the tumor. It is unclear whether the presence of solitary or multifocal lesions is a determinant for the survival time of patients with pancreatic metastatic malignant melanoma. The prognosis of patients reported in the literature is different[12-14,22,23]. The aim of this report is to add a new case of isolated pancreatic metastatic melanoma in a Chinese male, which was successfully treated with surgical operation, indicating that complete resection of a solitary pancreatic metastatic lesion can prolong the survival time of such patients[23].

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Abnormal colonic cholinergic and nitrergic activities in relation to elastosis in uncomplicated diverticular disease

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Abstract

I read with interest the review on the pathogenesis of diverticular disease by Commane *et al* in *World J Gastroenterol* 2009; 15(20): 2479-2488. However, I would like to discuss several important errors that the authors made whilst citing information from previously published work on the neuromuscular dysfunction in the disease.

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Key words: Diverticular disease; Neural; Acetylcholine; Nitric oxide; Elastin; Protein gene product

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TO THE EDITOR

I read with interest the review on the pathogenesis of diverticular disease by Commane *et al*[1] in *World J Gastroenterol* 2009 May 28; 15(20): 2479-2488. However, in the discussion, the authors made several important errors while citing information from previously published work on the neuromuscular dysfunction in the disease. The authors argued that methods for measuring the general nerve, and cholinergic and nitrergic activity in longitudinal muscle (LM) in the disease[2,3] may have been erroneous because of potential confounders of elastosis and smooth muscle shortening, and mentioned that it was not clear how these potential confounders were controlled.

First, the authors stated in error that these studies used prostaglandin as a marker of general nerve tissue, whereas in fact, both studies reported the antibody localization of protein gene product (PGP), a marker of general nerve tissue, which has been used extensively to localize nerves in histological sections[4].

As discussed previously[3], the reduction in immunoreactivity of PGP in LM in diverticular disease was probably a spurious finding, secondary to the associated 200% increase in the surface area of elastin in LM compared with normal controls. It may be not due to an increase in general nerve degeneration in the disease, as the qualitative signs of degeneration were similar between the disease and controls, and reflected the mean age of the patients.

The method that was used to semi-quantify cholinergic and nitrergic activity was specifically designed to overcome the potential problem of the effects of elastosis of LM in the disease. Choline-acetyl-transferase (ChAT), a marker of cholinergic activity, and nitric oxide synthase 1 (NOS1), a marker of nitrergic activity, were each co-localized with PGP on histological sections, which then underwent immuno-fluorescence analysis. The immuno-reactivities were expressed as % (surface area of ChAT)/% (surface area PGP) and (surface area of NOS1)/% (surface area PGP), respectively. The finding of lower immuno-reactivities of

both ChAT and NOS1 in diverticular LM, compared with controls, was therefore considered non-spurious, as any potential effect of elastosis in diluting immuno-reactivity would have been the same for both the neurotransmitters and PGP. These arguments were discussed in the respective articles[2,3].

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

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Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in tahe field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 37 October 7, 2010

EDITORIAL

4627 PTEN in liver diseases and cancer

Peyrou M, Bourgoin L, Foti M

4634 Mucin phenotype of gastric cancer and clinicopathology of gastric-type differentiated adenocarcinoma

Namikawa T, Hanazaki K

TOPIC HIGHLIGHT

4640 Era of Barrett's surveillance: Does equipment matter?

Mannath J, Ragunath K

OBSERVATION

4646 Surveillance for colitis-associated colon neoplasia

Freeman HJ

REVIEW

4652 Roles of liver innate immune cells in nonalcoholic fatty liver disease

Zhan YT, An W

ORIGINAL ARTICLE

4661 Endoscopic features and prognoses of mantle cell lymphoma with gastrointestinal involvement

Iwamuro M, Okada H, Kawahara Y, Shinagawa K, Morito T, Yoshino T, Yamamoto K

4670 Up-regulation of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in sentinel lymph nodes of colorectal cancer

He ZY, Wen H, Shi CB, Wang J

4677 Oncolytic adenovirus SG600-IL24 selectively kills hepatocellular carcinoma cell lines

Xue XB, Xiao CW, Zhang H, Lu AG, Gao W, Zhou ZQ, Guo XL, Zhong MA, Yang Y, Wang CJ

BRIEF ARTICLE

4685 ApoB-100, ApoE and CYP7A1 gene polymorphisms in Mexican patients with cholesterol gallstone disease

Jaime SC, Maribel AM, Eliakym AM, José RN, Julio G, Laura SM, Rosalío RP

4691 Alanine aminotransferase is an inadequate surrogate marker for detecting lamivudine resistance
Lim LG, Aung MO, Seet BL, Tan C, Dan YY, Lee YM, Sutedja DS, Fernandes M, Lee GH, Koay E, Lim SG

4697 Prospective study of MMP7 serum levels in the diagnosis of cholangiocarcinoma
Leelawat K, Narong S, Wannaprasert J, Ratanashu-ek T

4704 Usefulness of Y-shaped sheaths in CT angiography for examination of liver tumors
Ishikawa T, Higuchi K, Kubota T, Seki K, Honma T, Yoshida T, Nemoto T, Takeda K, Kamimura T

4709 Usefulness of magnifying endoscopy for iodine-unstained lesions in a high-risk esophageal cancer population
Choi IS, Jang JY, Cho WY, Lee TH, Kim HG, Lee BY, Jeong SW, Cho JY, Lee JS, Jin SY

4716 Loss of chromosome 9p21 and decreased p16 expression correlate with malignant gastrointestinal stromal tumor
Zhang Y, Cao H, Wang M, Zhao WY, Shen ZY, Shen DP, Ni XZ, Wu ZY, Shen YY, Song YY

4725 Undifferentiated liver embryonal sarcoma in adults: A report of four cases and literature review
Li XW, Gong SJ, Song WH, Zhu JJ, Pan CH, Wu MC, Xu AM

4733 Ultrasonic elastography in clinical quantitative assessment of fatty liver
Li YY, Wang XM, Zhang YX, Ou GC

4738 Mn-SOD and CuZn-SOD polymorphisms and interactions with risk factors in gastric cancer
Yi JF, Li YM, Liu T, He WT, Li X, Zhou WC, Kang SL, Zeng XT, Zhang JQ

CASE REPORT

4747 Gastrointestinal bleeding 30 years after a complicated cholecystectomy
Brechmann T, Schmiegel W, Nicolas V, Reiser M

4751 Completely obstructed colorectal anastomosis: A new non-electrosurgical endoscopic approach before balloon dilatation
Curcio G, Spada M, di Francesco F, Tarantino I, Barresi L, Burgio G, Traina M

World Journal of Gastroenterology
Volume 16 Number 37 October 7, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
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EDITORIAL

PTEN in liver diseases and cancer

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Abstract

The phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Akt axis is a key signal transduction node that regulates crucial cellular functions, including insulin and other growth factors signaling, lipid and glucose metabolism, as well as cell survival and apoptosis. In this pathway, PTEN acts as a phosphoinositide phosphatase, which terminates PI3K-propagated signaling by dephosphorylating PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. However, the role of PTEN does not appear to be restricted only to PI3K signaling antagonism, and new functions have been recently discovered for this protein. In addition to the well-established role of PTEN as a tumor suppressor, increasing evidence now suggests that a dysregulated PTEN expression and/or activity is also linked to the development of several hepatic pathologies. Dysregulated PTEN expression/activity is observed with obesity, insulin resistance, diabetes, hepatitis B virus/hepatitis C virus infections, and abusive alcohol consumption, whereas mutations/deletions have also been associated with the occurrence of hepatocellular carcinoma. Thus, it appears that alterations of PTEN expression and activity in hepatocytes are common and recurrent molecular events associated with liver disorders of various etiologies. These recent findings suggest that PTEN might represent a potential common therapeutic target for a number of liver pathologies.

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Key words: Phosphatase and tensin homolog; Obesity; Insulin resistance; Non-alcoholic fatty liver diseases; Steatosis; Steatohepatitis; Fibrosis; Hepatocellular carcinoma; Viral hepatitis; Alcohol

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INTRODUCTION

Obesity, metabolic syndrome, hepatitis virus infections, and abusive alcohol consumption are major etiological factors contributing together, or independently, to the development of severe liver diseases[1-3]. Interestingly, the hepatic pathologies induced by these various factors are associated with common metabolic disorders, i.e. insulin resistance and dysregulated lipid metabolism, and encompass similar histological abnormalities, ranging from hepatic steatosis to steatohepatitis, fibrosis, and cirrhosis[4]. Hepatocellular adenoma (HCA) or hepatocellular carcinoma (HCC) might then occur as a likely end stage of these diseases[5].

Deregulations of numerous signaling pathways leading to insulin resistance, steatosis, inflammation, fibrosis, aberrant cell proliferation, and resistance to cell death have been reported. Among these, abnormal signaling through the phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Akt pathway critically contributes to the development of non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic liver disease (ALD), viral hepatitis [hepatitis B virus (HBV) and hepatitis C virus (HCV)], and HCC[6-10]. Of particular interest in this signaling pathway is the role of PTEN, an important tumor suppressor having a protein and phosphoinositide phosphatase activity. Indeed, PTEN switches off signaling through the PI3K-Akt axis and by doing that, controls growth factor signaling, thereby acting as a potent tumor suppressor. In the context of hepatic metabolic disorders and cancer, increasing evidence now supports a crucial role of PTEN in the development of these diseases.

PTEN

The PTEN protein was first identified as a potent tumor suppressor that was frequently mutated or deleted in several human cancers, including HCC[11-16]. The best-characterized function of PTEN is its phosphoinositide phosphatase activity, where it dephosphorylates the PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 second messengers on the 3'-position of the inositol ring. PTEN thus antagonizes PI3K activation and acts as a potent regulator of growth factor signaling, in particular insulin/insulin-like growth factor (IGF)-1 signaling, in peripheral tissues such as the liver[8,17]. *In vitro* studies also suggested that PTEN might have a protein phosphatase activity; however additional studies are required to confirm this enzymatic activity[18-20].

Compared to other classical tumor suppressor, PTEN represents a particular case, as loss of heterozygosity, or partial inhibition of its expression/activity, is sufficient to promote carcinogenesis, in addition to affecting critical cellular functions, such as glucose and lipid metabolism. Consistent with these observations, PTEN expression and activity appears to be regulated by numerous and complex mechanisms. Among these mechanisms, epigenetic silencing by hypermethylation of its promoter[11,21] or histone deacetylase activity[22] strongly affect PTEN expression. Several transcription factors, including Egr1[23], p53[24], peroxisome proliferator-activated receptor γ[25], Spry2[26], Atf2[27], and Myc[28], have been shown to directly bind the PTEN promoter and to upregulate PTEN transcription. On the other hand, transcription factors such as nuclear factor (NF)-κB[29,30], p300/CBP[29], Hes-1[28], Cbf-1[31,32], and c-Jun[33] have been shown to negatively regulate PTEN transcription. Adding to the complexity of PTEN expression regulation, recent evidence also indicated that the PTEN mRNA undergoes post-transcriptional repression/degradation by specific microRNAs (miRNAs). Several miRNAs, including miR-21, miR-19a, miR-17-92, miR-214, miR-216a, and miR-217, have been shown to specifically modulate PTEN mRNA expression[34-39]. Finally, additional processes, whereby the PTEN protein level and activity are modulated, occur post-translationally. These include modifications of the protein, such as phosphorylation, acetylation, ubiquitination, and the REDOX state, which affect PTEN stability, degradation and enzymatic activity[21,40,41]. Similarly, PTEN sequestration in specific subcellular compartments or membranes through interactions with distinct proteins, i.e. FAK[42], MAGI proteins[43], MAST proteins[44], p53[45], NHERF1/2[46], and PICT1[47] likely represent additional mechanisms controlling PTEN stability and/or activity.

PTEN IN HEPATIC INSULIN RESISTANCE

Functional defects of key intracellular signaling proteins, in particular in the mammalian target of rapamycin (mTOR)/PI3K/PTEN/Akt pathways, are clearly associated with insulin unresponsiveness of peripheral tissues[48,49]. Indeed, the imbalance between propagation and termination of signaling through mTOR/PI3K/PTEN/Akt represents basal molecular dysfunctions triggering the development of insulin resistance and type Ⅱ diabetes. Given the enzymatic activity of PTEN, dysregulation of its activity/expression are potentially important mechanisms contributing to insulin resistance in tissues such as the liver[8].

Several *in vivo* studies, where PTEN expression was genetically altered in various organs of mice, support a crucial role for PTEN expression/activity in insulin sensitivity. PTEN haploinsufficiency and PTEN muscle-specific deletion were shown to improve skeletal muscle insulin sensitivity and to protect mice from insulin resistance and diabetes caused by high fat feeding, respectively[50,51]. Deletion of PTEN in the adipose tissue prevented the development of streptozotocin-induced diabetes[52]. Treatment of *db/db* mice with PTEN antisense oligonucleotides normalized plasma glucose levels in these animals[53].

Interestingly, liver-specific PTEN knockout mice also have an improved systemic insulin sensitivity and glucose tolerance[54,55]. However, whether this is related to increased insulin sensitivity specifically in the liver, or to a complex *in vivo* systemic crosstalk between a PTEN-deficient liver and other peripheral tissues, remains unclear. In support of this latter hypothesis, PTEN deletion in the liver is accompanied by decreased circulating levels of leptin and body fat content[55]. In addition, we observed that although constitutive Akt activity is increased in cultured hepatoma cells having downregulated PTEN, insulin signaling upstream of Akt, i.e. insulin receptor/IRS1 expression and phosphorylation, is impaired, as it has been previously described in cancer cells[30,56]. Consistent with these findings, we observed a lack of insulin responsiveness in terms of gene expression in PTEN deficient hepatocytes[30]. These data raise the hypothesis that PTEN downregulation in hepatocytes might, paradoxically, cause insulin resistance despite an increased activation of specific insulin effectors, such as Akt. Further studies are needed to clarify whether

PTEN downregulation in hepatocytes is a causal factor for insulin resistance in this organ.

PTEN IN NAFLD

Although liver-specific PTEN knockout mice have an overall improved systemic insulin sensitivity, they develop an important steatosis in the liver, suggesting that PTEN is required for an appropriate control of the hepatic lipid metabolism[54,55]. Consistent with these studies, hepatic PTEN expression is downregulated in obese and insulin resistant rat animal models and in humans having steatosis[30]. Further studies indicate that high levels of circulating free fatty acids, but not glucose or insulin, downregulate PTEN expression in hepatocytes[30]. PTEN downregulation by free fatty acids is triggered by an increase in miR-21, a miRNA targeting PTEN mRNA for degradation, through mTOR/NF-κB-dependent mechanisms[30,36]. In addition to an excess of circulating free fatty acids, a deregulated production of inflammatory cytokines by immune cells and/or of adipokines by the adipose tissue, as observed with NAFLD[57], can also independently, or synergistically with fatty acids, alter PTEN expression. Indeed, inflammatory cytokines, such as transforming growth factor β[39,58-60], tumor necrosis factor (TNF)α[29,61], interleukin (IL)-6[62], IL-1[63], or adipokines such as leptin, resistin and adiponectin[64,65], have been reported to either up- or downregulate PTEN expression or activity in various cells. Although most of these cytokines/adipokines are clearly involved in liver insulin sensitivity and steatosis/fibrosis/inflammation[57,66], it remains to be firmly established whether these factors modulate PTEN expression/activity in the liver and whether there is a causal relationship between potential PTEN alterations induced by these cytokines/adipokines and their beneficial/detrimental effects on the liver physiology.

PTEN loss of function in the liver leads to a progressive and step-wise development of steatohepatitis and fibrosis[54,55,67]. Consistent with studies using liver-specific PTEN knockout mice, decreased PTEN expression was also observed in the liver of rodents with hepatic fibrosis induced either by biliary stenosis or a choline-deficient diet[68,69]. Finally, PTEN depletion in hepatoma cells induces the expression of genes promoting inflammation, epithelial-to-mesenchymal transition, and fibrosis[70]. Taken together, these studies suggest that pathological dysregulation of PTEN expression/activity causing steatosis might also promote progression of this disorder towards different clinical stages of increasing severity. The molecular mechanisms by which PTEN deficiency triggers steatosis, inflammation, and fibrosis development in hepatocytes are still poorly defined. However, evidence indicates that *de novo* fatty acids synthesis is enhanced[54,55] in liver-specific PTEN knockout mice, whereas in cultured cells, accumulation of neutral lipids seems to rely mainly on increased fatty acids uptake and esterification[30]. These discrepant data might originate either from the different methodologies used to investigate fatty acid metabolism or, more likely, from the different extents of PTEN repression, i.e. complete deletion in knockout mice *vs* 40%-80% downregulation induced by fatty acids or silencing RNAs in cultured cells. Partial PTEN downregulation or total deletion can indeed mediate very different effects, as it was elegantly demonstrated in studies examining the role of PTEN in prostate tumor progression[41,71].

PTEN IN LIVER CARCINOGENESIS

The first evidence supporting a critical role for PTEN in liver cancer came from genetic studies in mice, where heterozygous deletion of PTEN was shown to induce atypical adenomatous liver hyperplasia[72]. Additional studies then demonstrated that PTEN deficiency in the liver induces hepatomegaly, HCA, and HCC with ageing[54,55]. Weak expression or mutation/deletion of PTEN, as well as upregulation of miRNAs specifically targeting PTEN for degradation, are also frequently observed in human HCC[11,13,16,34,73-76]. However, the tumor suppressor activity of PTEN seems to principally involve its antagonistic effects on the anti-apoptotic, proliferative, and hypertrophic activity of PI3K[77]; recent studies demonstrated that PTEN also plays an essential role in the nucleus to maintain chromosomal stability and for DNA repair[78]. In addition, there is evidence indicating that PTEN can modulate cancer cell invasiveness by stabilizing E-cadherin/β-catenin adherens junctional complexes[79,80]. Finally, we demonstrated that fatty acids-mediated PTEN downregulation in hepatocytes promotes cell proliferation, migration, and invasiveness, in addition to modulating a set of genes involved in cell cycle regulation and HCC[70]. As inflammation, EMT and genomic alterations are typical features of HCC[81,82], impaired PTEN expression or activity can thus represent an important step in progression of NAFLD towards HCC. Further studies are however still needed to confirm the relevance of PTEN as a prognostic marker for the risk of HCA/HCC development.

PTEN IN VIRAL HEPATITIS

Infections by HBV and HCV are major contributors to the high incidence of HCC, particularly in South-East Asia and Africa[83,84]. Similarly to NAFLD and NASH, HCV infection is strongly associated with liver insulin resistance and causes steatosis and fibrosis[85,86]. However, whether HBV infection causes similar liver disorders remains unclear[87].

Only a few studies have examined the involvement of PTEN in HBV/HCV-associated hepatocyte dysfunction. The HBV-X protein (HBx) was shown to trigger uncontrolled Akt activation by downregulating PTEN expression in Chang liver cells, thereby enhancing the invasive potential of these cells[88,89]. In accordance with these data, PTEN overexpression in Chang cells reversed pro-survival signaling and inhibited apoptosis induced by HBx[90]. In addition, PTEN was also shown to prevent HBx-mediated induction of IGF-Ⅱ expression in hepa-

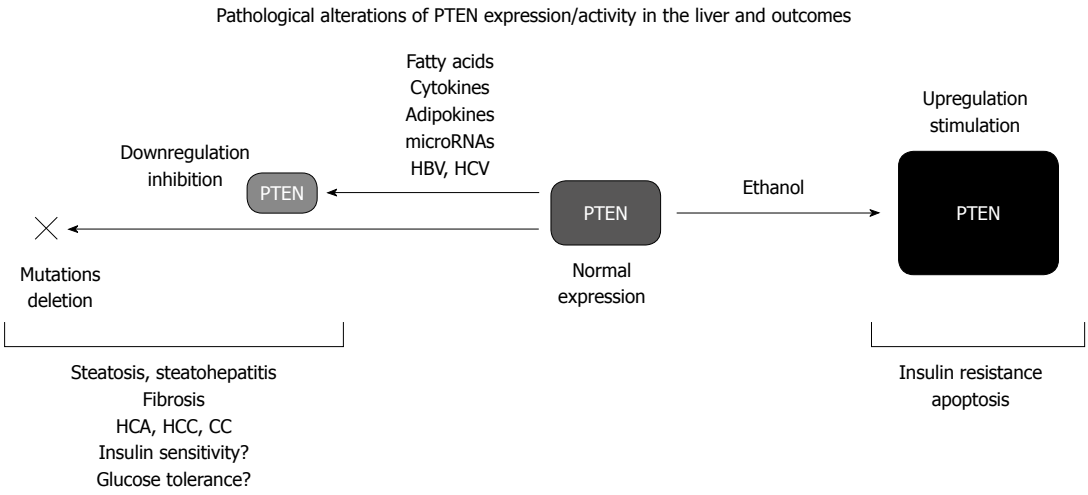

Figure 1 Alterations of phosphatase and tensin homolog expression/activity in the liver by various etiological factors and associated liver disorders. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCA: Hepatocellular adenomas; CC: Cholangiocellular carcinomas; HCC: Hepatocellular carcinomas; PTEN: Phosphatase and tensin homolog.

toma cell lines[91]. IGF-Ⅱ plays an essential role in HCC development; therefore, these data suggest that PTEN downregulation might make an important contribution to cell proliferation induced by HBx.

Direct evidence implicating a PTEN loss or gain of function in HCV-mediated liver diseases is scarce and only few studies investigate this issue. PTEN inactivation by post-translational phosphorylation was suggested to contribute to transactivation of SREBPs, which are major regulators of the lipid metabolism, in HCV-infected Huh-7 cells[92]. Correlative immunohistochemical analyses of human HCV-positive cirrhotic HCC also indicated that PTEN is downregulated in these tumors and that its expression was inversely correlated with expression of inducible nitric oxide synthase and cyclooxygenase Ⅱ. Interestingly, high PTEN expression was evaluated as a positive independent prognostic factor for the survival of HCV-positive cirrhotic HCC patients[93].

Further molecular, clinical, and epidemiological studies are now warranted to understand the detailed mechanisms by which HBV/HCV infections alter PTEN expression or activity in the liver, as well as the pathological outcomes of PTEN dysfunctions in HBV/HCV infections.

PTEN IN ALD

ALD also encompass a spectrum of histological and functional liver disorders, ranging from a relatively benign steatosis to alcoholic hepatitis, cirrhosis, and cancer[2]. Given the outcomes of alcohol abuse in the liver, and the effects of PTEN deletion or downregulation for the liver physiology, it could be expected that ethanol induces alterations of PTEN expression/activity in the liver. Surprisingly, increased apoptosis and decreased insulin signaling in hepatocytes of Long-Evans rats chronically fed with ethanol (serum ethanol levels is about 50 mmol/L) was associated with increased levels of PTEN mRNA and protein, thus suggesting that ethanol upregulates PTEN expression in the liver[94]. Increased hepatic PTEN expression was also observed in rats exposed to alcohol *in utero*[95]. Consistent with these studies, chronic exposure of hepatoma HepG2E47 cells to ethanol increased PTEN expression and subsequently increased the sensitivity of cells to TNFα-induced cytotoxicity and apoptosis[96]. In contrast, acute ethanol exposure did not affect PTEN expression in Huh-7 hepatoma cells. However, in these cells, ethanol increased the physical association between PTEN and the PI3K regulatory subunit p85a, which functionally resulted in a decreased Akt and downstream effectors activity[97].

Taken together, these studies indicated that, in contrast to the PTEN downregulation occurring with NAFLD, PTEN expression/activity is upregulated with ALD. This opposite regulation of a critical signaling effector strongly suggests that the mechanisms of insulin resistance and steatosis development in the context of NAFLD and ALD are distinct. In addition, PTEN might represent a differential diagnostic marker to distinguish between liver disorders with these different etiologies.

CONCLUSION

Accumulating evidence indicates that PTEN is a major dysregulated cellular factor contributing to the development of a broad spectrum of hepatic disorders, i.e. insulin resistance, steatosis, steatohepatitis, fibrosis, cirrhosis, and cancer (Figure 1). Indeed, hepatic PTEN expression/activity is altered in liver diseases associated with obesity, metabolic syndrome, viral infection, and alcohol consumption. Thus, it appears that dysregulation of PTEN expression/activity in hepatocytes represents an important and recurrent molecular mechanism contributing to the development of liver disorders with distinct etiologies.

Although hepatic steatosis is currently regarded as a benign disease, progression to inflammation, fibrosis, and cirrhosis can lead to liver failure and development of HCC. There are multiple molecular factors involved in the progression of hepatic steatosis towards more severe stages and among those, dysregulation of PTEN expres-

sion/activity, more than PTEN mutations or deletions, could be a critical step in the occurrence and development of these diseases. In addition, PTEN alterations induced by high levels of free fatty acids or inflammatory cytokines, provide an interesting link between insulin resistance and steatosis, which might also explain, at least in part, the high risk factor for HCA/HCC associated with diabetes and obesity[98,99]. Given the tumor suppressor activity of PTEN, the role of steatosis and steatohepatitis as preneoplastic states in the hepatocyte malignant transformation should also be re-evaluated. Additional studies are now required to carefully evaluate PTEN as a differential prognostic marker in liver pathologies with distinct etiologies and to assess the pertinence of future therapeutic interventions to restore physiological PTEN expression in the liver to prevent, or to alleviate, hepatic metabolic disorders and HCC.

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EDITORIAL

Mucin phenotype of gastric cancer and clinicopathology of gastric-type differentiated adenocarcinoma

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Abstract

Differentiated adenocarcinoma of the stomach is classified into gastric or intestinal phenotypes based on mucus expression. Recent advances in mucin histochemistry and immunohistochemistry have highlighted the importance of such a distinction, and it is important clinically to distinguish between gastric- and intestinal-type differentiated adenocarcinoma. However, a clinical and pathological diagnosis of this type is often difficult in early gastric cancer because of histological similarities between a hyperplastic epithelium and low-grade atypia. Furthermore, determining tumor margins is often difficult, even with extensive preoperative examination. It is therefore critical to consider these diagnostic difficulties and different biological behaviors with high malignant potential when treating patients with gastric-type differentiated adenocarcinoma.

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Key words: Differentiated adenocarcinoma; Gastric cancer; Gastric phenotype; Mucin core protein; Mucous phenotype

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INTRODUCTION

Gastric adenocarcinoma can be divided into intestinal and diffuse types using the Lauren classification system[1], or as differentiated and undifferentiated using the Nakamura classification system[2]. In general, intestinal-type adenocarcinoma is considered to be essentially equivalent to differentiated adenocarcinoma, as is diffuse-type and undifferentiated adenocarcinoma. These classifications are based on morphological characteristics centered largely on gland formation and histogenetic background.

These histologically different types of gastric tumors exhibit distinct biological behaviors. Intestinal metaplasia, which occurs as a result of *Helicobacter pylori* infection and consequent atrophic gastritis[3], is common in the human stomach and is associated with an increased risk of gastric cancer[4,5]. Generally, intestinal-type adenocarcinoma is preceded by metaplastic changes, whereas diffuse-type adenocarcinoma is thought to arise in normal gastric mucosa[2]. However, some cases of intestinal-type adenocarcinoma also arise from the gastric mucosa without intestinal metaplasia. Although these histological types of tumors can be distinguished using standard hematoxylin and eosin staining, recent advances in mucin histochemical and immunohistochemical methods using gastric and small intestinal cell markers have enabled the classification of gastric cancer into different phenotypes[6].

CLASSIFICATION ACCORDING TO MUCIN EXPRESSION

Mucins are heavily glycosylated glycoproteins that constitute most of the viscous gel that lines the gastrointestinal epithelium. Tumor phenotypes are generally classified on the basis of the expression various markers, including CD10 as a marker for the brush border on the luminal surface of small intestinal absorptive cells (enterocytes), mucin 2 (MUC2) as a marker of intestinal goblet cells, MUC5AC or human gastric mucin (HGM) as a marker of surface gastric epithelium (foveolar cells), and MUC6 as a marker for pyloric glands (pyloric gland cells, mucous neck cells, pseudopyloric gland cells). Several additional markers specific for the mucin core proteins encoded by the *MUC* genes are now also available[7].

Accordingly, CD10 and MUC2 are considered useful diagnostic markers of the intestinal phenotype, whereas MUC5AC, HGM, and MUC6 can be used to differentiate the gastric phenotype. Although goblet cells are present in the small and large intestine, enterocytes are limited to the small intestine. Gastric cancer phenotypes can be classified quite simply into four groups depending on the combinations of the expression of these markers as intestinal type, gastric type, combined type, and unclassified type (Figure 1A).

Intestinal metaplasia of the stomach can be divided on the basis of morphology into incomplete, characterized by goblet cells in the gastric gland, and complete, which has small intestinal absorptive cells in addition to goblet cells[8]. These types differ in cell components and in the role they play in gastric carcinogenesis. The incomplete type of intestinal metaplasia is closely associated with carcinoma, whereas complete-type intestinal metaplasia is not considered a precancerous lesion[9,10].

Based on the type of intestinal metaplasia, gastric cancer phenotypes can be classified into four groups depending on the marker combinations as complete intestinal type, incomplete intestinal type, gastric type, and unclassified type (Figure 1B). The complete intestinal type is positive for CD10 and MUC2, and negative for MUC5AC (Figure 2). The incomplete intestinal phenotype is positive for CD10 and MUC5AC simultaneously, or positive for MUC2 alone. The gastric type is positive for MUC5AC, and negative for CD10 and MUC2 (Figure 3). Unclassified phenotypes are negative for CD10, MUC2, MUC5AC, and MUC6. Classification of the mucin phenotype based on the type of intestinal metaplasia is useful for understanding the biological behavior of carcinomas and when considering various therapeutic strategies.

DIAGNOSTIC DIFFICULTIES FOR GASTRIC-TYPE DIFFERENTIATED ADENOCARCINOMA

Mucins specific to the gastric mucosa are defined as gastric-type mucins, although differentiated adenocarcinomas of the stomach change their mucin phenotype as they increase in size and depth of invasion. Based on recent reports, the incidence of gastric-type differentiated adenocarcinoma in early gastric cancer (EGC) appears to be 7.9%-23.9%[11-13].

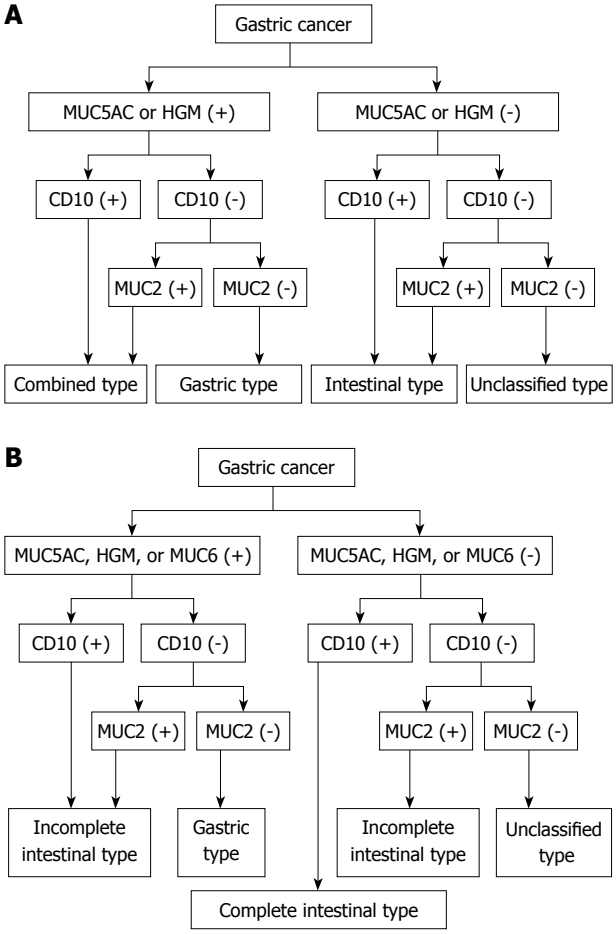

Figure 1 Classification of gastric cancer based on mucous expression. A: Classification of gastric cancer according to the phenotypic combination of mucin expression; B: Classification of gastric cancer by mucin-expression phenotype according to the type of intestinal metaplasia. HGM: Human gastric mucin.

Early gastric-type differentiated adenocarcinomas tend to be significantly larger tumors and exhibit higher rates of submucosal invasion than intestinal-type EGC[12]. An investigation into the macroscopic features of gastric-type differentiated adenocarcinoma by Higuchi *et al*[14] has revealed that the incidence of a discolored surface and non-wavy tumor margins is significantly higher than in cases of intestinal-type differentiated adenocarcinoma. In another study, gastric-type differentiated adenocarcinomas showed indistinct margins and an even coloring across the mucosal layer, whereas intestinal-type differentiated adenocarcinomas had an elevated, distinct margin and a red mucosa[15]. These findings may also reflect the difficulty in correctly diagnosing gastric-type differentiated adenocarcinoma at an early stage.

Esophagogastroduodenoscopy is highly reliable in defining the area of cancer infiltration in EGC, although it remains difficult to diagnose gastric cancer accurately in some cases if biopsy specimens do not reveal the presence of adenocarcinoma. Oda *et al*[16] have reported

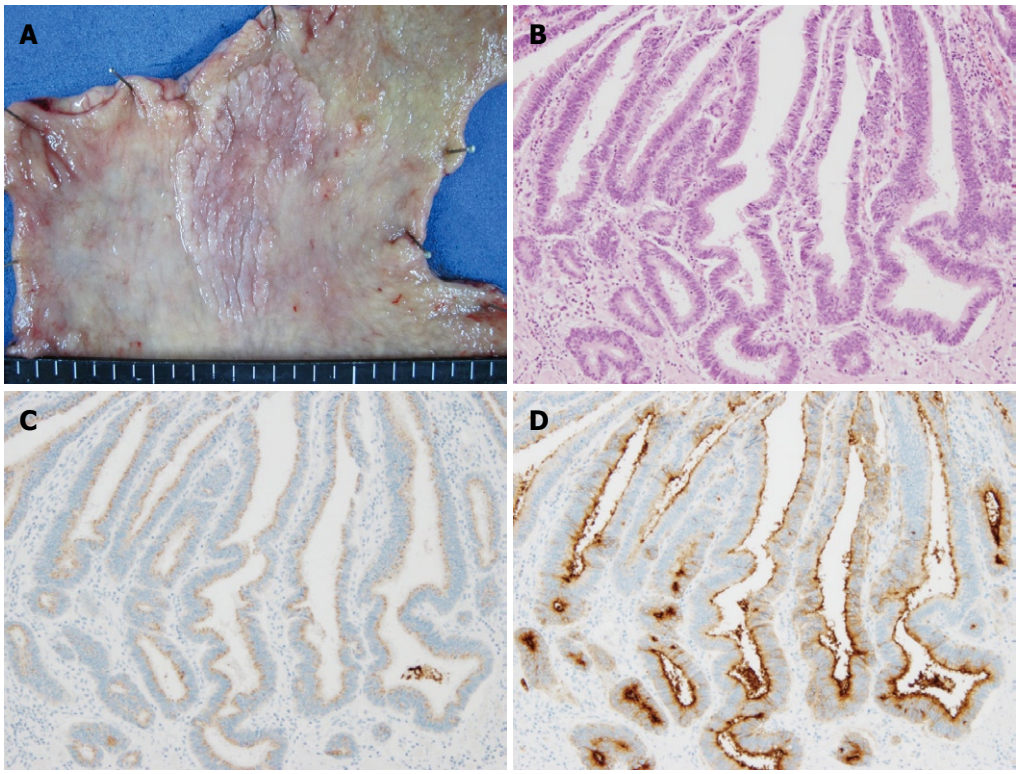

Figure 2 Complete intestinal-type differentiated adenocarcinoma. A: Macroscopic appearance showing a slightly elevated granular lesion with a distinct margin; B: Well-differentiated tubular adenocarcinoma (HE staining); C: No staining of MUC5AC was found in the carcinomatous gland; D: Positive staining of CD10 was apparent on the luminal side of the carcinomatous gland.

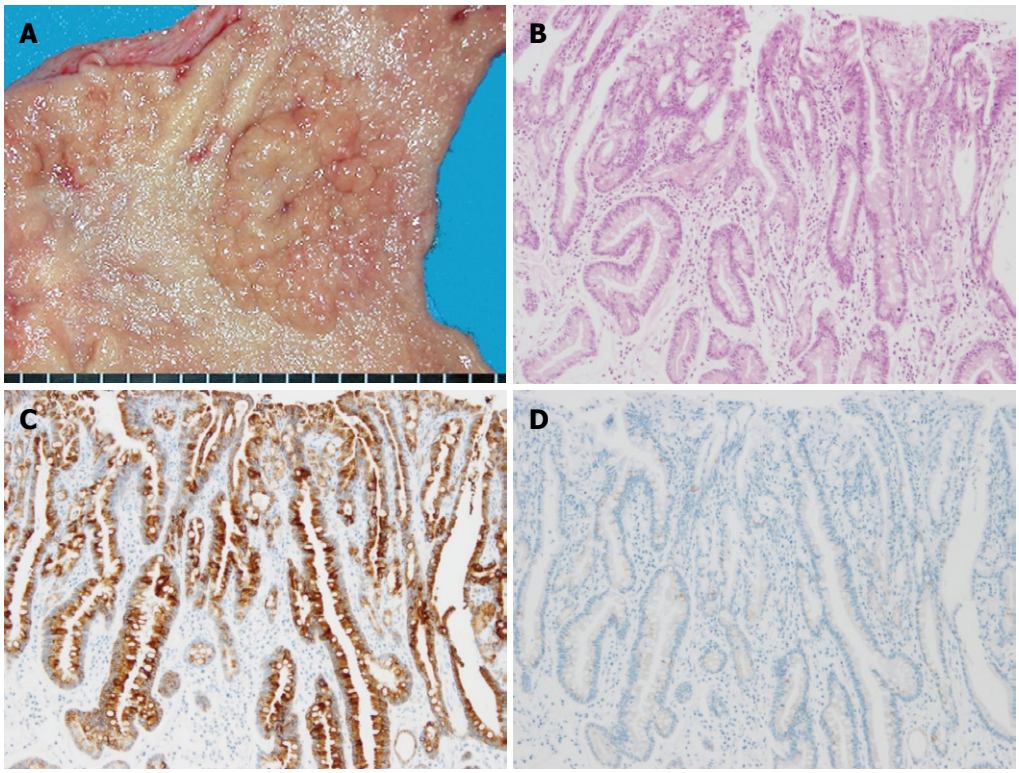

Figure 3 Gastric-type differentiated adenocarcinoma. A: Macroscopic appearance, showing a fine-granule aggregated lesion; B: Well-differentiated tubular adenocarcinoma (HE staining); C: Diffuse positive staining of MUC5AC was apparent in the carcinomatous gland; D: No staining of MUC2 was evident.

that, in terms of endoscopic features, unclear margins and an even color tone are more common in the mucosal layer of gastric-type differentiated adenocarcinoma compared with the intestinal type. Taking these difficul-

ties in endoscopic diagnosis into account[16,17], it is clear that a method enabling the precise diagnosis of gastric-type differentiated adenocarcinoma is needed.

Microscopically, it is often difficult to distinguish gastric-type differentiated lesions from regenerative or inflammatory changes to the intestinal epithelium. The high degree of differentiation and mild cellular atypia of gastric-type differentiated adenocarcinoma frequently causes diagnostic difficulties, especially with regard to presurgical biopsy specimens[17,18]. Despite the fact that these tumors are potentially highly malignant and associated with a high incidence of lymphatic or venous invasion and lymph node metastasis, the clinical and pathological diagnosis of this disease remains a challenge.

BIOLOGICAL BEHAVIOR OF GASTRIC-TYPE DIFFERENTIATED ADENOCARCINOMA

Gastric- and intestinal-types of differentiated adenocarcinoma exhibit some differences in terms of their biological behavior. Specifically, gastric-type tumors show scirrhous infiltration, whereas intestinal-type lesions show solid growth in the gastric wall[16,19]. In addition, although gastric-type differentiated adenocarcinomas commonly arise as well or moderately differentiated cancer, they often change histologically into a signet ring-cell carcinoma or poorly differentiated adenocarcinoma[11,20]. Tajima *et al*[21] have reported that, of patients with advanced gastric carcinoma, those with gastric-type tumors have a significantly poorer prognosis than those with intestinal-type tumors. Thus, gastric-type differentiated adenocarcinomas can be distinguished from other types of differentiated adenocarcinomas on the basis of their increased malignant potential in the incipient phase of invasion and metastasis[11].

Recent advances in limited surgery, including endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), or minimal surgical therapy such as laparoscopic surgery, now offer a better quality of life to patients with EGC. However, even in the early stages of gastric-type differentiated adenocarcinoma, it would seem that the decision to proceed with EMR, ESD, or minimal surgical procedures as a curative treatment should be made with care. Koseki *et al*[11] have reported a significantly higher incidence of lymphatic invasion, venous invasion, and lymph node metastasis in gastric-type compared with intestinal-type adenocarcinoma, whereas Kabashima *et al*[13] have found no significant difference between the different subtypes based on lymphatic or venous invasion and lymph node metastases. Accordingly, the malignant potential of gastric-type adenocarcinoma confined to the mucosa may not be obvious compared with tumors that invade deeper than the submucosa.

Cases of undifferentiated gastric adenocarcinoma show no clinicopathological differences between gastric and intestinal types[22]. However, gastric-type undifferentiated tumors display different growth patterns compared with tumors that have an intestinal phenotype[22]. Specifically, gastric-type undifferentiated adenocarcinomas tend to spread through the middle layer of the mucosa more frequently than intestinal-type lesions, and because the carcinoma cells do not appear on the surface of the mucosa in the gastric-type lesions, the margins of carcinoma are considered to be unclear.

GENETIC BACKGROUNDS ASSOCIATED WITH MUCIN PHENOTYPES

Recent studies have revealed that the genetic background of patients with differentiated adenocarcinoma differs among mucin phenotypes[12,23-25]. The *p53* gene is a tumor suppressor. Overexpression of p53 protein is widespread in differentiated adenocarcinoma regardless of the mucin phenotype, but is rare in undifferentiated adenocarcinoma[12,23]. The microsatellite instability (MSI) status of a tumor represents mutations of short tandem repeat sequences distributed throughout the genome. Endoh *et al*[25] have demonstrated that MSI is closely related to expression of the gastric phenotype. Yamazaki *et al*[26] also have reported that MSI is significantly associated with gastric phenotype and, furthermore, that it is inversely associated with CD10 expression, whereas *APC* mutations are significantly associated with CD10 expression and the intestinal phenotype and inversely associated with expressions of HGM and MUC6. These results suggest that differentiation to gastric gland cells is related to MSI, whereas differentiation to intestinal epithelial cells reflects mutations in APC[27].

The c-*erb*B-2 oncogene encodes the receptor for an epidermal growth factor-like growth factor with tyrosine kinase activity. Overexpression of c-erbB-2 protein indicates a poor prognosis for patients with gastric cancer[28]. Overexpression of c-erbB-2 has been reported in some differentiated adenocarcinomas, but not in gastric-type differentiated adenocarcinoma[12]. Furthermore, Sugai *et al*[23] have suggested that the mucin phenotype of a differentiated adenocarcinoma of the stomach is dependent on distinct genetic profiling based on chromosomal allelic losses, MSI, and overexpression of the p53 protein[23]. Thus, classification according to phenotypic expression may provide us with an understanding of the genetic basis that underlies the carcinogenesis of differentiated adenocarcinomas.

RELATIONSHIP WITH BACKGROUND MUCOSA

The development of differentiated adenocarcinomas could be closely related to intestinal metaplasia. In general, the phenotype of gastric cancers tends to imitate the surrounding mucosa[29], with gastric-type gastric cancers developing in areas expressing gastric-type or mixed-type mucins. However, Matsuoka *et al*[12] have indicated that the mucin phenotype of an early-stage differentiated adeno-

carcinoma may not reflect the mucin type in the surrounding gastric mucosa, because pure intestinal metaplasia was observed in 11.8% of gastric-type differentiated adenocarcinomas[12].

Kabashima *et al*[29] have reported a higher incidence of gastric-type carcinomas and incomplete intestinal-type background mucosa among cases of multiple-type EGCs than among cases with a solitary type of cancer. Intestinal metaplasia surrounding differentiated adenocarcinoma with a gastric phenotype is proposed to be immature and incomplete compared with differentiated adenocarcinoma with a gastric-intestinal or intestinal phenotype[30]. The instability of differentiated adenocarcinomas and the background mucosa in multiple-type early gastric carcinomas have been implicated in the high neoplastic potential and multiple occurrences of these carcinomas[29].

CONCLUSION

The biological behavior of gastric cancer and the relationship with background mucosa reveal different characteristics depending on mucin phenotype expression. Namely, the disease entity of gastric-type differentiated adenocarcinoma has unique clinicopathological characteristics that distinguish it from the intestinal type, including malignant biological behavior and diagnostic difficulties. It is important to consider this type of carcinoma in diagnoses of gastric cancer, and mucin immunohistochemical staining should be conducted in addition to traditional macroscopic examinations to ensure a precise diagnosis. Furthermore, different genetic backgrounds associated with different mucin phenotypes may affect the pathway of gastric carcinogenesis.

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TOPIC HIGHLIGHT

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Era of Barrett's surveillance: Does equipment matter?

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Abstract

Barrett's esophagus is a consequence of long standing gastro-esophageal reflux disease and predisposes to the development of esophageal adenocarcinoma. Regular surveillance endoscopies can detect curable early neoplasia in asymptomatic patients, which in turn could improve the prognosis compared to symptomatic cancer. Early neoplastic lesions, which are amenable for local therapy, could be treated endoscopically, avoiding a major surgery. However, in the absence of obvious mucosal lesions, random four quadrant biopsies are done, which is associated with significant sampling error. Newer imaging modalities, such as autofluorescence endoscopy, are helpful in detecting subtle lesions that could be examined in detail with narrow band imaging to characterize and target biopsies. This has the potential benefit of reducing the number of random biopsies with a better yield of dysplasia. Confocal endomicroscopy provides "optical biopsies" and is a valuable tool in targeting biopsies to improve dysplasia detection; however, this is technically challenging. Fuji intelligent chromoendoscopy and I-Scan are recent additions to the imaging armamentarium that have produced notable early results. While all these additional new imaging techniques are promising, a thorough examination by high resolution white light endoscopy after clearing the mucosa with mucolytics should be the minimum standard to improve dysplasia detection during Barrett's surveillance.

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Key words: Barrett's esophagus; Endoscopy; Autofluorescence imaging; Narrowband imaging; Early diagnosis of cancer

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INTRODUCTION

Barrett's esophagus (BE) develops as a consequence of long standing gastro-esophageal reflux diseases (GERD) and is characterized by replacing the distal stratified squamous epithelium by columnar lined mucosa containing specialized intestinal metaplasia (SIM). The diagnosis of Barrett's is established with endoscopic presence of salmon colored mucosa proximal to the gastric folds and associated histopathological examination confirming intestinal metaplasia. BE is a pre-malignant condition

which predisposes to the development of esophageal adenocarcinoma (EAC). These patients carry a cancer risk that is 30-125 times higher than that of an age-matched population[1]. The metaplastic epithelium acquires genetic changes over a period of time and malignant transformation occurs in a stepwise manner progressing through low grade dysplasia (LGD), high grade dysplasia (HGD), and finally cancer[2]. Medical and surgical therapy for GERD has not been shown to prevent development of EAC or dysplasia. However, some observational studies suggested that the use of proton pump inhibitors decreased the incidence of dysplasia[3]. In the absence of any preventive strategy, regular surveillance to identify early neoplasia is the most pragmatic approach and hence most of the international gastroenterological societies advise surveillance programmes in patients with BE[4]. Endoscopic surveillance can detect curable early neoplasia, and asymptomatic cancers discovered during surveillance are less advanced than those found in patients who present with cancer symptoms, such as dysphagia and weight loss[5,6]. Early neoplastic lesions can be treated by endotherapy, avoiding the morbidity and mortality associated with major surgery. In the absence of mucosal abnormalities, random four quadrant biopsies every 1-2 cm is the standard practice; however the yield of dysplasia with such a labor intensive endoscopic biopsy protocol is suboptimal[7,8].

HGD and early cancer are often difficult to identify, as many of them will be flat lesions with no obvious mucosal irregularity or nodules. The use of mucolytic agents, such as N-Acetyl Cysteine (NAC), has been shown to improve visibility during endoscopy[9] and should be considered in all cases undergoing a thorough mucosal examination. In this article we will address newer imaging modalities that have been developed with the hope of improving dysplasia detection.

ERA OF ENDOSCOPIC TECHNOLOGICAL ADVANCES

Conventional video endoscopes have a focal distance between 1 and 2 cm from the tip of the endoscopes and use less than 200 000 pixels to construct an image. A close examination of the area of interest would be compromised due to blurring of image if moved close to the mucosa. Technological advances over the past decade have allowed enhancement of the endoscopic image by increasing the resolution of the charge coupled devices (CCD) and have improved the clarity of the images by using high definition monitors. Currently, endoscopes with integrated zoom lenses and microscopes are available, and with these techniques, tissue can be imaged at the cellular and nuclear levels, which provides in-vivo optical histology. Image enhancement using dye (chromoendoscopy) or optical methods [Narrow Band Imaging (NBI), Fuji Intelligent Chromo Endoscopy (FICE) and I-Scan] could allow improved detection and characterization of dysplastic lesions in BE.

USE OF MAGNIFICATION AND HIGH RESOLUTION ENDOSCOPY IN BE SURVEILLANCE

Optical magnification is closely related to the concept of resolution, which is the ability to discriminate between two points, and in an electronic image, this is the function of the pixel density. Current magnifying, or "zoom", endoscopes enlarge the image up to 150 fold by optical magnification using a mechanically or electronically movable lens controlled by a lever at the head of the endoscope (optical zoom). This is different to the electronic magnification, where the images are magnified by only up to 1.5 times. Availability of high resolution endoscopes equipped with high density CCD (600 000-1 000 000 pixels) make high magnification possible without loss of resolution. These endoscopes also have variable focal distances, which helps to move the endoscope very close to the mucosal surface, thus providing a magnified image.

Use of magnification with indigocarmine chromoscopy was found to correctly identify specialized intestinal metaplasia (SIM) and high grade dysplasia (HGD)[10]. However, low grade dysplasia (LGD) was shown to have similar patterns to SIM. Various mucosal pit patterns, such as ridged/villous, circular and irregular/distorted patterns were identified in this study[10]. The presence of irregular/distorted patterns were found to be specific for HGD in a later multicenter study by the same investigators[11]. Acetic acid and methylene blue are also used as contrast agents with magnification endoscopy, but the results are inconsistent[12-14]. There was also a high reported inter-observer variability in some studies, questioning the accuracy of these techniques[13]. The role of magnification high resolution white light endoscopy without contrast agents is not well studied in this context.

NARROW BAND IMAGING

NBI is a relatively new technology of image enhanced endoscopy that was first described in 2004 by a Gono *et al*[15]. In endoscopic systems with NBI, an additional filter is activated by pressing a button on the hand control of the endoscope. This filter narrows the band widths of the emitted blue (440-460 nm) and green light (540-560 nm) and the relative contribution of blue light is increased. By narrowing the bandwidths of blue and green light, the superficial mucosal details are better visualized. Also, the blue light is absorbed by hemoglobin, enabling visualization of superficial vasculature. NBI is user friendly and provides uniform visualization of the endoscopic field without the need for any additional dyes.

NBI could be useful in detecting Barrett's dysplasia compared to standard resolution white light endoscopy (WLE). In a prospective tandem endoscopy study of 65 patients, higher grades of dysplasia were detected by NBI compared to WLE. NBI directed target biopsies yielded more dysplasia than WLE directed biopsies and the number of biopsies taken by WLE were significantly

more than that of NBI[16]. An earlier study by Kara *et al*[17] compared the dysplasia detection rates of high resolution endoscopy (HRE), indigocarmine chromoscopy (ICC), and NBI with magnification. Targeted biopsies with HRE alone had a sensitivity of 79% in detecting HGD. The addition of chromoscopy and NBI did not improve the yield significantly. The difference in observations could be related to the use of high resolution endoscopy in the latter study compared to standard WLE in the former study. A recent randomized cross-over trial presented as an abstract showed that NBI did not improve dysplasia detection rates on a per-patient analysis, but more neoplastic lesions were detected by NBI[18]. More well-designed studies are necessary to comment on the ability of NBI in detecting dysplastic lesions.

NBI with magnification, however, could help in assessing the micro-structural (pit) and vascular patterns of any suspicious areas detected in the Barrett's segment. Various studies have identified different pit patterns and capillary patterns in BE[19-21]. Regular pit patterns include round, linear, tubular/ridged, and villous types. Irregular patterns and absent pit patterns are also reported. Micro-vascular patterns are classified as either regular or irregular. The sensitivity and specificity of the irregular micro vascular and pit patterns for prediction of HGD was as high as 90% and 100% in an observational study[19]. Similarly, the villous/ridged/absent pit patterns were thought be highly suggestive of specialized intestinal metaplasia (SIM) and the round patterns associated with columnar lined epithelium[21].

NBI is widely available for clinical use and magnification endoscopes are commercially available. The role of NBI in detecting dysplastic lesions remains controversial, but there are a number of studies that have used NBI to characterize the suspicious lesions[19-23]. These studies have shown good overall accuracy in diagnosing the lesions, especially so in cases of HGD and early cancer depicted by irregular pit patterns and/or vascular patterns. A recent meta-analysis confirmed a high diagnostic accuracy in characterizing HGD using NBI with magnification[24]. This would help in reducing the number of random biopsies and help in targeting lesions. We believe that, by adopting a standardized pit pattern and vascular classification, it is possible to improve the diagnostic accuracy of NBI with magnification in diagnosing dysplasia and SIM.

AUTOFLUORESCENCE ENDOSCOPY

The phenomenon of autofluorescence occurs when a light of shorter wavelength interacts with a tissue containing endogenous fluorophores, which in turn emits light of longer wavelength. A number of biological substances in the gastrointestinal tract, such as collagen, elastin, nicotinamide, and flavins, can act as endogenous fluorophores. Earlier autofluorescence imaging (AFI) systems used fiber optic endoscopes that failed to produce sufficient image quality for clinical utility. However the emergence of high resolution video endoscopy with a second CCD for autofluorescence imaging has made it possible to obtain pseudo-color images with a significant improvement in quality. AFI offers an easy way to distinguish between normal and dysplastic tissue, by combining an autofluorescence image on irradiating with a blue light of wavelength of 390-470 nm. The image of green reflected light depicts the absorbed light of hemoglobin, so that normal tissue appears pale green and dysplastic tissue appears magenta.

The role of AFI in Barrett's esophagus has been widely studied. One of the earliest studies used a fiber based laser induced fluorescence system, which could be passed through the accessory channel of the endoscopes. Panjehpour *et al*[25] studied this system in 36 patients with BE. They found that 96% of non-dysplastic Barrett's was classified as benign and 90% of HGD as pre-malignant. 5-aminolevulinic acid induced protoporphyrin IX fluorescence was found to identify areas of HGD with a modest sensitivity of 70% in a later study[26]. *In vitro* studies on surgical specimens showed that the highest fluorescence ratio was obtained in areas of adenocarcinoma, compared to dysplastic Barrett's and non-dysplastic Barrett's[27]. The next generation of light induced autofluorescence endoscopes (LIFE) was investigated at the turn of this century. In a randomized crossover trial, Kara *et al*[28] investigated the role of AFI in the detection of dysplasia in BE compared to WLE. The sensitivity of WLE targeted biopsies was better than that of AFI in this study (85% *vs* 69%)[28]. Thus, AFI did not improve dysplasia detection rates using this system. This resulted in the introduction of a video autofluorescence endoscope, which was studied in 2005 by the same group. Twenty-two patients with HGD were examined with AFI and WLE. AFI detected additional lesions in three patients compared to WLE. The use of AFI was found to be feasible and promising in detecting dysplasia[29].

These earlier studies prompted the use AFI as a "red flag" technique to highlight suspicious areas in Barrett's that could be closely examined with WLE or NBI with and without magnification. One of the disadvantages observed was the high false positive rates for AFI, and it was hypothesized that this could be improved by additional NBI use. Twenty patients with suspected HGD were observed with AFI and suspected areas were examined closely with NBI. All 28 lesions in this cohort were picked up by AFI; however, there was a false positive rate of 40%. This was reduced to 10% by the use of NBI, thus making a combined approach more specific[30]. In a randomized trial comparing AFI and WLE in Barrett's surveillance patients, AFI was found to improve dysplasia detection rates. However, this did not suggest replacing the standard four quadrant biopsy protocols, because in 11/19 patients, dysplasia was detected only on random sampling[31]. The combined use of WLE, AFI, and NBI is possible with commercially available endoscopes with magnification (Figure 1). The value of this so called 'trimodal imaging' was investigated in a multicenter study. AFI was superior to WLE in detecting dysplastic lesions, but the false positive rate was high as reported before

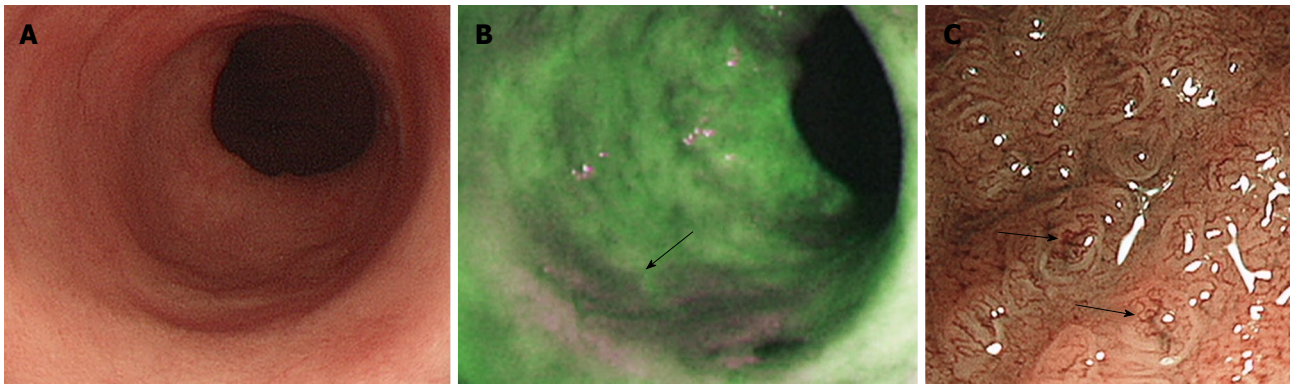

Figure 1 Trimodal imaging of Barrett's esophagus. A: High resolution endoscopy showing Barrett's segment with no conspicuous lesions; B: Autofluorescence imaging shows a low intensity abnormal area in magenta (arrow) suggestive of dysplasia; C: Narrow band imaging with magnification showing irregular vascular patterns (arrows) consistent with dysplasia.

(81%). This was reduced to 26% after inspection with NBI[22]. Contrary to these findings, a recent smaller study found that the sensitivity of AFI was suboptimal, but that NBI had a good negative predictive value[32]. Thus, the current available evidence is inconsistent, but on balance, trimodal imaging seems to improve the dysplasia detection rates in BE.

FICE AND I-SCAN

These techniques are based on a new computed spectral estimation technology. FICE (Fujinon endoscopy®) and I-Scan (Pentax Medical®) transforms an ordinary endoscopic image taken from the video processor and arithmetically processes the reflected photons to reconstitute virtual images by increasing the relative intensity of narrowed blue light to a maximum and by decreasing narrowed red and green light to a minimum. This leads to better delineation of microvasculature and mucosal pit patterns due to the differential absorption of light by hemoglobin in the mucosa. A recent study has found that Barrett's esophagus can be easily diagnosed with FICE compared to standard endoscopy, with a clear demarcation between the Barrett's segment and gastric mucosa[33]. A randomized crossover trial by Pohl *et al*[34] compared the accuracy of FICE to acetic acid chromoscopy (AAC) in detection of HGD/early cancer and found that FICE is comparable to AAC. I-Scan was studied in patients with reflux symptoms and was noted to help in identifying reflux associated lesions[35]. More studies are necessary in Barrett's esophagus to assess the utility of these new techniques.

CONFOCAL LASER ENDOMICROSCOPY

The concept of "optical biopsy" had been achieved in its true sense by the confocal laser endomicroscopy (CLE). An integrated confocal microscope (Pentax Medical®), and a probe based confocal microscope that can be passed through the working channel of an ordinary endoscope (Mauna Kea technologies®) are available commercially. To create confocal images, blue laser light is focused on the desired tissue *via* the distal end of confocal endoscope. Fluorescent materials are used intravenously, which are excited by laser lights and the confocal optical unit detects this in a defined horizontal level. Extreme magnification (up to 1000 times) is obtained with this technology acquiring images at the cellular/nuclear level, mimicking histopathology sections, thereby allowing targeted biopsy and reducing the number of random biopsies.

During endomicroscopy, the columnar lined epithelium could be easily identified and goblet cells appear as dark cells within the intestinal metaplasia. It is possible to distinguish the gastric type epithelium from the intestinal type, and any suspicious areas could be targeted[36]. CLE was used in Barrett's esophagus to study the mucosal morphology and predict dysplasia. The sensitivity in predicting intestinal metaplasia and dysplasia compared to targeted histology was 98% and 93%, with a specificity of 94% and 98%[37]. They have proposed a classification for detection of Barrett's esophagus and associated neoplasia comprising of criteria for vessel and crypt architecture. CLE with optical biopsies or targeted biopsies have been shown to improve the yield of endoscopically inapparent BE dysplasia in a randomized trial, compared to non-targeted biopsies[38]. However, scanning a long segment of Barrett's with this technique is challenging and may not be appropriate in routine surveillance.

All the above modalities could take significant additional time during the procedure and in routine clinical practice this needs to be considered against resources. In our experience, "trimodal imaging" adds around 5-10 min to routine examination and biopsies. The dysplasia detection studies described are potentially assessing sensitivities of various techniques against four quadrant or targeted biopsies. However, this is not the true sensitivity of the modality, as we know that random biopsies are associated with significant sampling error. Nevertheless, most studies have investigated the additional value of these new techniques over WLE in improving dysplasia detection.

CONCLUSION

In summary, the detection of dysplasia in Barrett's esoph-

agus has been improved by the newer imaging techniques, such as autofluorescence endoscopy and narrow band imaging. NBI with magnification is particularly useful in characterizing suspicious lesions. However, most of these studies are conducted in centers of excellence and whether similar results could be reproduced in centers with less experience needs to be ascertained. Large randomized trials are necessary before advocating these techniques for routine surveillance. Nevertheless, we believe that a thorough examination using a high resolution endoscope and a high definition monitor with the use of mucolytics should be the minimum standard for routine Barrett's surveillance.

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OBSERVATION

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Surveillance for colitis-associated colon neoplasia

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Abstract

The risk of developing colon cancer is increased in colitis patients, particularly if the disease is extensive and its duration long-standing. Endoscopic guidelines have been developed with the goal of detecting early neoplastic changes prior to development of advanced malignancy. Unfortunately, the natural history of this superimposed neoplastic process in colitis appears to be very heterogeneous and poorly understood. Moreover, there are numerous confounding variables in colitis patients that limit accurate assessment of the surveillance effectiveness of colonoscopy and multi-site biopsy protocols. Although the clinical challenge posed to even the most experienced clinicians remains significant, evolving methods of endoscopic imaging may facilitate better evaluation of this highly select group of patients.

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Key words: Ulcerative colitis; Crohn's disease; Colon cancer; Surveillance colonoscopy; Colon biopsies

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INTRODUCTION

In long-standing and extensive colitis, the risk of colon cancer is increased[1]. Supportive evidence for this increased colon cancer risk in colitis initially came from observational studies in tertiary centers in the United Kingdom and the United States during the pre-surveillance era. In these tertiary centers, more severe disease, some already complicated by carcinoma, would have influenced risk estimates. Later, studies using data from different community-based clinical practices or population-based studies suggested that this risk was increased, but the magnitude of this risk was less. Recent data estimates from a referral-based population in a long-standing and uniform program of colonoscopy surveillance in the United Kingdom noted that the overall cumulative incidence of colitis-associated colon cancer was about 2.5% after 20 years of disease, 7.6% after 30 years, and 10.8% after 40 years[2].

Risk factors that may contribute to the eventual development of colorectal cancer in colitis have become increasingly apparent. Some of these risk factors are listed in Table 1, although precise risk estimates for each factor have only been defined to a limited extent. Overall, the underlying cause for this increased cancer risk has been hypothesized to be the ongoing chronic and persistent colonic mucosal inflammatory process[6], but the actual molecular mechanisms involved still require definition. In recent years, a novel, but still hypothetical "inflammation to carcinoma sequence" has been conceptualized to more

precisely separate this process from the well enunciated "adenoma-carcinoma sequence" proposed for sporadic colon cancer[16-18]. A number of differences in the molecular changes of sporadic compared to colitic cancer have been noted[19]. APC loss of function appears to be less frequent in colitis-associated colon cancer, while p53 mutations seem to occur earlier[19]. Finally, CpG-island methylation also appears to be accelerated in colitis[20].

RATIONALE FOR COLONOSCOPY SURVEILLANCE FOR NEOPLASIA

In the past, prophylactic proctocolectomy was sometimes performed in selected cases to reduce subsequent risk of colorectal cancer. Although this undoubtedly reduced colon cancer risk in this setting, most colon resections were not required, and, in themselves, probably resulted in reduced quality of life and created significant morbidity, and likely, some mortality. Although this approach may still have merit in some selected situations, a different clinical approach based on surveillance has emerged in recent decades; in part, owing to the increased availability of colonoscopy to permit detection of neoplasia. In the setting of inflammatory bowel disease, the goal to detect either precancerous changes or early stage invasive carcinoma has been pursued to permit curative colon resection. Although there are no randomized controlled clinical trials to show that surveillance colonoscopy is an effective approach, 3 case-control studies have appeared[21-23]. As a result, enthusiasm exists for development of surveillance programs in chronic colitis, but surveillance colonoscopy *per se* may not actually prolong survival, even in extensive colitis[24]. Possibly, cancers are detected at an earlier stage with a resulting better prognosis, but it has been suggested that this likely reflects, in large part, the phenomenon of lead-time bias[24]. Guidelines for surveillance have been developed based on the rationale that detection of these early neoplastic changes in the colon could result in a significant reduction or elimination of the morbidity and mortality from colon cancer in selected high risk patients with inflammatory bowel disease[25,26]. These guidelines suggest that colonoscopy should be carried out on a regular basis (for some, on an annual basis) and also urge that biopsies be performed throughout the colon to include "flat" areas of mucosa as well as visibly abnormal mucosa (or macroscopically-defined lesions). It has also been emphasized that the optimal surveillance interval has not been defined and that there are no prospective studies on the optimal number of biopsy specimens from different sites in the colon[25].

CONFOUNDING VARIABLES IN SURVEILLANCE PRACTICE

Studies have also shown that patient and physician compliance to published surveillance colonoscopy guidelines or recommendations varies. In part, this likely reflects the presence of many other confounding issues. For example, pre-scheduled procedures may be completed during periods of active inflammatory disease which, from a pathological perspective for histological evaluation, may not be optimal. Guidelines do not clearly define the duration that surveillance studies should be delayed, if moderate to severe symptomatic disease is present. Differences in methodology are also evident and include (but are not limited to): biopsy site, size and numbers; forceps type (e.g. "jumbo"); biopsy methods (i.e. "multiple-bite single pass" biopsies *vs* "single-bite multiple pass" biopsies); and, fixation methods (i.e. formalin *vs* picric acid or mercury-based fixatives, such as Bouin's or Hollande's). Some of these fixatives, for example, may significantly impact on cellular (particularly nuclear/nucleolar) detail in a colonic biopsy section and the resultant histological appreciation and interpretation of neoplastic changes. Studies have also shown that much of the benefit attributed to colonoscopy in a surveillance population may be due, in part, to the intensified degree of follow-up (compared to a no-surveillance population) rather than the precise frequency of procedures (annual or otherwise) or numbers of colonoscopic biopsies *per se*. Assuming similar biological behavior of the disease (which may not be appropriate in an essentially heterogeneous population), patients with inflammatory bowel disease who are followed frequently and regularly are thought to more likely have a positive outcome than those not followed in a defined protocol. Guidelines suggested for long-standing and extensive ulcerative colitis have also been extended to Crohn's disease[25,26] since prolonged disease duration in extensive Crohn's colitis appears to result in increased colon cancer risk[27,28], but data supporting a role for a program of surveillance colonoscopy in Crohn's disease are still needed. Finally, the development of sporadic or "non-colitic" colon cancers in patients with inflammatory bowel disease may also be critically influenced by other underlying genetic, geographic and environmental factors. Some of these factors may confound data analysis and prevent direct translation of published data to immediate clinical practice.

Table 1 Risk factors for cancer in colitis

Epithelial cell dysplasia (high-grade > low-grade)
Extent of mucosal involvement (pancolitis > distal colitis > proctitis)[3,4]
Extended duration of ongoing disease (> 8-10 yr)[1,2,5]
Severity of histologic inflammation (?linked to compliant 5-ASA use)[6,7]
Onset in childhood (?linked to underlying duration of disease)[3,4,8]
Primary sclerosing cholangitis[9,10]
Liver transplantation, usually for primary sclerosing cholangitis[11-13]
Underlying familial colon cancer risk[14,15]
Other (?immunosuppression, ?biologic agents)

EPITHELIAL DYSPLASIA

The key histopathological lesion in bowel disease surveillance categorized by standard classification is epithelial dysplasia[29]. An alternative classification has also been more recently devised[30]. Dysplasia (from the Greek,

translated roughly as "bad formation") is a pathological term used to describe a neoplastic process that is hypothesized to be restricted to epithelial cells, not other mucosal cell types, and occurring in this case in the colon. These epithelial cells display features of both delayed maturation and differentiation, but have not invaded through the underlying basement membrane. From a practical perspective, dysplasia is considered the earliest recognizable form of the neoplastic process with the potential for invasive cancer. Eventual development of cancer has been hypothesized to be related to the degree or grade of dysplasia. Essentially, dysplasia represents a histopathologically-defined risk marker for carcinoma. However, the precise risk for an individual focus of low-grade epithelial dysplasia to ultimately transform into a focus of high-grade dysplasia, and eventually into an invasive carcinoma, is not known although it is probably low. Nevertheless, from a clinical perspective, the detection of dysplasia, an unequivocal neoplastic lesion, is thought to represent a histopathological marker of increased risk for eventual development of invasive cancer, or even concurrent cancer elsewhere in the colon. Importantly, colon cancer may also occur in colitis even if dysplasia is not detected[26].

Indeed, the long-term natural history of epithelial dysplasia is poorly understood, especially in the setting of colitis. Additionally, there are many considerations that might influence this hypothetical biological process. Specifically, it is not known if a tiny focus of epithelial dysplasia in the colonic mucosa remains irreversibly present or if a focus of dysplasia can spontaneously regress, or even disappear. Furthermore, it is not known if a persistent focus of dysplasia, if given enough time, inevitably reaches a higher grade or remains static. Moreover, it is not known if there is a biological qualitative difference between a tiny single focus of dysplasia in flat mucosa compared to a larger "field change" in flat mucosa. Also, it is not precisely known if dysplasia in flat mucosa differs from dysplasia in visibly abnormal mucosa. If dysplasia is associated with a mass lesion, the presence of an associated cancer is thought to be higher, particularly in the mass *per se*. Indeed, underlying malignancy (below the overlying mucosa) has been detected in patients with mucosal biopsies that show dysplasia but no invasive carcinoma. Finally, it is conceivable that dietary, pharmacological or other therapeutic variables, including biological agents, used to treat the colonic inflammatory process may positively or negatively affect this histologically-defined biological change in the epithelial cell.

CLASSIFICATION OF DYSPLASIA

Dysplasia may occur in flat or elevated mucosa. Evaluation of a colonic biopsy for dysplasia results in 3 possible pathological conclusions: negative, indefinite or positive. Changes negative for dysplasia include normal mucosa, regenerative changes and mucosa with active inflammatory change. Changes indefinite for dysplasia include epithelial changes too aberrant to be classified as negative, but insufficient to fulfill criteria for positive. Positive refers to nuclear, cellular and architectural epithelial changes (Table 2). Positive for dysplasia may be further subdivided into low-grade and high-grade dysplasia depending on the predominant location of the nuclei in the epithelial cell layer. In low-grade dysplasia, the nuclei occupy the basal half of the cell. In high-grade dysplasia, the nuclei extend into the luminal half of the cell or the nuclei simply appear in a particularly disorganized pattern. Most pathologists define the degree of dysplasia (low-grade, high-grade) based on the most severe changes detected. Finally, adenomatous polyps with varying degrees of epithelial dysplasia may be seen in colitis. If these are sessile, it may be particularly difficult to distinguish these from other visible lesions, such as the so-called dysplasia-associated lesion or mass.

Table 2 Changes positive for dysplasia[1]

Nuclear changes	Nuclear enlargement
	Pleomorphism
	Hyperchromatism
	Chromatin fragmentation
	Increased mitotic numbers
	Nuclear stratification
Cellular changes	High nuclear to cytoplasm ratios
	Enlarged nuclei
	Reduced or absent mucus production
Architectural changes	Gland-like arrangement of epithelial cells

[1]Dysplasia may be subdivided into low-grade dysplasia and high-grade dysplasia based largely on the nuclear localization in the cells of the epithelial layer. Based on Riddell *et al*[29].

INTERPRETATION ISSUES IN DYSPLASIA

Unfortunately, expert gastrointestinal pathologists may not agree in defining dysplasia or its severity or grade[29,31-34]. Early studies demonstrated good inter-observer agreement for "negative" but limited agreement for grading the "positive" category. Later studies have shown limited agreement for low-grade "positive" compared to the "indefinite" category, compared to high-grade "positive" and "negative" categories. For histological definition of dysplasia, review by a second expert pathologist has also been recommended. In this situation, a second opinion that confirms the initial assessment may be helpful, but the impact of disagreement on the clinical decision-making process has not been thoroughly evaluated. Finally, many clinicians feel that the definition of a single focus of low-grade dysplasia may not be sufficient to recommend colectomy and may increase the frequency of surveillance to confirm the presence of dysplasia or seek an additional site of dysplastic change.

OTHER METHODS FOR PREDICTION OF DYSPLASIA

Other methods have been reported to have potential value in predicting or corroborating dysplasia. For example,

flow cytometry showing DNA aneuploidy in a group of high risk patients without detectable dysplasia prospectively predicted an increased rate of dysplasia development later in the same group (although not in specific individuals within the same group). In addition, use of immunohistochemical staining methods have been advocated as another approach to support the pathological definition of dysplasia (p53, Ki-67, β-catenin). Indeed, a mutation of the p53 tumor suppressor gene, often detected in colon cancer, has been reported in some patients with inflammatory bowel disease before dysplasia is detected[35,36]. α-methylacyl-CoA racemase may also be a useful marker of dysplasia[37] and further confirmatory studies to evaluate its specificity and sensitivity are needed.

ANEUPLOIDY STUDIES AND MATHEMATICAL MODELING

While it is believed that surveillance colonoscopy and biopsy sampling *per se* may either categorize the degree of risk (i.e. low-grade or high-grade dysplasia) for cancer or define an early cancer, it is hoped that this process might actually reduce the morbidity and mortality associated with delayed recognition of a late stage colon cancer. It has been hypothesized that programs of surveillance colonoscopy with defined biopsy protocols might permit accomplishment of this goal. Unfortunately, there are no controlled studies available. Some published guidelines have been based, in part, on an approach taken in an earlier research study[38] largely designed for the different purpose of detection of DNA aneuploidy prior to or during development of dysplasia in ulcerative colitis. In that study, "jumbo" forceps biopsies were obtained from 4 quadrants at 10 cm intervals for an average of 40 separate biopsies for each procedure, in those with disease extending beyond the rectosigmoid region. In addition, visible lesions other than inflammatory polyps were also removed. The biopsies were each estimated to approximate 5 mm in size. Similar studies were performed on colectomy specimens, although the samples were larger (up to 1 cm in diameter) and removed every 3 cm for an average of 100 specimens per colon. A portion of some, but not all, biopsies were used for DNA aneuploidy studies. The study concluded that aneuploidy correlated with histological grade and might define a patient subset without dysplasia potentially at higher risk for later development of dysplasia. Although not designed to determine an optimal biopsy protocol, detection of neoplasia, either dysplasia or cancer, was mathematically estimated to require 18 biopsy samples, possibly obtained during 2 or more colonoscopies over 4 to 6 years, a time estimated for progression to high-grade dysplasia or cancer in ulcerative colitis. Although there are guidelines that have appeared to suggest annual or biannual colonoscopies with multiple biopsies taken in 4 quadrants every 10 cm in extensive colitis, evidence for this approach is not available. Indeed, most biopsies taken with standard forceps measure only about 2 mm × 2 mm. Not surprisingly, a recent study confirmed that jumbo forceps were superior to standard, although large-capacity, forceps in obtaining diagnostically adequate surveillance specimens[39]. If anything, random biopsy sampling only serves to emphasize the potentially high miss rate for focal areas of dysplasia since the total colonic surface area has been estimated, on average, to be about 1600 cm^2. As a result, it is not surprising to find that specialist endoscopists differ substantially in the actual practice of surveillance, including the number of biopsies obtained during a surveillance procedure for dysplasia detection. Recent evidence suggests that evolving technology, including chromoendoscopy with magnification[40], narrow band imaging or confocal endomicroscopy[41,42], autofluorescence imaging[43] and other emerging refinements using new molecular markers[44], may permit more precise definition of neoplastic change in long-standing and extensive colitis, rather than labor-intensive (and time-intensive) procurement of "blind" biopsies from multiple areas of otherwise flat mucosa. It is likely that these newer methods for cancer surveillance (e.g. chromoendoscopy for biopsy targeting) will eventually be incorporated into emerging guidelines[45]. These newer methods of enhancement, however, raise fresh issues underlined by recent mathematical modeling studies suggesting that enhanced endoscopic methods may not necessarily translate into improved patient outcomes[46].

OTHER LIMITATIONS IN NEOPLASIA SURVEILLANCE

Although dysplasia surveillance may be regularly performed, other confounding variables may make evaluation of surveillance programs difficult. The operator may be less experienced and the potential for missing lesions, particularly flat lesions, proximal to the hepatic flexure has been noted. Fortunately, in patients with long-standing chronic colitis, complete evaluation to include the cecum may be more readily accomplished (compared to screening non-colitic colons for polyps); in part, because the colon is often more tubular, fibrotic and foreshortened. Other factors may play a role. Firstly, the biological behavior of a neoplastic lesion in the setting of an extensive and chronic inflammatory process may differ substantially from a neoplastic lesion that develops without a background of inflammatory disease, or may biologically differ depending on the site within the colon (right *vs* left). Secondly, some neoplastic lesions that occur in patients with inflammatory bowel disease, such as neuroendocrine carcinomas, may have rapidly progressive growth. Even though these are very rare, some have been detected within months of surveillance studies that failed to define dysplasia[47,48]. Since colon cancers in this setting of inflammatory bowel disease may be very heterogeneous, detection of dysplasia only suggests increased risk, but cannot predict rate of progression to cancer. Thirdly, neoplastic lesions may also initially develop insidiously in "hidden sites", such as the appendix, where surveillance biopsies cannot normally be procured[49,50]. Fourthly, other underlying diseases followed

by concomitant or new treatments may significantly influence the immunological status of patients in surveillance programs. For example, increased colon cancer rates appear to develop after liver transplantation for sclerosing cholangitis[12,13].

PRACTICAL EVALUATION

Recognizing these inherent limitations, surveillance with multiple site biopsies, at least in well documented extensive disease, has merit as a potentially powerful tool for prevention of colonic neoplasia. Although guidelines have appeared, data to support a precise evaluative approach related to procedural frequency and numbers of biopsies performed during each procedure remain difficult to define, even after 8 to 10 years of ongoing disease. If colonic disease is extensive and long-standing, but the patient has entered clinical remission, then colonoscopic evaluation might reasonably be carried out every 3 years with biopsies from different sites, particularly from macroscopically abnormal mucosa. Part of the value of surveillance, however, also relates to increased frequency of clinical review, especially in those with few or no symptoms. Paradoxically, clinically well patients are often those most likely to become relaxed regarding their ongoing medical care and surveillance. Conversely, patients who remain continuously (or intermittently) symptomatic, especially if relapses are frequent, are more likely to require more significant medical therapy (and more intensive clinical evaluation) for disease control. In these, frequent procedural re-evaluation may become a significant element in their management, and so surveillance will essentially be accomplished. In those with the most clinically significant disease, colectomy should result, reducing (or removing) the colon cancer risk. In time, this approach will evolve as improvements in technology occur and their effectiveness continues to be evaluated.

CONCLUSION

Surveillance colonoscopy in inflammatory bowel disease, particularly in extensive long-standing ulcerative colitis, represents a challenge for the clinician. Assessment of its effectiveness is especially difficult and has been limited because there are numerous confounding variables that play a role in the individual patient. Finally, there is an evolving appreciation that inflammatory bowel disease *per se* represents a truly heterogeneous inflammatory process, even in those classified with long-standing and extensive disease.

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REVIEW

Roles of liver innate immune cells in nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in the United States and other developed countries and is expected to increase in the next few years. Emerging data suggest that some patients with NAFLD may progress to nonalcoholic steatohepatitis (NASH), cirrhosis and even hepatocellular carcinoma. NAFLD can also promote the development and progression of disease in other organ systems, such as the cardiovascular and endocrine (i.e. diabetes) systems. Thus, understanding the pathogenesis of NAFLD is of great clinical importance and is critical for the prevention and treatment of the disease. Although the "two-hit hypothesis" is generally accepted, the exact pathogenesis of NAFLD has not been clearly established. The liver is an important innate immune organ with large numbers of innate immune cells, including Kupffer cells (KCs), natural killer T (NKT) cells and natural killer (NK) cells. Recent data show that an imbalance in liver cytokines may be implicated in the development of fatty liver disease. For example, Th1 cytokine excess may be a common pathogenic mechanism for hepatic insulin resistance and NASH. Innate immune cells in the liver play important roles in the excessive production of hepatic Th1 cytokines in NAFLD. In addition, liver innate immune cells participate in the pathogenesis of NAFLD in other ways. For example, activated KCs can generate reactive oxygen species, which induce liver injury. This review will focus primarily on the possible effect and mechanism of KCs, NKT cells and NK cells in the development of NAFLD.

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Key words: Innate immune cells; Nonalcoholic fatty liver disease; Kupffer cell; Natural killer T cell; Natural killer cell

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in the United States and other developed countries[1,2]. Approximately 30% of adults in the USA have NAFLD[3], and the incidence of NAFLD in Shanghai, Guangzhou and Hong Kong of China is roughly 15%[4]. With the rise in the incidence of metabolic syndrome in recent years, the incidence of NAFLD is expected to increase in many countries[5]. In the past, NAFLD was considered to be a benign liver dis-

ease. However, recent data have shown that some patients with NAFLD can progress to nonalcoholic steatohepatitis (NASH) and then to cirrhosis and even hepatocellular carcinoma (HCC). One study showed that 20% of patients with NASH eventually progressed to cirrhosis, and among them, 8% would go on to develop a potentially fatal liver disease, such as liver cancer[2]. In the United States, about 2%-3% of adults have NASH, and approximately 3% of patients diagnosed with NAFLD develop cirrhosis or a liver-related complication[6,7]. The World Health Organization estimates that at least two million patients will develop cirrhosis following hepatic steatosis in the years to come[8]. Additionally, NAFLD can promote the development and progression of diseases in other organs. Recent studies show that NAFLD is associated with a higher prevalence of cardiovascular disease and that this association is independent of classical risk factors, such as the presence of metabolic syndrome[9,10]. NAFLD also significantly increases the risk of diabetes[11] and the development of chronic kidney disease in individuals with type 2 diabetes[12]. Thus, understanding the pathogenesis of NAFLD is of great clinical importance and is critical for the prevention and treatment of the disease.

The pathogenesis of NAFLD is described by the "two-hit" hypothesis. The "first hit" (i.e. fat accumulation) sensitizes the liver to the injurious effects of one or more additional factors, while the "second hit" leads to the development of steatohepatitis and fibrosis[13]. A variety of factors that may be involved in the development of inflammation and fibrosis may comprise the "second hit". These factors include cytokine overproduction, hepatocyte organelle (particularly mitochondria) malfunction, lipid peroxidation, reactive oxygen species (ROS) and peroxisome proliferator-activated receptor (PPAR) dysfunction in the cell nucleus[14,15]. The exact pathogenesis of NAFLD is, to date, not well understood. The innate immune system is responsible for the rapid, initial response of the organism to potentially dangerous stressors, including pathogens, tissue injury and malignancy[16]. As pathophysiological research on NAFLD continues, a considerable amount of the current data shows that innate immune processes both within and outside the liver are involved with NAFLD[17]. In this review, we will summarize the information concerning the contributions of liver innate immune cells, such as Kupffer cells (KCs), natural killer T (NKT) cells and natural killer (NK) cells to the development of NAFLD and will discuss the possible role of their involvement in the disease. This is the first paper to comprehensively review the role of various liver innate immune cells in NAFLD.

LIVER IS AN IMPORTANT INNATE IMMUNE ORGAN

The innate immune system responds to potential attacks by pathogens against the organism and is the first line of defense against infection. Innate immunity consists of lymphocytic cells, phagocytic cells, physical barriers, chemical barrier and humoral factors. Recent evidence suggests that the liver is a major immune organ and functions predominantly in innate immunity. The liver is an important part of the body's immune response[18,19]. From the perspective of its anatomical location, the liver is exposed to a large variety of antigens from the gastrointestinal tract, including dietary antigens, pathogens and toxins. The liver rapidly removes these harmful particles from the intestinal tract. Studies have shown that liver lymphocytes are primarily located around the portal tracts. This distribution of lymphocytes in the liver aids in the rapid removal of gastrointestinal antigens from the circulation. The liver is responsible for the biosynthesis of 80%-90% of innate proteins, including acute-phase proteins (APPs), complement factors and secreted pattern recognition receptors. APPs are the key to the innate defenses against infection and reduce tissue damage through inactivation of proteinases, which are released by pathogens and dead or dying cells. Over 35 proteins and protein fragments make up the complement system, including serum proteins, serosal proteins and cell membrane receptors. These proteins are synthesized primarily in the liver and account for about 5% of the globulin fraction of blood serum. These proteins interact with each other to protect against infection. In addition, the complement system contributes to the pathogenesis of many liver disorders, including liver fibrosis and alcoholic liver disease. The liver contains large numbers of innate immune cells, including phagocytic cells (e.g. KCs) and lymphocytic cells. Liver KCs account for about 80%-90% of the total fixed tissue macrophages in the body. In an average human liver of 1.5 kg, there are about 1×10^{10} lymphocytes[20]. The liver lymphocyte population is enriched with NK cells and NKT cells[21]. For example, in the mouse liver, NK and NKT cells account for approximately 10% and 30% of all lymphocytes; however, in the rat and human liver, NK cells account for 30%-50% of all lymphocytes[22]. In comparison with other organs, such as the spleen, the proportion of innate immune cells is much higher in the liver. The large quantities of these cells comprise the cellular basis of the liver's innate immune response. Several studies in both experimental animal models and human clinical studies have shown that nearly all innate immune cells in the liver are involved in liver injury[23,24]. It has been proposed that an imbalance of liver cytokines (e.g. Th1 cytokine excess) produced by liver innate immune cells could be the common pathogenic mechanisms for hepatic insulin resistance and NASH[21].

Th1-PREDOMINATED CYTOKINE RESPONSE IN NAFLD

T helper cells (Th cells) are a sub-group of lymphocytes that play an important role in maximizing the capability of the immune system. Th cells can be categorized into two groups: (1) T helper 1 (Th1), which produces proinflammatory cytokines including tumor necrosis factor-β (TNF-β) and induces cellular immunity; and (2) T helper 2 (Th2), which produces anti-inflammatory cytokines and induces humoral immunity primarily due to antibody production[25]. The balance between Th1 and Th2 is be-

lieved to play an important role in the immune response against invading microbes[26]. Recently, more studies have addressed the role of proinflammatory cytokines in fatty livers. Although NASH is not classically considered to be a Th1-polarized disease, several recent studies show that an imbalance between a relative excess in proinflammatory Th1 cytokines and a relative deficiency of anti-inflammatory cytokines can affect fatty liver disease[17]. Hepatic innate immune cell activation due to Th1-predominated cytokine responses, therefore, could be one mechanism by which NAFLD occurs. Food-derived fatty acids, intestinal bacteria-derived fatty acids and adipose tissue-derived fatty acids contribute to activation of innate immune cells in the liver[27]. Fatty acids bind to toll-like receptors (TLRs) expressed on immune cells, resulting in activation of the immune system. Adipose tissue-derived cytokines may also promote activation of innate immune cells in the liver. For example, TNF-α activates the KCs by interacting with its specific receptors on these cells. In addition, the effects of different types of liver innate cells on each other also play an important role in the activation of hepatic innate cells. KCs are able to activate liver NK cells directly by interaction between retinoic acid early inducible-1 (Rae1) on KCs and natural killer group 2, member D (NKG2D) on NK cells; they also indirectly interact with liver NK cells *via* interleukin (IL)-12, IL-18 and TNF-α[28].

KCs

KCs reside in liver sinusoids and are derived from circulating monocytes that probably originate from bone marrow progenitors. The liver contains a large number of KCs, which constitute approximately 20% of hepatic nonparenchymal cells (hepatic nonparenchymal cells include endothelial cells, KCs, lymphocytes, hepatic stellate cells and biliary ductal cells)[19]. KCs possess scavenger receptors which are responsible for eliminating blood-borne pathogens[29] and are essential in the clearance of bacteria from the blood-stream. KCs also generate various mediators, including proinflammatory cytokines and ROS. These mediators can act either locally or systemically to mediate immune responses[16]. These immune responses directly leads to hepatocyte injury.

KCs are closely involved in the liver's response to infection, toxins, transient ischemia and a variety of other stressors[30]. Recent studies have revealed that KCs also participate in the pathogenesis of NAFLD. For example, in a rat model of NASH induced by a high fat diet KCs are largely recruited and activated[31]. Indeed, the number of KCs seen in the liver of rats with NAFLD has been shown to be high[32]. Adachi *et al*[33] reported that KCs were inactivated by gadolinium chloride, and the inactivation of KCs could prevent the development of fatty liver and inflammation in rats chronically exposed to ethanol *via* intragastric feeding. In experimental liver transplantation, Frankenberg *et al*[34] observed that depletion of Kupffer cells in donor animals prevents primary nonfunction of fatty livers after transplantation and diminishes amino acid release at harvest. Meanwhile, the increased expression of the adhesive molecule Intercellular Adhesion Molecule-1 was inhibited only after transplantation, indicating that the increased proteolysis in marginal donor livers is not induced by cytokines, but is Kupffer cell-dependent. In experimental models of NASH in mice, Rivera *et al*[35] found that destruction of Kupffer cells can attenuate the histological appearance of hepatic steatosis, inflammation and necrosis. These results suggest that KCs contribute to the pathogenesis of NAFLD.

The characteristics of macrophages include plasticity and functional polarization. The macrophage phenotype has been defined at two separate polarization states, i.e. M1 and M2. M1 (i.e. classically activated macrophages) is induced by proinflammatory mediators, such as interferon-γ (IFN-γ). M1 macrophages have a high capacity to present antigen, to induce the release of large amounts of some cytokines (IL-12, IL-6, TNF-α, and IL-23) and to activate polarized Th1 responses; M1 macrophages also produce ROS. M2 (i.e. alternatively activated) macrophages respond to IL-4 and IL-13, thus promoting a Th2 response. M2 cells express high levels of the anti-inflammatory cytokines IL-10 and IL-1 decoy receptor. Recent studies show that adipose tissue macrophages from lean mice have the characteristics of the M2 phenotype, while macrophages from obese mice present the characteristics of the M1 phenotype. KCs also display great plasticity in their activation programs, ranging from the proinflammatory classical state to the anti-inflammatory alternative state[36]. It is possible that M1 or "classically activated" KCs play an important role in the development of NAFLD by producing TNF-α, IL-12, IL-6, and ROS. TNF-α is critical to the pathogenesis of NASH. Crespo *et al*[37] demonstrated that NASH patients with significant fibrosis exhibited increased expression of TNF-α mRNA when compared with those with minimal or non-existent fibrosis. Li *et al*[38] reported that treatment with anti-TNF-α antibodies can improve NAFLD induced by a high-fat diet in ob/ob mice. The mechanism of TNF-α's effect on NAFLD may include the following: (1) TNF-α induces hepatocyte cell death; (2) TNF-α causes insulin resistance, which results in hepatocyte steatosis; and (3) TNF-α regulates KCs's activation through an autocrine mechanism[39]. While KCs are the primary source of hepatic TNF-α, hepatic TNF-α also comes from visceral adipose tissue, especially in obese human subjects. TNF-α interacts with two specific receptors, TNF receptor 1 (p55) and TNF receptor 2 (p75). KC depletion reduces liver IL-12 expression in choline-deficient diet-induced fatty liver, suggesting that KCs are important cellular sources of liver IL-12 in NAFLD. The fact that hepatic IL-12 mRNA levels significantly increase in choline-deficient diet (CDD)-induced mice suggests that IL-12 participates in the development of NAFLD. IL-12 promotes the production of hepatic Th1-associated cytokines and is involved in hepatic NKT cell depletion. Using IL-12-deficient mice fed with CCD, however, Kremer *et al*[40] reported that IL-12 does not influence the progression of hepatosteatosis, suggesting an indirect role of KC-derived IL-12 in NAFLD. NAFLD

patients with increased systemic IL-6 usually have a higher prevalence of inflammation and fibrosis. IL-6 causes insulin resistance locally. Local insulin resistance may be linked to systemic insulin resistance. The results from considerable investigations suggest that IL-6 is a potential mediator of insulin resistance. In contrast to its role in the liver, IL-6 is believed to be beneficial for insulin-regulated glucose metabolism in muscle. A few studies have shown that IL-6 administration alleviates fatty livers in mice, protects hepatocytes from cellular necrosis and apoptosis, ameliorates hepatic microcirculation and inhibits hepatocyte death, suggesting a beneficial effect of IL-6 on NAFLD[41,42]. The effects of IL-6 are seemingly influenced by whether it is present acutely or chronically; the latter is the setting associated with insulin resistance. KCs also generate hepatic ROS which is involved in the development of NAFLD, as demonstrated by Wei *et al*[43]. ROS has a causal role in multiple forms of insulin resistance, which can further promote exacerbation of oxidative stress[44]. Oxidative stress increases the release of lipid peroxidation products and cytokines, which together can trigger the liver lesions of NASH[45]. NAFLD is usually caused by two "hits": the "first hit" is peripheral insulin resistance, which causes steatosis, while the "second hit" is caused by ROS. which induces vicious cycles that lead to inflammation[46]. Therefore, ROS plays an important role in the conversion of simple hepatic steatosis to NASH. However, the molecular mechanism of ROS in NASH formation is still unclear. ROS can directly activate inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) and Jun N-terminal kinase (JNK). JNK can stimulate the transcription of inflammatory target genes through the activation of protein-1, while IKK can stimulate the transcription of inflammatory target genes through the activation of NF-κB[17]. The activated KCs secrete transforming growth factor (TGF)-β1[47], which is one of the key fibrogenic factors of NASH. Other nonparenchymal liver cells, such as hepatic stellate cells and sinusoidal endothelial cells, also produce TGF-β1[48]. All these data suggest that KCs may be implicated in the development of NAFLD *via* multiple pathways (Figure 1).

The mechanism of KC activation in the development of NAFLD remains unclear. A number of studies have suggested that a gut-derived endotoxin could play a role in the pathogenesis of insulin resistance and NAFLD. In patients with NAFLD, the increase of gut permeability may induce bacterial overgrowth in the small intestine and lead to endotoxin production[49]. Gut-derived endotoxin can bind to TLR4 to induce KC activation[50]. Endotoxin can also activate the complement system. The activated complement system releases the anaphylatoxins C3a and C5a, which activate KCs through their receptors, C3aR and C5aR[51]. Visceral adiposity tissue in patients with NAFLD produces numerous proinflammatory cytokines, including TNF-α and IL-6, and these cytokines are involved in the recruitment and activation of liver KCs[52]. Therefore, we speculate that gut-derived endotoxin and visceral adiposity tissue-derived proinflammatory cytokines may contribute to M1 phenotype activation of KCs. While PPARδ plays a critical role in alternative phenotype activation of KCs, the KCs prepared from PPARδ-deficient mice (PPARδ$^{-/-}$) are unable to maintain the alternative phenotype[36]. NAFLD results in a high level of serum fatty acids. Fatty acids and their metabolites are ligands for the PPARs[53]. It is possible that binding of fatty acid to PPARδ on KCs can result in the alternative phenotype activation of KCs. Effective mechanism of PPARs agonists on NAFLD may be involved in the alternative phenotype activation of KCs.

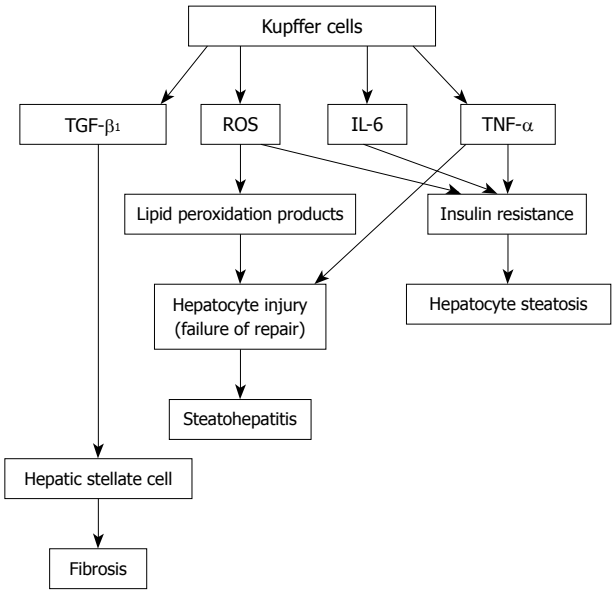

Figure 1 Mechanisms for the role of Kupffer cells in the development of nonalcoholic fatty liver disease. (1) Activated Kupffer cells (KCs) produce reactive oxygen species (ROS), interleukin-6 and tumor necrosis factor-α (TNF-α), which induce insulin resistance, leading to hepatocyte steatosis; (2) Activated KC-derived ROS causes lipid peroxidation production. Lipid peroxidation products and KC-derived TNF-α result in hepatocyte injury. Failure to repair hepatocytes after injury promotes the development of steatohepatitis; and (3) Activated KCs produce transforming growth factor-β1, which activates hepatic stellate cells. Activated hepatic stellate cells produce large quantities of extracellular matrix, leading to fibrosis.

NKT CELL

NKT cells are a unique subset of lymphocytes that express NK cell markers, such as CD161 and CD94, as well as a T-cell receptor (TCR) α/β[54]. NKT cells differentiate from NK cells and are different from NK cells in that NKT cells have a restricted repertoire and have TCR. NKT cells can be categorized into three subclasses: class Ⅰ (classical, Vα14-Jα18^{+}TCR/CD1-dependent); class Ⅱ (non-classical, all other CD1d-dependent T cells); and class Ⅲ, i.e. NKT-like cells (CD1d-independent NK1.1^{+} T cells)[51]. Evidence suggests that NKT cells develop in the thymus and migrate to peripheral organs, including the spleen and the liver. Reconstitution of adult thymectomized irradiated mice with syngeneic bone marrow cells gives rise to NKT cells in the recipient organs, including the liver[55]. Hence, liver NKT cells may originate from both the thymus and bone marrow. Because NKT cells have numerous functions related to innate and adaptive immunity, NKT cells are critical in the immune

response against viral infections and malaria, as well as in tumor immunity and autoimmune diseases[54,56]. The current studies show that NKT cells modulate inflammatory and fibrogenic responses in various liver diseases, such as viral hepatitis, various autoimmune liver disease, metabolic liver disease and hepatic malignant tumor[57].

In recent years, there has been increasing evidence of NKT cell population or function abnormalities in NAFLD patients. Observation suggests an inverse correlation between hepatic NKT cell populations and the accumulation of hepatic lipid. Guebre-Xabier *et al*[58] showed that NKT cells were selectively reduced in the fatty livers of obese, leptin-deficient ob/ob mice. This reduction was also confirmed in different models of diet-induced hepatic steatosis[59]. Utilizing biopsies from patients with mild to severe hepatic steatosis, Kremer *et al*[40] found that the NKT cell population in the human liver decreased when hepatosteatosis was moderate to severe. Xu and colleagues[57] also demonstrated a reduction in the numbers of peripheral NKT cells in patients with NAFLD. Adoptive inoculation of a relatively small aliquot (1×10^6 cells) of NKT cells into ob/ob mice leads to a significant reduction in hepatic fat content. Within 12 d of transplantation, an estimated 12% of hepatic fat content in NKT cell-transplanted mice was decreased, as compared with the control-treated ob/ob mice, and resulted in a shift from a mixed microvesicular-macrovesicular steatosis pattern to a microvesicular steatosis pattern[60]. These results further suggest that there is a negative correlation between hepatic NKT cell populations and the severity of NAFLD and that enhancing the activity of NKT cells may become a therapeutic tool for the treatment of NASH.

It remains unclear how hepatic steatosis reduces hepatic and peripheral NKT cells. The fact that apoptosis of hepatic NKT cells is significantly increased in ob/ob mice suggests that apoptosis of this cell line may be critical in NAFLD. Deng found that a high-fat diet triggers an accumulation of immature myeloid cells in B6 mice livers, e.g. $CD11b^+Ly6C^{hi}Ly6G^-$ cells, and that these cells can induce NKT cell apoptosis[61]. In addition, IL-12 can promote NKT cell death. A study of CDD-induced mice fatty liver shows that up to 98% of the hepatic NKT cell population in wild-type mice was depleted after 20 wk. However, hepatic NKT cells in IL-12-deficient ($IL\text{-}12^{-/-}$) mice was preserved. Further observations show that KC depletion blunts hepatic IL-12 and leads to a complete repopulation of hepatic NKT cells in CDD-induced mice fatty liver. Accordingly, KC-derived IL-12 may be involved in the loss of NKT cells in fatty livers[40]. Additionally, hepatic lipid accumulation might be directly responsible for the reduced NKT cell population in NAFLD.

Activated NKT cells express FAS ligand on their cell surface. FAS ligand can interact with FAS on hepatocytes, resulting in hepatocyte apoptosis. NKT cells can also release perforin and granzyme from cytoplasmic granules which can destroy hepatocyte cells[62]. Based on this finding, reductions in NKT cell accumulation in the liver could potentially become a therapeutic strategy to minimize liver damage in patients with NASH.

Hepatic NKT cells modulate liver injury primarily by balancing local production of Th1 and Th2 cytokines. NKT cells can generate a lot of Th1 cytokines, such as IFN-γ, TNF-α and Th2 cytokines, such as IL-4, IL-10 and IL-13[63]. Hepatic NKT cell-derived Th2 cytokine quantity may be greater than NKT cell-derived Th1 cytokine quantity. Therefore, hepatic NKT cell depletion or reduction may lead to Th1 polarization of hepatic cytokine production, increasing TNF-α, IL-12 and IFN-γ[64]. The Th1 cytokine polarization caused by hepatic NKT cell reductions appears to play an important role in the pathogenesis of NASH. Therefore, the net effect of hepatic NKT cell reductions may be harmful for NASH patients.

However, in contrast to the past studies, recent studies support the concept that NKT cells accumulate in progressive fatty liver disease. Tajiri *et al*[65] noted that hepatic $CD3^+CD56^+$ NKT cells increased as NAFLD progressed. Most recently, Syn *et al*[66] reported that NKT cells expressing CD56/CD3 or CD57/CD3 were largely accumulated in the livers of patients with progressive and chronic diseases, suggesting that NKT cells contribute to the progression of NASH into cirrhosis. Different study results in the NKT cell population of livers with NAFLD may be due to multiple factors, including different fatty liver models used and the different NKT cell subtypes checked. The different study results suggest a complex role of NKT cells in the development of NAFLD.

NK CELL

NK cells are an important component of the innate immune response against many viruses. NK cells have the ability to lyse virus-infected cells and to secrete cytokines. Cytokines can inhibit viral replication and activate and recruit cells of the adaptive immune response[67]. NK cells play a critical role in bridging the innate and adaptive arms of the immune response[68]. NK cells originate from the bone marrow; undergo a complex maturation process, which leads to the acquisition of their effector functions; and then redistribute from the bone marrow and lymph nodes to blood, spleen, liver and lung[69]. NK cells are abundant in the liver and are relatively rare in peripheral lymphoid organs. Hepatic NK cells are large granular cells in the liver sinusoids and were originally termed Pit cells. Many studies have shown that liver NK cells may be involved in the pathogenesis of liver injury, fibrosis and regeneration[70-72]. Recent findings revealed that NK cells may also participate in the development of NAFLD. Obesity is the most common cause of NAFLD. By determining the cytotoxic activity of peripheral blood NK cells, Lamas *et al*[73] found that rats with diet-induced overweight had significantly lower NK cytotoxic activity compared with control rats. O'Shea *et al*[74] also demonstrated that obese human subjects had significantly lower circulating NK ($CD56^+CD3^-$) cells (7.6% of all lymphocytes) when compared with lean healthy controls (16.6% of all lymphocytes). In addition, the cytotoxic function of NK cells was significantly lower in obese human subjects compared

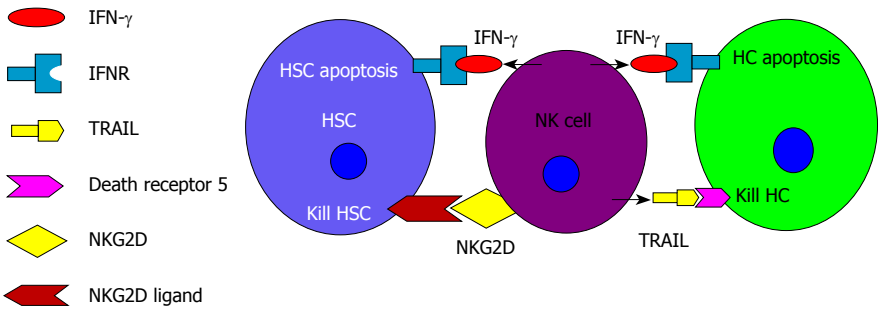

Figure 2 Potential roles of hepatic natural killer cells in the development of nonalcoholic fatty liver disease. Hepatic natural killer (NK) cells may have two different roles in the pathogenesis of nonalcoholic fatty liver disease. (1) NK cells have anti-fibrotic effects. NK cells release interferon-γ (IFN-γ), which combines with its receptor to induce hepatic stellate cell (HSC) apoptosis. Early activated HSCs express increased levels of natural killer group 2, member D (NKG2D). NK cells can kill early activated HSCs by binding NKG2D on NK cells with NKG2D ligand on HSCs; and (2) NK cells induce hepatocyte injury. NK cell derived IFN-γ results in hepatocyte apoptosis. NK cell derived-tumor necrosis factor-related apoptosis-inducing ligand combines with its death receptor 5 to kill hepatocytes. HC: Hepatocyte; IFNR: Interferon-γ receptor; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand.

with lean healthy controls (30% *vs* 42% tumor cells lysed). However, Kahraman and colleagues[75] recently reported that hepatic NK cells were increased in patients with NASH, while only a few of these cells were found in patients with NAFLD, and almost no NK cells were found in healthy controls. It is possible that there is a difference in the NK cell population found in peripheral blood and that found in the liver of patients with NAFLD.

Hepatic NK cells may have two different roles in the pathogenesis of NAFLD (Figure 2). First, a recent study on liver fibrosis in mice suggests that NK cells have an anti-fibrotic effect[76]. The mechanisms of NK cells on anti-fibrosis may be two-fold. NK cells can directly kill early activated hepatic stellate cells (HSCs), which are the principal fibrogenic cell type in the liver. Activation of the HSC is the central event in hepatic fibrosis[77-79]. Activated HSCs can produce a great deal of extracellular matrix, which leads to hepatic fibrosis. Second, hepatic NK cells release IFN-γ, inducing HSC cell cycle arrest and apoptosis[80]. Of immune cells in the liver, NK cells are the primary producers of IFN-γ[81]. A recent study showed that IFN-γ is effective in inhibiting hepatic fibrosis induced by intraperitoneal injections of dimethylnitrosamine in non-obese diabetic mice[82]. In addition to their anti-fibrotic effect, NK cells may also protect hepatocytes from NASH injury. Kahraman *et al*[75] reported that mRNAs expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was significantly enhanced in NASH livers. Activated NK cells are able to kill hepatocytes and cholangiocytes *via* TRAIL. Because hepatic apoptosis is one of the prominent features of liver injury during the pathogenesis of NASH[83], IFN-γ-induced hepatocyte apoptosis in response to activation of NK cells seems to be a primary mechanism explaining liver injury in this disease. IL-12 and IL-18 can induce IFN-γ production and the secretion of NK cells[84]. Many recent studies have identified the critical role of IL-18 as a potent IFN-γ inducer in NK cells[85].

Cytokines are involved in NASH *via* modulation of the activation of NK cells. IL-18 is the most important KC-derived cytokine, which in turn promotes NK cell activation. IL-12 is another cytokine that activates NK cells. Accumulation of IL-12 or IL-18 in the liver can stimulate the local expansion of cytotoxic NK cell subpopulations, which produce large amounts of IFN-γ within the hepatic microenvironment[30]. IL-15 is an important cytokine that maintains NK cell activation. Numerous *in vitro* and *in vivo* studies have shown that IL-15 plays a critical role in the regulation of development, survival and function of the NK cell lineage[86]. IL-15 prevents NK cells from undergoing apoptosis[87]. In mice lacking IL-15, the numbers of NK cells are found to be severely decreased[88]. However, IL-10 can suppress the activation of the NK cells. NK cells can also be activated through expression of the NKG2D ligand by macrophages or tumor cells and its interaction with NKG2D[89]. The increased level of NKG2D ligands in KCs activates NK cells and induces hepatocyte damage. Likewise, because NKG2D ligands are expressed in HCC and cholangiocarcinoma, the tumor cells could be eradicated by this specific anti-tumor immune response[90].

DYSLIPIDAEMIA, IMMUNE AND INSULIN RESISTANCE

The incidence of dyslipidemia is high in NAFLD patients. Dyslipidemia includes hypertriglyceridemia, hypercholesterolemia and abnormal increases in low-density lipoprotein or decreases in high-density lipoprotein cholesterol. Hypertriglyceridemia is the most common dyslipidemia in NAFLD patients. Triglycerides hydrolyze to form fatty acids, and many studies have found that fatty acids have immunologic effects[91]. Fatty acids can drive macrophages to reside in adipose tissue, where they can recruit more macrophages from the circulation. Macrophages that "reside" in tissue in this manner can produce high levels of TNF-α, which may worsen the insulin resistance. In addition, excessive fat or fatty acids may also affect insulin resistance through the following mechanisms: (1) Stimulation of the IKK and JNK signaling pathway[17]. IKK and JNK can cause insulin resistance by promoting aberrant serine phosphorylation of insulin receptor substrates 1 (IRS-1) and IRS-2, which in turn inhibit insulin receptor

signaling. Fatty acids combined with TLR activate IKK and JNK downstream, leading to insulin resistance; (2) Activation of protein kinase C (PKC). Obese diabetic rats have insulin resistance and likely also have elevated free fatty acids (FFAs). Data shows that hepatic PKC activity is greater in obese diabetic rats than in lean rats[92], suggesting that FFAs induce hepatic insulin resistance through the activation of PKC. PKC causes insulin resistance by increasing aberrant serine phosphorylation of IRS-1 and IRS-2 and by activation of the IKK and JNK signaling pathways[17]; (3) Formation of ROS. Oxidation of fatty acids generates ROS, which can activate IKK and JNK[93] to induce insulin resistance; and (4) Endoplasmic reticulum (ER) stress. Excessive fat can promote ER stress. which can increase the JNK-dependent serine phosphorylation of IRS-1 to inhibit insulin receptor signaling[94].

CONCLUSION

NAFLD has become a very common liver disease worldwide. However, the pathogenesis of NAFLD remains unclear. In recent years, hepatologists have studied the roles of liver innate immune cells in the development of NAFLD. However, to our best knowledge, no paper has provided a comprehensive review regarding the roles of liver innate immune cells in the development of NAFLD. Therefore, we reviewed prior studies and provide a holistic framework concerning the relationship between liver innate immune cells and NAFLD. The liver contains a large number of innate immune cells, which are associated with the pathogenesis of NAFLD. Serum fatty acids, adipose tissue-derived cytokines and gut-derived endotoxin could affect liver innate immune cells, and different types of liver innate immune cells affect each other, together leading to the functional abnormalities seen in fatty liver diseases. The Th1 cytokine excessive production in NAFLD results in hepatic insulin resistance and NASH. Activated KC-derived ROS also plays an important role in the development of NAFLD. Additionally, activated NKT cells can directly induce hepatocyte injury. Further studies on the effects and mechanisms of liver innate immune cells in NAFLD will help us better understand the pathogenesis of NAFLD and identify novel targets for the prevention and treatment of NAFLD.

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ORIGINAL ARTICLE

Endoscopic features and prognoses of mantle cell lymphoma with gastrointestinal involvement

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Abstract

AIM: To evaluate the endoscopic manifestations and prognoses of gastrointestinal (GI) mantle cell lymphoma (MCL).

METHODS: A database search at the Department of Pathology of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences revealed 57 MCL patients with GI involvement. Clinical records were available for 35 of the 57 patients from 21 institutions, and those 35 patients were enrolled in this study. We summarized the gross types of endoscopic features, event-free survival (EFS), and overall survival (OS) of those patients.

RESULTS: Of the 35 patients, GI involvement in the esophagus, stomach, and duodenum was found in 2 (5.7%), 26 (74.3%), and 12 (34.3%) patients, respectively. Twenty-one of the 35 patients underwent colonoscopy; among them, GI involvement in the ileum, cecum, colon, and rectum was found in 10 (47.6%), 3 (14.3%), 12 (57.1%), and 10 (47.6%), respectively. Various lesions, such as superficial, protruded, fold thickening, or ulcerative, were found in the stomach, whereas multiple lymphomatous polyposis (MLP) was dominant from the duodenum to the rectum. Twelve patients were treated with a hyper-CVAD/MA regimen, and they had better OS (3-year rate, 88.3% *vs* 46.4%, $P < 0.01$) and better EFS (3-year rate, 66.7% *vs* 33.8%, $P < 0.05$) than the remaining 23 patients who were not treated with this regimen.

CONCLUSION: MLP was a representative form of intestinal involvement, whereas a variety of lesions were found in the stomach. The hyper-CVAD/MA regimen may improve survival in these patients.

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Key words: Chemotherapy; Gastrointestinal lymphoma; Mantle cell lymphoma; Multiple lymphomatous polyposis; Non-Hodgkin's lymphoma

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INTRODUCTION

Mantle cell lymphoma (MCL) is a small B-cell neoplasm that may affect the gastrointestinal (GI) tract[1]. In 1961, Cornes[2] reviewed 22 case reports of multiple lymphomatous polyposis (MLP), and introduced that term as a unique form of malignant GI lymphoma. Two decades later, studies revealed that the neoplastic cells of MLP originate from the mantle zone of the lymphoid follicle in most cases, hence these days MLP is a representative intestinal manifestation of MCL[3]. On the other hand, the gastric lesions of MCL vary from pale folds[2,4] to nodules and inflammation[5,6]. Previously, the frequency of GI involvement was reported to be up to 30%[7,8]. However, recent reports based on endoscopic examinations have revealed that 46%-49% of MCL patients had esophagogastroduodenal involvement, and that 38%-62% had colorectal involvement[5,6]. As these previous reports were conducted by hematologists, the endoscopic findings and data on frequency, particularly of the gastric lesions, have not yet been investigated thoroughly.

Importantly, MCL is clinically more aggressive and has a shorter median survival, only 3-5 years, compared with other types of small B-cell neoplasms, such as extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) and follicular lymphoma. However, due to the rarity of MCL (it represents only about 3%-10% of non-Hodgkin lymphomas)[1], the prognoses of cases with GI MCL have not been discussed sufficiently.

In this report, we identified 35 MCL patients with GI involvement from 21 institutions and summarized their endoscopic manifestations, chemotherapy regimens, and prognoses.

MATERIALS AND METHODS

A database search at the Department of Pathology of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences found 57 patients who were histologically diagnosed as MCL with GI involvement from August 1999 to July 2009. The diagnosis of MCL was made according to the World Health Organization classification[9]. Briefly, histological diagnosis was made by morphologic and immunophenotypic analyses on surgically resected specimens or on endoscopically biopsied specimens[1]. All cases were blindly reviewed and the diagnosis was confirmed by a single pathologist (Morito T). The typical features of MCL were monomorphic proliferation of small to medium-sized lymphoid cells with a vaguely nodular, diffuse, or mantle zone growth pattern accompanying architectural destruction and the expression of cyclin D1 on immunohistochemical study (Figure 1). CD5 staining was also performed, and its positivity supported the diagnosis of MCL. All samples were also positive for BCL2 protein. Clinical records were available for 35 of the 57 patients from 21 institutions, and those 35 patients were enrolled in this study. Nine of the 35 patients were examined as subjects of our previous study[10]. For all patients, the results of endoscopic, radiological, and biological examinations, as well as clinical information including treatment regimens and prognoses, were retrospectively reviewed from clinical records.

Invaded GI organs were evaluated by esophagogastroduodenoscopy and colonoscopy. Neither capsule endoscopy nor double-balloon/single-balloon enteroscopy was performed to evaluate the jejunum and ileum. The GI tract was subdivided into three parts: the esophagus, the stomach, and the intestines (including the duodenum, ileum, cecum, colon, and rectum). GI lesions in each part were classified into the following six subtypes by gross findings: (1) the protruded type (solitary or fewer than 10 elevated lesions forming tumorous nodules; these lesions often resemble submucosal tumors and sometimes accompany ulcers on their tops); (2) the fold thickening type (thickened mucosal folds like large cerebroid folds; typically seen only in the stomach); (3) the MLP type (multiple micropolyps with or without some large polyps; the number of polyps is 10 or more); (4) the ulcerative type (solitary or multiple lowered lesions due to ulcers); (5) the superficial type (changes in the mucosal color and/or changes in mucosal morphology); and (6) the mixed type (combinations of these five subtypes). All cases were reviewed and their subtypes were classified by at least two board certified endoscopists (Iwamuro M and Okada H).

The Lugano staging system for the classification of GI tract lymphoma[11,12] was used to determine the patients' clinical stages. Patients were diagnosed with primary GI MCL based on a set of criteria established by Dawson *et al*[13]. The response evaluation included a physical examination, a complete blood count, serum biochemistry profile, endoscopic examination, bone marrow aspirate and biopsy, chest X-ray, abdominal ultrasound, and computed tomography scans of the neck, chest, and abdomen. A complete response (CR), partial remission, stable disease, and progressive disease were defined according to the International Lymphoma Workshop response criteria[14]. Overall survival (OS) was measured from diagnosis until death from any cause, and event-free survival (EFS) was measured from diagnosis until documented progression/relapse, death from any cause, or off-protocol treatment for any reason.

Therapeutic regimens, international prognostic index (IPI)[15], a recently introduced prognostic index for advanced-stage MCL (MCL IPI score: MIPI score)[16], and mitotic rates in the histological specimens were investigated as predictive prognostic factors[17,18]. The MIPI score was calculated as follows: [0.03535 × age (years)] + 0.6978 (if the Eastern Cooperative Oncology Group performance status[19] > 1) + [1.367 × log10 (LDH/upper limits of normal LDH)] + [0.9393 × log10 (white blood cell count per 10^{-6} L)]. Patients were classified as low risk (score < 5.7), intermediate risk (5.7 ≤ score < 6.2), or high risk (score ≥ 6.2) according to their MIPI scores, as previously reported[16]. To estimate the mitotic rate, Ki-67 staining was performed. Positivity of 0%-10% was classified as a low mitotic rate, while more than 10% positivity was classified as high. Cox proportional hazards regression analysis was used to ana-

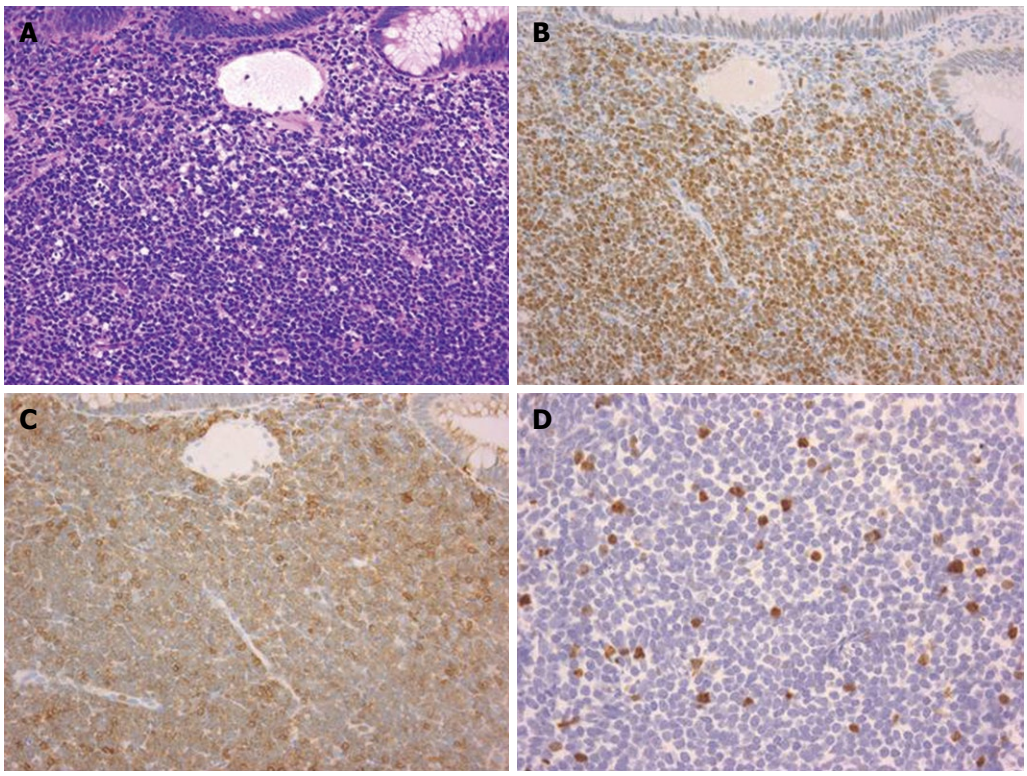

Figure 1 Histological features of mantle cell lymphoma. A: Rectal biopsied specimen demonstrated monomorphic proliferation of medium-sized lymphoid cells (Hematoxylin and eosin staining). This case illustrated typical immunohistochemical features of mantle cell lymphoma, showing positivity for cyclin D1 and CD5 staining; B: Immunohistochemical staining for cyclin D1; C: Immunohistochemical staining for CD5; D: Approximately 5% of neoplastic cells were positive for Ki-67 staining; consequently, this case was classified as a low mitotic rate. A-C: Original magnification, × 200; D: × 400.

lyze the prognostic factors. Factors exhibiting significant values in the univariate analysis were further analyzed by multivariate analysis. Kaplan-Meier curves were generated for OS and EFS. We compared the curves for the two groups with the log-rank test. Statistical analyses were performed by JMP 8.0.1 software (SAS Institute, Cary, NC, USA), and $P < 0.05$ was considered significant.

RESULTS

Clinical features

Thirty-five patients (32 male, 3 female) were enrolled; their characteristics are summarized in Table 1. The median age at diagnosis was 67 years (range: 47-86 years). Twenty-nine of 35 patients (82.9%) were Lugano stage Ⅳ, whereas only one patient was stage I with gastric involvement. Only this case fulfilled Dawson's criteria for primary GI MCL[13]. Essentially all the patients in this study had GI lesions. Other extranodal sites involved were bone marrow (n = 12), spleen (n = 5), liver (n = 3), kidney (n = 3), Waldeyer's ring (n = 3), peripheral blood (n = 3), skin (n = 2), tongue (n = 1), and ureter (n = 1).

Table 1 Characteristics of the 35 patients

	n (%)
Male sex	32 (91.4)
Median age (range, yr)	67 (47-86)
Lugano staging system	
Stage Ⅰ	1
Stage Ⅱ-1	1
Stage Ⅱ-2	4
Stage Ⅳ	29
Involved site of gastrointestinal tract	
Esophagus	2/35 (5.7)
Stomach	26/35 (74.3)
Duodenum	12/35 (34.3)
Ileum	10/21 (47.6)
Cecum	3/21 (14.3)
Colon	12/21 (57.1)
Rectum	10/21 (47.6)
Mitotic rate (Ki-67 index) (n = 19)	
Low mitotic rate	9
High mitotic rate	10
MIPI score (n = 31)	
Average score (range)	6.01 (5.31-6.85)
Low risk	6
Intermediate risk	15
High risk	10

MIPI: Mantle cell lymphoma international prognostic index.

Endoscopic features

All patients underwent esophagogastroduodenoscopy, and 21 of the 35 patients underwent colonoscopy. Of the 35 patients, GI involvement in the esophagus, stomach, and duodenum was found in 2 (5.7%), 26 (74.3%), and 12 (34.3%) patients, respectively. Among the 21 patients who also received colonoscopy, 10 (47.6%), 3 (14.3%), 12 (57.1%), and 10 (47.6%) patients showed GI involvement in the ileum, cecum, colon, and rectum, respectively

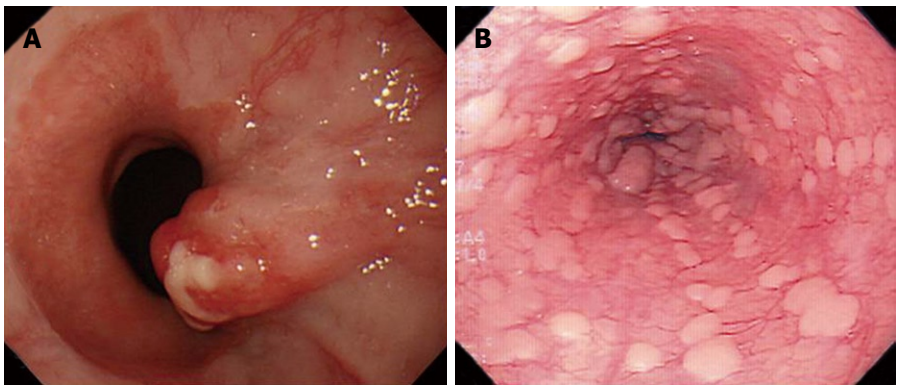

Figure 2 Esophageal lesions of mantle cell lymphoma. A: A protruded tumor was seen in the esophagogastric junction; B: In another patient, multiple whitish plaques were seen throughout the whole esophagus.

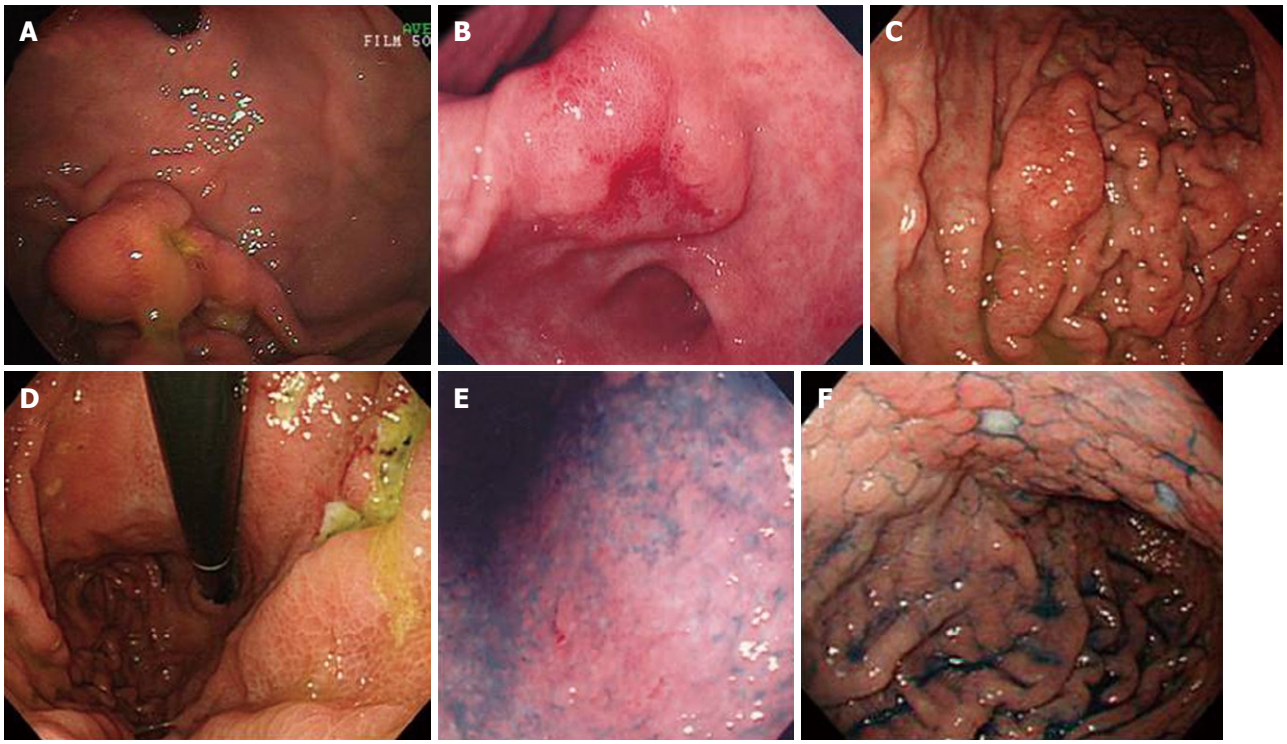

Figure 3 Gastric lesions of mantle cell lymphoma. A: Protruded type (n = 6) often resembled submucosal tumor; B: One patient with a protruded type lesion was diagnosed with stage I primary gastrointestinal mantle cell lymphoma; C: Fold thickening type (n = 6); D: Ulcerative type (n = 6); E: In one patient, only a change in mucosal color with redness was seen. This case was classified as the superficial type (n = 7); F: Among superficial type lesions, cobblestone-like mucosa with a few shallow ulcers was also seen.

Table 2 Gross findings of the gastrointestinal tract

	Esophagus (n = 2)	Stomach (n = 26)	Intestines (n = 22)
Protruded	1	6	4
Fold thickening	-	6	-
MLP	-	-	17
Ulcerative	-	6	-
Superficial	1	7	1
Mixed	-	1	-

The intestines include the duodenum, ileum, cecum, colon, and rectum. MLP: Multiple lymphomatous polyposis.

(Table 1). Twenty-two patients had at least one site of involvement in the intestines (from duodenum to rectum). Endoscopic features are summarized in Table 2. Gross findings of the esophageal lesions were the protruded type in one patient and the superficial type in another. The former lesion was a solitary nodule in the esophagogastric junction, and measured about 7 mm in diameter (Figure 2A). The latter lesion had a unique form showing slightly elevated multiple white plaques resembling glycogenic acanthosis (Figure 2B). The number of these plaques increased 13 mo after the initial endoscopy, and a biopsied specimen revealed infiltration by MCL cells. Gastric lesions varied morphologically: the superficial type was found in 7 cases (26.9%), the protruded type in 6 (23.1%), the fold thickening type in 6 (23.1%), the ulcerative type in 6 (23.1%), and the combined (protruded and ulcerative) type in 1 (3.8%) (Figure 3). In stark contrast to

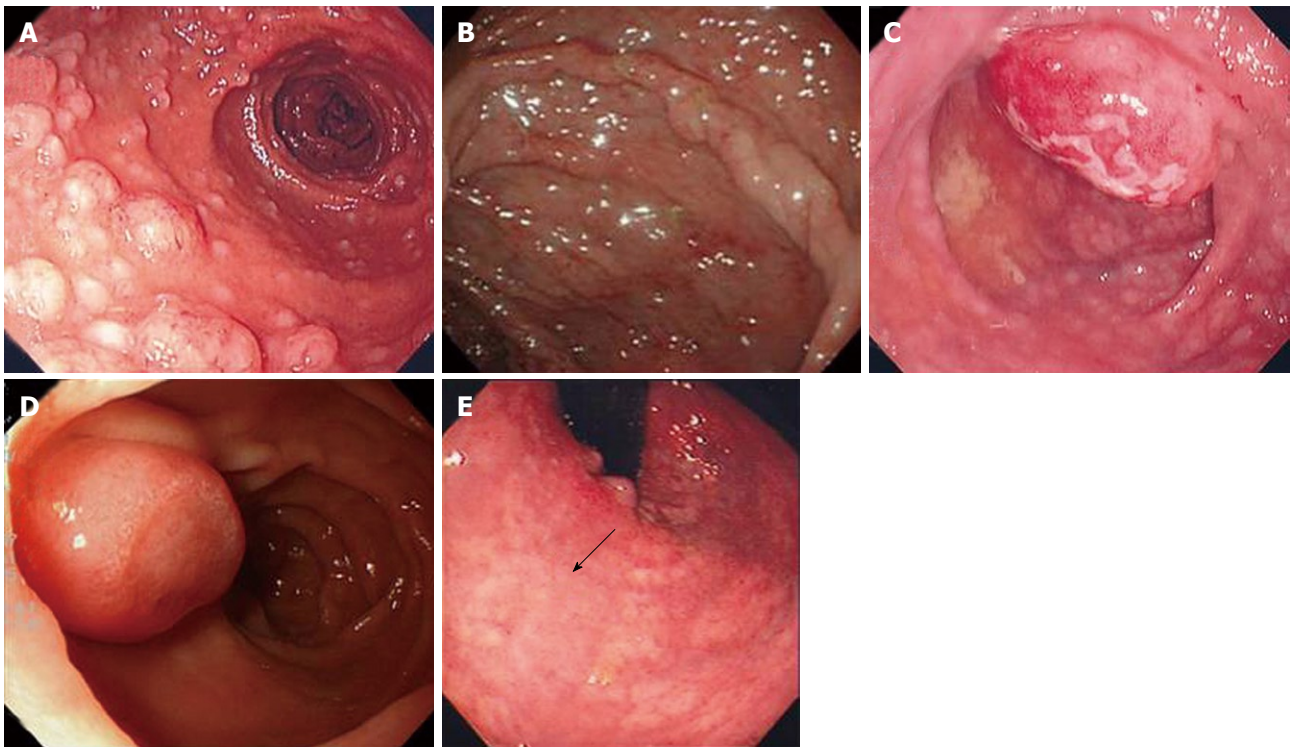

Figure 4 Intestinal lesions of mantle cell lymphoma. From the duodenum to the rectum, multiple lymphomatous polyposis (MLP) was dominant (*n* = 17). A, B: Typical features of MLP were diffuse multiple micropolyps; C: Some large tumorous polyps were sometimes found together with micropolyps; D: Protruded type (*n* = 4); E: Superficial type (*n* = 1, arrow).

the morphological variety of the gastric lesions, MLP was dominant in the intestines; it was identified in 17 of the 22 cases (77.3%). The remaining patients showed protruded type lesions in 4 (18.2%) and the superficial type in 1 (4.5%) (Figure 4).

Prognosis and therapeutic regimens

Various regimens were employed for treatment of the 35 patients, because they were treated at 21 different institutions. CHOP-like regimens, which included CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) and THP-COP (pirarubicin, cyclophosphamide, vincristine, and prednisone), were used for 16 patients, and rituximab was also administered to 10 of those 16 patients. The hyper-CVAD/MA regimen (fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone, alternating with high doses of methotrexate and cytarabine) was used for 12 patients, and rituximab was added to this regimen in 7 of the 12 patients. In the hyper-CVAD/MA regimen, cycles 1, 3, 5, 7 (Hyper-CVAD) consisted of cyclophosphamide (300 mg/m^2, iv every 12 h for 6 doses) on Days 1-3, doxorubicin (16.6 mg/m^2 per day, iv continuous infusion for 72 h) on Days 4-6, vincristine (1.4 mg/m^2 iv, maximum 2 mg) on Days 4 and 11, and dexamethasone (40 mg/d, iv or orally) on Days 1-4 and Days 11-14. Cycles 2, 4, 6, 8 (MA) consisted of methotrexate (200 mg/m^2, iv) on Day 1, methotrexate (800 mg/m^2, iv continuous infusion for 22 h) on Day 1, and cytarabine (3000 mg/m^2, reduced to only 1000 mg/m^2 if age > 60 years or creatinine > 1.5 mg/dL, iv every 12 h for 4 doses) on Days 2-3. Cycles 1 and 2 were alternated every 21 d. After the hyper-CVAD/MA regimen, high-dose chemotherapy with autologous peripheral blood stem cell transplantation (PBSCT) was performed in 8 patients. Allogeneic PBSCT was carried out in one patient for relapse after the hyper-CVAD/MA regimen.

For OS, only the therapeutic regimen (hyper CVAD/MA regimen *vs* other treatment) had a significant impact in the univariate Cox regression analysis (Table 3). For EFS, neither sex, Lugano stage (Ⅰ and Ⅱ *vs* Ⅲ and Ⅳ), LDH levels, white blood cell count, bone marrow involvement, Ki-67 positivity, IPI (high risk *vs* other), nor MIPI score showed prognostic relevance in the univariate analysis. In contrast, age and therapeutic regimen (hyper CVAD/MA regimen *vs* other treatment) had a significant impact on EFS (Table 4), whereas in the multiple Cox regression, neither age nor the therapeutic regimen was of prognostic relevance (Table 5).

Survival curves are shown in Figure 5. Median OS had not been reached after a median follow-up of 33.8 mo, and the 3-year OS rate was 61.1%. Median EFS was 15.2 mo, and the 3-year EFS rate was 44.4%. As shown in Figure 5, patients treated with the hyper-CVAD/MA regimen had both markedly longer OS and longer EFS than the group without hyper-CVAD/MA; the 3-year OS rate and 3-year EFS rate of the patients treated with hyper-CVAD/MA were 83.3% and 66.7%, respectively, whereas those of the patients without hyper-CVAD/MA were 46.4% and 33.8%. The differences between the two groups were statistically significant.

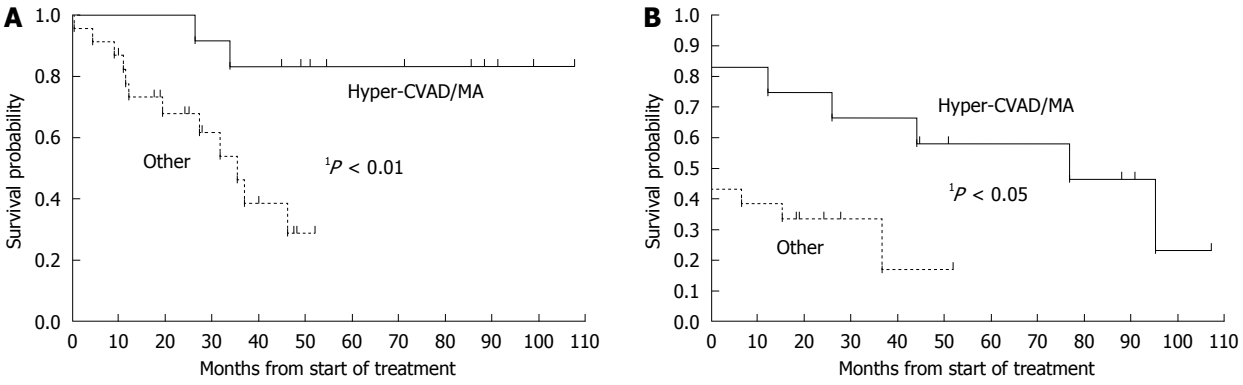

Figure 5 Overall survival (A) and event-free survival (B) according to administration of the hyper-CVAD/MA regimen. [1]Log-rank test.

Table 3 Prognostic relevance of overall survival according to the univariate Cox regression

Prognostic factor	Comparison	*n*	RR	95% LCL	95% UCL	*P*
Age (yr)	≥ 60 *vs* < 60	35	3.00	0.81	19.42	0.107
Sex	Female *vs* male	35	3.42	0.53	12.88	0.167
LDH	Elevated *vs* normal	32	1.72	0.57	4.97	0.324
WBC count	Elevated *vs* normal	32	1.32	0.36	4.06	0.649
Stage	Ⅲ and Ⅳ *vs* Ⅰ and Ⅱ	32	0.34	0.07	2.37	0.237
Bone marrow involvement	Present *vs* absent	35	1.21	0.40	3.51	0.720
Ki-67 positivity	High *vs* low	19	1.17	0.19	8.91	0.863
IPI	High risk *vs* other	32	0.49	0.03	2.55	0.450
MIPI	Other *vs* low risk	31	1.38	0.35	9.04	0.674
MIPI	High risk *vs* other	31	1.12	0.29	3.73	0.857
Treatment	Other *vs* hyper-CVAD/MA	35	6.18	1.64	40.21	0.005

RR: Relative risk; LCL: Lower confidence limit; UCL: Upper confidence limit; IPI: International prognostic index; MIPI: Mantle cell lymphoma international prognostic index; WBC: White blood cell; LDH: Lactate dehydrogenase.

Table 4 Prognostic relevance of event-free survival according to the univariate Cox regression

Prognostic factor	Comparison	*n*	RR	95% LCL	95% UCL	*P*
Age (yr)	≥ 60 *vs* < 60	35	5.27	1.47	33.77	0.007
Sex	Female *vs* male	35	2.67	0.61	8.39	0.170
LDH	Elevated *vs* normal	32	1.64	0.67	3.88	0.274
WBC count	Elevated *vs* normal	32	1.53	0.57	3.76	0.377
Stage	Ⅲ and Ⅳ *vs* Ⅰ and Ⅱ	32	0.83	0.27	3.60	0.772
Bone marrow involvement	Present *vs* absent	35	1.24	0.49	2.95	0.639
Ki-67 positivity	High *vs* low	19	0.62	0.16	2.19	0.459
IPI	High risk *vs* other	32	1.07	0.25	3.24	0.918
MIPI	Other *vs* low risk	31	2.54	0.72	16.09	0.164
MIPI	High risk *vs* other	31	0.75	0.24	1.98	0.580
Treatment	Other *vs* hyper-CVAD/MA	35	2.81	1.02	9.25	0.044

RR: Relative risk; LCL: Lower confidence limit; UCL: Upper confidence limit; IPI: International prognostic index; MIPI: Mantle cell lymphoma international prognostic index; WBC: White blood cell; LDH: Lactate dehydrogenase.

Table 5 Prognostic relevance of event-free survival according to the multiple Cox regression

Prognostic factor	Comparison	RR	95% LCL	95% UCL	*P*
Age (yr)	≥ 60 *vs* < 60	4.17	0.95	29.25	0.059
Treatment	Other *vs* hyper-CVAD/MA	1.51	0.52	5.5	0.474

RR: Relative risk; LCL: Lower confidence limit; UCL: Upper confidence limit.

DISCUSSION

In our patients, the gastric lesions varied in form; superficial, protruded, fold thickening, and ulcerative lesions appeared equally. On the other hand, intestinal involvement showed a clear predominance of MLP. Cornes described gastric lesions as pale folds, like the convolutions of the brain, with some larger lobules standing out like solitary tumors[2]. Ruskoné-Fourmestraux *et al*[4] also characterized gastric lesions as having large cerebroid folds; this is equal

to the fold thickening type in our study. Romaguera *et al*[5] reported that the most frequent abnormal findings in the upper GI tract in MCL cases were nodules and inflammation. A recent report by Salar *et al*[6] described 3 cases of gastric lesions, with antral gastritis in 2 and pangastritis in 1. Thus, the gastric involvement of MCL shows diverse forms in endoscopic examinations.

In this study, esophageal, gastric, and intestinal lesions were found by endoscopic examinations in 2 (5.7%), 26 (74.3%), and 22 (62.9%) patients, respectively. The prevalence of gastric lesions seems to be higher than the reported incidence of 46%-49%, whereas the prevalence of intestinal lesions is compatible with the reported rate of 38%-62%[5,6]. Importantly, histological evidence of MCL involvement reportedly exists in most cases even those with endoscopically intact mucosa[5,6]. Therefore, random biopsies by endoscopy at the time of diagnosis will probably reveal a higher incidence of GI involvement in MCL patients. Nevertheless, the impact of microscopic involvement in the outcome of MCL patients has not yet been elucidated[6].

MALT lymphomas and follicular lymphomas are classified as small B-cell neoplasms as well as MCL. It is well known among endoscopists that MALT lymphomas exhibit various lesions in the stomach including erosions, ulcers, polyps, protruded tumors, and swollen mucosal folds, but the involvement of other parts of the GI tract, such as the small intestine, colon, and rectum, is uncommon[20-23]. Follicular lymphomas often arise in the duodenum around the ampulla of Vater with multiple whitish granules, and they rarely form bulky masses or ulcers[24]. Endoscopists should bear in mind that MALT lymphomas and follicular lymphomas can affect the entire GI tract and even form MLP[25-27]. Thus, evaluation of the entire GI tract enables endoscopists to discriminate MCL from other small B-cell neoplasms, although a differential diagnosis is sometimes difficult and requires immunostaining for CD5, CD10, cyclin D1, and BCL2.

The prognosis of patients with GI MCL has not yet been thoroughly discussed. The present study revealed that the hyper-CVAD/MA regimen plus rituximab and PBSCT is effective for MCL patients with GI involvement as well as for systemic MCL. The prognosis of MCL patients is poorest among those with B-cell lymphoma[28]. Conventional chemotherapeutic regimens such as CHOP, with or without rituximab, obtained only short (less than 2 years) remission periods, despite a high remission rate (75%-96%)[29-32]. To overcome the unfavorable outcomes of CHOP-like regimens, a hyper-CVAD/MA regimen was established as an effective cytoreductive regimen for MCL patients[33,34]. An excellent response rate, over 90%, and a CR rate of 38%-68% were achieved by this intensified initial chemotherapy. Additionally, rituximab in combination with hyper-CVAD/MA has augmented CR rates to 87%[35]. High-dose chemotherapy with PBSCT after hyper-CVAD/MA with or without rituximab could extend the CR period and achieve 3-year OS of 72%-92%[33,36]. In this study, the hyper-CVAD/MA regimen exhibited a superior response and higher rates of survival among MCL patients over other regimens. Therefore, this regimen is a promising therapeutic option for MCL patients with GI involvement as well as for systemic MCL. We believe the hyper-CVAD/MA regimen plus rituximab and PBSCT should be administered to MCL patients with GI involvement who are young (< 60-65 years) and fit (no relevant co-morbidity).

A small subset of patients with MCL may show indolent behavior and have extended survival even with little or no treatment[28]. To identify this subset of patients and classify MCL patients according to their prognoses, several researchers have attempted to establish a prognostic index. Ki-67 positivity, which represents cell proliferation, has been reported as a predictor; high mitotic rates were associated with adverse prognoses[17,18]. The recently introduced MIPI scoring system successfully differentiated OS based on four independent prognostic factors: age, performance status, LDH, and leukocyte count[16]. In this study, however, Ki-67 positivity and MIPI score failed to classify our patients' prognoses. We speculate that any one of several factors may explain this. First, MCL patients with GI involvement might have different prognoses from all other MCL patients. In this study, all but 3 patients were at advanced stages (Ⅱ-2 or Ⅳ). GI involvement of MCL might represent a more advanced disease status, but the impact of these GI findings on the outcomes of MCL patients is not known[6]. Second, because this study was retrospective and the treatment regimens administered to patients were not uniform, the non-uniform backgrounds might have distorted analysis. Further investigation concerning the prognosis of MCL with GI involvement is required in a study using a larger patient group.

In conclusion, 35 cases of MCL with GI involvement were included in this report. Esophageal, gastric, and intestinal lesions were identified in 2 (5.7%), 26 (74.3%), and 22 (62.9%) patients, respectively. MLP was a representative form of intestinal involvement, whereas a variety of lesions were found in the stomach. Prompt diagnosis based on the above findings and administration of initial cytoreductive chemotherapy, such as the hyper-CVAD/MA regimen followed by PBSCT, may improve the survival of MCL patients.

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COMMENTS

Background

Mantle cell lymphoma (MCL) is clinically more aggressive and has a shorter median survival, only 3-5 years, as compared with other types of small B-cell neoplasms. MCL can affect the gastrointestinal (GI) tract and its frequency is reportedly up to 60% in patients undergoing endoscopic examinations. However, the prognosis of MCL patients with GI involvement has not yet been revealed in detail.

Research frontiers

Recently, the hyper-CVAD/MA regimen was established as an effective cytoreductive regimen for MCL patients. An excellent response rate, over 90%, and a complete remission (CR) rate of 38%-68% were achieved by this intensified initial chemotherapy. Additionally, rituximab in combination with hyper-CVAD/MA has augmented the CR rates to 87%. High-dose chemotherapy with autologous peripheral blood stem cell transplantation (PBSCT) after hyper-CVAD/MA with or without rituximab could extend the CR period and achieve 3-year overall survival (OS) of 72%-92%.

Innovations and breakthroughs

Patients treated with the hyper-CVAD/MA regimen had both markedly longer OS and longer event-free survival (EFS) than the group without hyper-CVAD/MA; the 3-year OS rate and 3-year EFS rate of the patients treated with hyper-CVAD/MA were 83.3% and 66.7%, respectively, whereas those of the patients without hyper-CVAD/MA were 46.4% and 33.8%, respectively. The differences between the two groups were statistically significant.

Applications

Hyper-CVAD/MA regimen plus rituximab and PBSCT is effective for MCL patients with GI involvement as well as for systemic MCL. The hyper-CVAD/MA regimen plus rituximab and PBSCT should be administered to MCL patients with GI involvement who are young (< 60-65 years) and fit (no relevant comorbidity).

Terminology

In the hyper-CVAD/MA regimen, cycles 1, 3, 5, 7 (Hyper-CVAD) consisted of cyclophosphamide, doxorubicin, vincristine, and dexamethasone. Cycles 2, 4, 6, 8 (MA) consisted of methotrexate and cytarabine. Cycles 1 and 2 were alternated every 21 d. Rituximab is a chimeric monoclonal antibody against the protein CD20, which is primarily found on the surface of B cells. Rituximab is used for the treatment of many lymphomas, leukemias, and some autoimmune disorders.

Peer review

This is a multi-institutional retrospective review of 35 MCLs of the GI tract. Its strength is the multiple institutions that patients were treated allowing for comparison of different therapeutic regimens.

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ORIGINAL ARTICLE

Up-regulation of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in sentinel lymph nodes of colorectal cancer

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Abstract

AIM: To investigate the early metastasis-associated proteins in sentinel lymph node micrometastasis (SLNMM) of colorectal cancer (CRC) through comparative proteome.

METHODS: Hydrophobic protein samples were extracted from individual-matched normal lymph nodes (NLN) and SLNMM of CRC. Differentially expressed protein spots were detected by two-dimensional electrophoresis and image analysis, and subsequently identified by matrix assisted laser desorption/ionization-time of flight mass spectrometry-mass spectrometry and Western blotting, respectively.

RESULTS: Forty proteins were differentially expressed in NLN and SLNMM, and 4 metastasis-concerned proteins highly expressed in SLNMM were identified to be hnRNP A1, Ezrin, tubulin β-2C and Annexin A1. Further immunohistochemistry staining of these four proteins showed their clinicopathological characteristics in lymph node metastasis of CRC.

CONCLUSION: Variations of hydrophobic protein expression in NLN and SLNMM of CRC and increased expression of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in SLNMM suggest a significantly elevated early CRC metastasis.

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Key words: Colorectal cancer; Micrometastasis; Proteomics; Sentinel lymph node

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INTRODUCTION

At present, colorectal cancer (CRC) is the third most common cause of cancer-related death worldwide[1]. Its incidence in China has increased rapidly during the past few decades[2]. Since CRC metastasis has a great effect on the survival of its patients and selection of its treatment mo-

dalities, it is therefore important to understand the molecular basis of metastasis in order to develop better preventive and therapeutic procedures. CRC development is a multistep process that spans 10-15 years, with different proteins involved in different steps[3], it is thus of great significance to find out the proteins involved in micrometastasis for early detection and treatment of CRC.

Sentinel lymph nodes (SLN) provide the primary lymphatic drainage of a tumor, thus metastatic cancer cells first spread into the lymph nodes. It has been shown that the prognosis of CRC patients is related to sentinel lymph node micrometastasis (SLNMM)[4,5]. SLN techniques, such as SLN biopsy[6-8] and SLN mapping[9-11], have been used in diagnosis of CRC and can better stage CRC than standard HE analysis. Since SLN is the most intensively exposed to bioactive tumor cell products, it is important to know which proteins play a role in micrometastasis. Therefore, detection of differentially expressed proteins in SLNMM is of great significance in understanding the molecular mechanism underlying early CRC metastasis.

Comparative proteome techniques allow the characterization of global alterations in protein expression during cancer development and has been widely used in many kinds of tumors, including CRC[12]. Current studies on proteomics in CRC are mainly focused on comparison between primary CRC foci, normal tissue, and distant metastasis[13-16], or between different tumor cell lines[17,18], but the technology has not yet been used in comparison between SLNMM and normal lymph nodes (NLN). In this study, the technique was used to identify the differentially expressed proteins in SLNMM in order to find out the early metastasis-associated proteins in CRC.

MATERIALS AND METHODS

Tissue sample collection

Forty-three cases of moderately differentiated colorectal adenocarcinoma (24 males and 19 females) at the age of 39-80 years (mean ± SD = 51.2 ± 12.6 years), who underwent operation from January 2007 to January 2008, were randomly collected from Department of General Surgery, Second Affiliated Hospital of Nanjing Medical University, China. Endoscopic ultrasonography was carried out 1 d before operation to identify the invasion extent and 0.1% isosulfan blue was injected circumferentially around the neoplasm to mark SLN[19].

A set of lymph nodes were collected during operation and stained with HE and cytokeratin-20 immunohistochemistry (CK-IHC) immediately by two experienced pathologists. Based on HE staining and CK-IHC, the lymph nodes were divided into NLN and SLNMM. All samples were snap frozen in liquid nitrogen and stored at -80℃ until further analysis. All patients recruited in this study received neither chemotherapy nor radiotherapy before surgery. Permission for this study was obtained from the Ethics Committee of Second Affiliated Hospital of Nanjing Medical University. All specimens were anonymous and handled according to the ethical and legal standards.

Protein sample preparation

Protein was extracted from 50 mg of frozen tissue by homogenization in lysis buffer containing 4% CHAPS, 2 mol/L thiourea, 7 mol/L urea, 2% NP-40, 1% Triton X-100, 100 mmol/L DTT, 5 mmol/L PMSF, 0.5 mmol/L EDTA, 2% pharmalyte, 1 mg/mL DNase I, 0.25 mg/mL RNase A, and 40 mmol/L tris-HCl, at pH 8.5, and incubated at room temperature for 2 h. The mixture was centrifuged at 40 000 × *g* for 1 h at 4℃. The supernatant was saved and stored at -70℃. Supernatants from 10 individual specimens corresponding to each group were pooled to minimize the individual variations, and the protein concentration in each mixed sample was measured with the bicinchoninic acid method using PBS as the standard.

Two-dimensional gel electrophoresis and image analysis

Three hundred micrograms protein of each group was loaded onto a 240 mm linear IPG strip (pH3-10, Amersham Biosciences, Piscataway, NJ) for first-dimensional isoelectric focusing. Protein separation in the second dimension SDS-PAGE (Bio-Rad, Hercules, CA) was carried out on vertical systems, IPG strips were loaded and run on a 125 g/L acrylamide SDS-PAGE gel in electrode buffer (Tris 0.025 mol/L, glycine 0.192 mol/L, SDS 1 g/L, pH8.3). Electrophoresis was performed with a current of 30 mA/gel for 15 min, followed by 60 mA/gel for 4 h. Each sample was subjected to 2D gel electrophoresis three times to avoid procedural errors. After electrophoresis, the gels were stained with silver nitrate and scanned with an Imagescanner (Amersham Biosciences). The software of PD-Quest 7.3.1 (Bio-Rad) was employed for image analysis, including background abstraction, spot intensity calibration, spot detection, and matching.

Protein identification

Differential protein spots selected were excised from 2-DE gels and cut into small pieces, which were destained, reduced and digested with trypsin overnight. Tryptic digests were extracted and analyzed in a matrix assisted laser desorption/ionization-time of flight mass spectrometry-mass spectrometry (MALDI-TOF-MS) (Bruker, Daltonics, Billerica, MA, USA). The resultant MS data were then screened against NCBInr and SWISS-PROT databases using the MASCOT search program (Matrix Science, London, UK; http://www.matrixscience.com). Protein identities were assigned if at least 4 peptide masses were matched within a maximum of 100 ppm error spread across the data set and the candidate agreed with the estimated pI and molecular weight from the 2-DE gel.

Western blotting

Tissue samples were lysed following the method for 2-DE described above. Aliquots of protein extracts (50 mg) were separated on a 12.5% SDS-polyacrylamide gel. Sub-

sequently, the protein was electrophoretically transferred onto a PVDF membrane (Bio-Rad). After blocked with TBS-Tween 20 (TBST) containing 10% skim milk, the membranes were incubated with mouse monoclonal antibodies against mouse hnRNP A1 and tubulin β-2C, and rabbit polyclonal antibodies against mouse Annexin A1 and Ezrin for 1 h, respectively, followed by peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:10000 in TBST for 1 h. Finally, blots were developed with chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA). In order to equal protein loading, blots were re-stained using anti-actin antibody (Santa Cruz Biotechnology) as a control.

Immunohistochemistry analysis

Formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated using xylene and a series of graded alcohol, respectively. Tissue sections were treated with 3% hydrogen peroxidase for 15 min at room temperature, followed by incubation overnight at 4℃ with anti-hnRNP A1 (1:50 Gmbh, Forckenbeckstr, Aachen, Germany), anti-tubulin β-2C (1:50 Saier Biotechnology Inc, Wuhan, China), anti-Annexin A1 (1:100 Saier Biotechnology Inc, Wuhan, China), and anti-Ezrin (1:50 Gmbh, Forckenbeckstr, Aachen, Germany) antibodies, respectively. Finally, the tissue sections were incubated with ready to use peroxidase-conjugated goat anti-rabbit antibody (MaiXin, Fuzhou, China), developed with diaminobenzidine as chromogen, and counterstained with hematoxylin.

Statistical analysis

Experimental data were analyzed by Student's *t*-test and χ^2 test using SPSS 10.0. $P < 0.05$ was considered statistically significant.

RESULTS

Harvesting and identification of SLNMM

A total of 62 NLN and 126 blue-stained lymph nodes from 43 patients were excised and processed. As a result, 37 and 54 blue-stained lymph nodes were considered to be SLNMM with HE staining (29.36%, Figure 1A) and CK-IHC (42.85%, Figure 1B), respectively, and at least one SLNMM was detected in each case.

Differential expression of proteome in NLN and SLNMM

Sixty-three protein spots were differentially expressed in NLN and SLNMM (Figure 2). Among the 63 protein spots, some could not be identified with incomplete polypeptide fragments, and some were too low in abundance to obtain useful data. Finally, 40 protein entries were identified by MALDI-TOF-MS analyses (Table 1). The expression was up-regulated and down-regulated in 15 and 25 protein entries, respectively.

The 15 proteins with their expression up-regulated were then grouped and classified according to their biological functions (http://www.geneontology.org/) into cytoarchitecture reorganization- concerned proteins including VCL, PDZD8, LMNA, RUVBL1, TCP11, CLIC1,

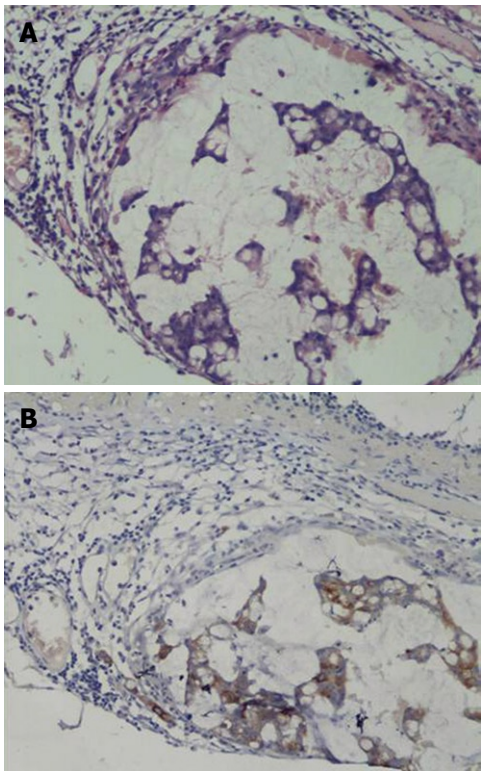

Figure 1 HE staining (A) and cytokeratin-20 immunohistochemistry (B) of sentinel lymph nodes (× 200).

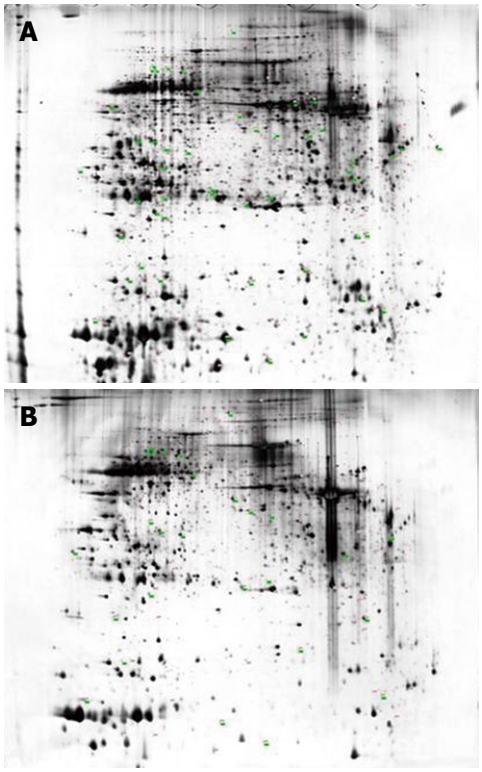

Figure 2 Representative 2-DE maps of normal lymph nodes (A) and sentinel lymph node micrometastasis (B). The numbered spots represent differentially expressed proteins.

Table 1 Identification of differentially expressed proteins in normal lymph nodes and sentinel lymph node micrometastasis of colorectal cancer

Spot ID	International protein index accession No.	Protein	Score	Sequence coverage (%)	Mr/pI
Up					
185	IPI00307162	VCL Isoform 2 of Vinculin	98	11	124292/5.50
482	IPI00168698	PDZD8 PDZ domain-containing protein 8	198	15	129681/5.78
515[1]	IPI00027834	HNRNPL heterogeneous nuclear ribonucleoprotein L isoform a	119	17	64720/8.46
501	IPI00216952	LMNA Isoform C of Lamin-A/C	190	41	65153/6.40
530	IPI00289499	ATIC Bifunctional purine biosynthesis protein PURH	252	46	65089/6.27
741	IPI00021187	RUVBL1 Isoform 1 of RuvB-like 1	133	39	50538/6.02
1071	IPI00027444	SERPINB1 Leukocyte elastase inhibitor	94	38	42829/5.90
1266[1]	IPI00007752	TUBB2C Tubulin β-2C chain	134	30	50255/4.79
1411	IPI00103433	TCP11 Isoform 1 of T-complex protein 11 homolog	108	24	56675/5.08
1449[1]	IPI00218918	ANXA1 Annexin A1	196	50	38918/6.57
1649	IPI00554767	CLIC1 18 kDa protein	96	52	17927/4.71
1650[1]	IPI00746388	EZR Ezrin	133	21	69353/5.88
1967	IPI00878282	ALB 23 kDa protein	94	36	23414/5.93
2102	IPI00220766	GLO1 Lactoylglutathione lyase	107	48	20992/5.12
2410	IPI00216691	PFN1 Profilin-1	62	40	15216/8.44
Down					
391	IPI00021405	LMNA Isoform A of Lamin-A/C	204	42	74380/6.57
509	IPI00022463	TF Serotransferrin precursor	65	19	79280/6.81
701	IPI00878282	ALB 23 kDa protein	127	52	23414/5.93
719	IPI00553177	SERPINA1 Isoform 1 of α-1-antitrypsin precursor	247	52	46878/5.37
994	IPI00027223	IDH1 Isocitrate dehydrogenase (NADP) cytoplasmic	150	50	46915/6.53
1042	IPI00021926	PSMC6 26S protease regulatory subunit S10B	85	25	44430/7.10
1204	IPI00298497	FGB Fibrinogen β chain precursor	204	46	56577/8.54
1433	IPI00455315	ANXA2 Annexin A2	130	41	38808/7.57
1442	IPI00295889	SRP19 Signal recognition particle 19 kDa protein	129	38	16374/9.87
1550	IPI00872780	ANXA4 Annexin A4	217	60	36088/5.84
1572	IPI00745868	ANXA3 Uncharacterized protein ANXA3 (Fragment)	121	41	36623/5.53
1715	IPI00394878	C1QTNF1 C1q and tumor necrosis factor related protein 1	273	68	22841/8.40
1856	IPI00003766	ETHE1 ETHE1 protein, mitochondrial precursor	124	45	28368/6.35
1863	IPI00465028	TPI1 Isoform 1 of Triosephosphate isomerase	292	80	31057/5.65
1890	IPI00025512	HSPB1 Heat shock protein β-1	86	48	22826/5.98
1919	IPI00853525	APOA1 Apolipoprotein A1	191	64	28005/5.80
1954	IPI00220766	GLO1 Lactoylglutathione lyase	107	48	20992/5.12
1961	IPI00219622	PSMA2 Proteasome subunit α type-2	275	70	25996/ 6.92
1995	IPI00003815	ARHGDIA Rho GDP-dissociation inhibitor 1	91	43	23250/5.02
2104	IPI00014832	PDK2 [Pyruvate dehydrogenase kinase isozyme 2,	152	19	51389/7.67
2237	IPI00096066	SUCLG2 Succinyl-CoA ligase [GDP-forming]	184	23	46824/6.15
2245	IPI00384679	RNF170 20 kDa protein	184	54	20773/6.40
2349	IPI00796366	MYL6B 16 kDa protein	97	51	16451/4.56
2540	IPI00295844	RP11-429E11.3 Novel protein	153	65	15089/5.49
2564	IPI00796636	HBB Hemoglobin (Fragment)	98	64	11554/5.90

[1]Over-expressed proteins in sentinel lymph nodes and their function-related metastasis of cancer cells.

ALB and PFN1, cytometabolism-concerned proteins including SERPINB1, PURH and GLO1, and metastasis-concerned proteins including hnRNP A1, Ezrin, tubulin β-2C and Annexin A1. The expression and distribution of these 4 metastasis-concerned proteins were further studied to assess the role they play in the progress of early CRC metastasis.

Detection of differentially expressed proteins by Western blotting

Western blotting showed that the expression level of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1, was significantly higher in SLNMM than in NLN (Figure 3A). The quantitation of protein bands showed that the expression level of the four proteins was about 2-fold higher in SLNMM than in NLN (Figure 3B).

Immunohistochemical analysis of proteins in NLN and SLNMM

The representative immunohistochemistry staining of different proteins in each group is shown in Figure 4. The positive expression rate was 54.8% and 69.8% respectively for hnRNP A1, 8.1% and 87.3% respectively for Ezrin, 19.3% and 74.6% respectively for tubulin β-2C, and 14.5% and 53.9% respectively for Annexin A1, in NLN and SLNMM. Statistical analysis demonstrated that the positive expression rate for the 4 proteins was significantly higher in SLNMM than in NLN (Table 2).

Furthermore, these four proteins were negatively or weakly expressed in NLN, but strongly expressed in SLNMM (Figure 4). Annexin A1 and hnRNP A1 were mainly expressed in cell nuclei and cytoplasm, and tubulin β-2C was mainly expressed in cell membrane. Ezrin was

Table 2 Expression of hnRNP A1, Ezrin, tubulin β-2C, and Annexin A1 in normal lymph nodes and sentinel lymph node micrometastasis

Group	Case	hnRNP A1			Ezrin			Tubulin β-2C			Annexin A1		
		N	P	Rate (%)	N	P	Rate (%)	N	P	Rate (%)	N	P	Rate (%)
NLN	62	28	34	54.8	57	5	8.1	50	12	19.3	53	9	14.5
SLNMM	126	38	88	69.8	16	110	87.3	32	94	74.6	58	68	53.9
χ^2 value			4.11			109.84			51.57			26.75	
P value			0.05 > *P* > 0.01			< 0.01			< 0.01			< 0.01	

NLN: Normal lymph nodes; SLNMM: Sentinel lymph node micrometastasis; N: Negative; P: Positive.

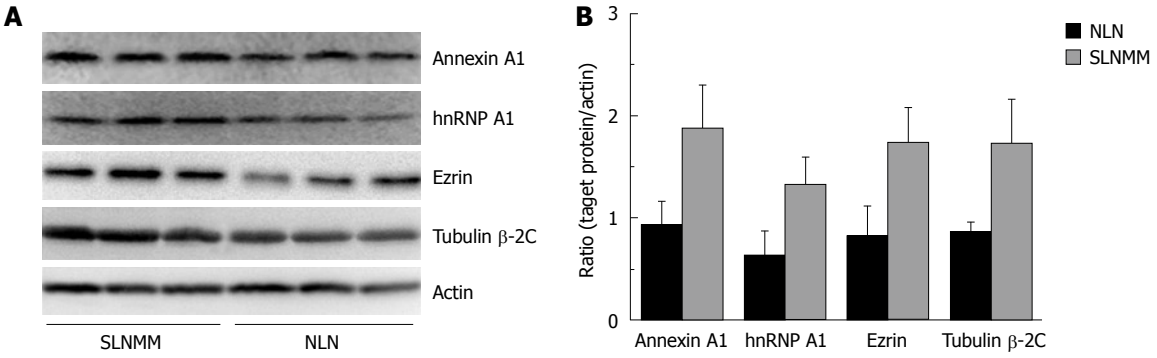

Figure 3 Western blotting (A) and quantitation of protein bands (B) showing differentially expressed hnRNP A1, Ezrin, tubulin β-2C, and Annexin A1 in normal lymph nodes and sentinel lymph node micrometastasis. NLN: Normal lymph nodes; SLNMM: Sentinel lymph node micrometastasis.

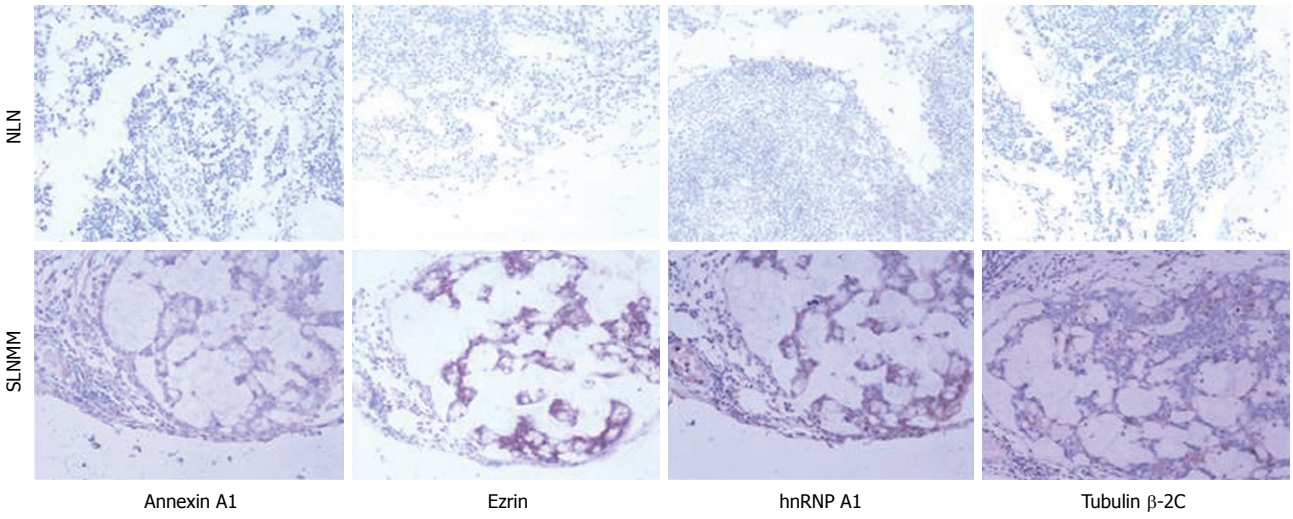

Figure 4 Immunohistochemistry analysis showing differentially expressed hnRNP A1, Ezrin, tubulin β-2C, and Annexin A1 in normal lymph nodes and sentinel lymph node micrometastasis (× 200). NLN: Normal lymph nodes; SLNMM: Sentinel lymph node micrometastasis.

enriched on cell membrane surface of SLNMM, but its distribution in cytoplasm of NLN was uniform.

DISCUSSION

The proteome approach, applied in this study is of clinical importance to identify the differentially expressed proteins in NLN and SLNMM of CRC, since these proteins can be potentially used as tumor markers and anticancer targets.

We marked SLN using isosulfan blue and identified micrometastasis of CRC with HE staining and CK-IHC. The total positive rate was 42.85%, which is consistent with the reported data[20]. A total of 40 proteins were differentially expressed in NLN and SLNMM. Of these 40 proteins, 15 were up-regulated and 25 were down-regulated. The 15 proteins with their expression up-regulation were then divided into 3 groups according to their functions. Western blotting and immunohistochemistry analysis showed that the expression and distribution of 4 metastasis-concerned proteins in NLN and SLNMM were significantly different.

The Annexins are a family of calcium-regulated phospholipid-binding proteins with a diverse role in cell biol-

ogy[21]. To date, 12 Annexins have been found in higher vertebrates. Although no exact physiological function of Annexins has been described, there is evidence that they are differentially expressed in various carcinomas. For example, expression of Annexins at mRNA and protein level is sharply up-regulated in many cancers[22,23], while some data indicate that declined expression of Annexins may play a significant role in tumorigenesis and metastasis[24]. So, the precise role of Annexin expression in pathogenesis of tumors is still unknown. In this study, Western blotting and IHC showed that the expression level of Annexin A1 was significantly higher in SLNMM than in NLN, suggesting that up-regulated expression of Annexin A1 may contribute to early CRC metastasis.

Ezrin, a membrane-cytoskeleton anchor, can affect cell adhesion and regulate tumor cell invasion and metastasis. Wang *et al*[25] reported that Ezrin expression level is obviously higher in CRC tissue than in normal colorectal mucosa tissue, which is closely related to CRC invasion and metastasis. Elzagheid *et al*[26] found that intense Ezrin immunoreactivity in cytoplasm can predict poor survival of CRC patients, thus providing clinically valuable information on the biological behavior of CRC. In this study, Ezrin was expressed on cell membrane surface or in cytoplasm, but not uniformly expressed in cytoplasm, which is consistent with the reported findings in pancreatic cancer[27], indicating that membrane translocation of Ezrin may also play an important role in early CRC metastasis.

Cell locomotion, including cancer cell invasion, is closely associated with dynamics of cytoskeletal structures. Tubulin isotype composition may affect polymerization properties and dynamics of microtubules. Portyanko *et al*[28] showed that tubulin β (Ⅲ) is associated with tumor budding grade, and changes in tubulin isotypes can modulate the invading activity of CRC cells. In our study, the expression of tubulin β-2C was about 2-fold higher in SLNMM than in NLN, and IHC showed that the staining of tubulin β-2C was weak and mostly gathered around nuclei of NLN but stronger and diffused in cytoplasm of SLNMM, suggesting that the expression and distribution of tubulin β-2C are different in NLN and SLNMM of CRC, and the increased expression of tubulin β-2C is associated with early lymph node micrometastasis, thus leading to poor prognosis of CRC.

HnRNP is most abundantly expressed in nuclear protein of mammalian cells, which is associated with pre-mRNA processing and other aspects of mRNA metabolism and transport[29]. As a class of protein family, many of its subtypes are related to the occurrence of different tumors, and hnRNP A2/B1 subtype is now used as an indicator in early diagnosis of lung cancer[30]. In our study, Western blotting and IHC showed the expression level of hnRNP A1 was higher in SLNMM than in NLN, indicating that hnRNP A1 plays an important role in the occurrence and development of CRC[31,32] and can thus be considered a potential molecular indicator/biomarker of tumorigenesis in CRC.

In summary, comparative proteomics technologies can be used in study of protein profiles in NLN and SLNMM and in identification of early CRC metastasis-related proteins. Increased expression of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in SLN suggests a significantly elevated incidence of early CRC metastasis. However, further study is needed to verify their role in therapeutic target of CRC.

COMMENTS

Background

Tumor metastasis severely affects the prognosis and therapeutic procedures of colorectal cancer (CRC), so early detection of CRC metastasis is of great significance in improving the survival rate of CRC patients. However, no effective protein indicators of early CRC metastasis are available. Sentinel lymph nodes (SLN) provide the primary lymphatic drainage of a tumor, using proteomics approach to the identification of differentially expressed proteins in SLN may be of important significance in early detection of lymph node metastasis of CRC.

Research frontiers

Comparative proteome allows the characterization of global alterations in protein expression during cancer development and has been widely used in many kinds of tumors, including CRC. Currently, studies on proteomics in CRC are mainly focused on comparison between primary CRC foci, normal tissue, and distant metastasis, or between different tumor cell lines, but the technology has not yet been used in comparison between SLN and normal lymph nodes (NLN).

Innovations and breakthroughs

Comparative proteomics technologies were used to study the protein profiles of SLN and NLN of CRC, and a number of early CRC metastasis-related proteins were identified.

Applications

Increased expression of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in SLN suggests a significantly elevated incidence of early CRC metastasis, which may contribute to the diagnosis of CRC and selection of its treatment modalities.

Peer review

Comparative proteomics technologies were used in this study to identify differentially expressed proteins in SLN, which may be of important significance in detection of early CRC metastasis.

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ORIGINAL ARTICLE

Oncolytic adenovirus SG600-IL24 selectively kills hepatocellular carcinoma cell lines

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Abstract

AIM: To investigate the effect of oncolytic adenovirus SG600-IL24 and replication-incompetent adenovirus Ad.IL-24 on hepatocellular carcinoma (HCC) cell lines and normal liver cell line.

METHODS: HCC cell lines (HepG2, Hep3B and MHCC97L) and normal liver cell line (L02) with a different p53 status were infected with SG600-IL24 and Ad.IL-24, respectively. Melanoma differentiation-associated (MDA)-7/interleukin (IL)-24 mRNA and protein expressions in infected cells were detected by reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and Western blotting, respectively. Apoptosis of HCC cells and normal liver cells was detected by cytometric assay with Hoechst33258 staining. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to investigate proliferation of HCC cells and normal liver cells, and cell cycle was assayed by flow cytometry.

RESULTS: RT-PCR, ELISA and Western blotting showed that the exogenous MDA-7/IL-24 gene was highly expressed in cells infected with SG600-IL24. MTT indicated that SG600-IL24 could suppress the growth of HepG2, Hep3B, MHCC97L, with an inhibition rate of 75% ± 2.5%, 85% ± 2.0%, 72% ± 1.8%, respectively ($P < 0.01$), promote the apoptosis of HepG2, Hep3B, MHCC97L, with an apoptosis rate of 56.59% ± 4.0%, 78.36% ± 3.5%, 43.39% ± 2.5%, respectively ($P < 0.01$), and block the HCC cell lines in the G2/M phase with a blocking rate of 35.4% ± 4.2%, 47.3% ± 6.2%, 42% ± 5.0%, respectively ($P < 0.01$) but not the normal liver cell line in a p53-independent manner.

CONCLUSION: SG600-IL24 can selectively suppress the proliferation and apoptosis of HCC cell lines *in vitro* but not normal liver cell line L02 in a p53-independent manner. Compared with Ad.IL-24, SG600-IL24 can significantly enhance the antitumor activity in HCC cell lines.

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Key words: Oncolytic adenovirus; Hepatocellular carcinoma; Cancer gene therapy; p53-independent; Melanoma differentiation-associated-7/interleukin-24

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common lethal malignant diseases in the world, is responsible for a significant number of deaths annually[1]. The HCC-associated annual morbidity and mortality rank second among all tumors[2]. In addition, the prognosis of HCC patients is poor. Gene therapy has emerged as an attractive option in treatment of HCC, and its strategies include transgenic therapy with anti-oncogenes such as p53 and Rb, anti-sense nucleotide therapy, drug gene therapy (e.g. suicide gene like HSV-TK), and tumor vaccines[3]. However, its current strategies destroy both tumor and normal cells simultaneously, thus limiting their clinical application.

Melanoma differentiation-associated (MDA)-7/interleukin (IL)-24 was identified by a combination of recombinant fibroblast interferon-β and protein kinase C activator mezerein subtraction hybridization by Fisher in 1995[4]. It has been shown that MDA-7/IL-24 can suppress the growth of melanoma, inhibit the proliferation and apoptosis of other cancer cells[5-8], and has thus been hailed as a novel and interesting development in experimental tumor therapy in the early 21st century[9]. Our previous study showed that Ad.IL-24 can selectively destruct a variety of liver cancer cells without any toxic effects and inhibit the proliferation of normal liver cell line L02. Ad.IL-24 can facilitate release of CytC and Smac from mitochondria into cytoplasm, independent of the Fas pathway[10]. MDA-7/IL-24 can also inhibit the growth and metastasis of high-transfer (HCCM6) liver xenograft tumors, with a synergistic effect when combined with doxorubicin. However, replication-defective adenovirus is limited in cancer gene therapy due to its poor targets, low transfection efficiency, low gene expression effect, and severe immune reaction. Oncolytic adenovirus, a non-defective virus, has a higher transfection efficiency because the exogenous gene can selectively replicate in tumor cells and kill tumor cells but not normal cells[11]. Besides, it combines the antitumor effect of viral vector and therapeutic gene. Therefore, we used this strategy to construct replication-competent oncolytic adenovirus SG600-IL24 carrying human MDA-7/IL-24. However, its effects on HCC cells with a different p53 status remain largely unknown. To clarify its effects, we first determined the expression of SG600-IL24 in HCC cell lines HepG2, Hep3B, MHCC97L, and normal human liver cell line L02 *in vitro*.

MATERIALS AND METHODS

Cell lines and culture conditions

HCC cell lines HepG2 and Hep3B, MHCC97L and normal human liver cell line L02 were purchased from the Institute of HCC, Fudan University (Shanghai, China). Human embryonic kidney cells (HEK 293) were a gift from Professor Qi-Jun Qian, Laboratory of Viral and Gene Therapy, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China). The cell lines were cultured in a high glucose DMEM (HyClone, USA) supplemented with 10% FBS (Gibco, USA) at 37℃ in a humidified incubator containing 5% CO_2 and 95% air. L02 was cultured in RPMI-1640 (HyClone, USA) supplemented with 10% FBS.

Virus construction, identification and purification

Oncolytic adenoviruses SG600-IL24 and SG600-EGFP were constructed and amplified. In brief, IL-24 expression cassette was released from pZD55-IL-24 and introduced into pClon9-INS to produce pClon9-INS-IL24. Using a plasmid transfection method, we co-transfected pSG600-IL24 and adenovirus skeletal plasmid pPE3 into HEK293 cells to construct the recombinant adenovirus vector SG600-IL24 carrying the MDA-7 gene. The genomes were analyzed to confirm the recombinant structure, and the virus was plaque purified and amplified in HEK293 cells. The recombinant replication-defective Ad.MDA-7 virus was constructed and amplified as previously described[11]. The titer of SG600-IL24, SG600-EGFP and Ad.IL-24 was 2.25×10^{10} PFU/mL, 2.79×10^{10} PFU/mL, and 7.1×10^{9} PFU/mL, respectively.

Virus infection

Six-well plates for each cell line were divided into control group, Ad.IL-24 group, SG600-EGFP group and SG600-IL24 group. Control group was treated with a serum-free DMEM. The cells were infected with oncolytic adenovirus with its multiplicity of infection (MOI) = 10.

Expression of MDA-7/IL-24 detected by reverse transcription-polymerase chain reaction

Cells were harvested at 24 h following SG600-IL24 infection for detection of MDA-7/IL-24 mRNA expression. Total RNA was extracted and reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described[10,12]. The sequences of primers of MDA-7 mRNA are sense: 5'-GGGCTGTGAAAGACACTAT-3', antisense: 5'-GCATCCAGGTCAGAAGAA-3'. The length of amplified fragments was 381 bp. The sequences of primers of β-actin are sense: 5'-CCTTCCTGGGCAATGGAGTCCT-3', antisense: 5'-GGAACAATGATCTTGATCTT-3'. The length of amplified fragments was 201 bp. The PCR conditions were as follows: denaturation at 94℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, at 56℃ for 30 s, at 72℃ for 30 s, a final extension at 72℃ for 10 min. The PCR products were subjected to 1% agarose gel electrophoresis.

Concentration of MDA-7/IL-24 in supernatant

Two-antibody sandwich ELISA was developed for detection of human MDA-7/IL-24. The antibodies used were monoclonal mouse anti-human IL-24 antibody (R&D Systems) and peroxidaseconjugated rabbit anti-goat IgG antibody (R&D Systems). Cell culture supernatant was col-

lected after 24, 48 and 72 h, respectively and stored at -20℃. Concentrations of MDA-7/IL-24 in supernatant were measured by ELISA at different time points. Absorbance was read at a wavelength of 450-nm. Concentration of IL-24 was measured according to the standard curve.

Cell proliferation determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells were seeded into 96-well tissue culture plates (1×10^3 cells per well), and treated with PBS, 10 MOI of Ad.IL-24, SG600-EGFP, SG600-IL24, respectively, on the next day. After cultured for 24 h, the medium was replaced with a fresh medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were incubated at 37℃ for 4 h. After about 150 μL of a solution was added to each well, the cells were incubated for an additional 10 min at 37℃ with gentle shaking. Absorbance was read on a Bio-Rad microplate reader at a wavelength of 595 nm.

Western blotting analysis

Cell lines were cultured in 6-well plates, and cells after different treatments were collected at the indicated time points. The cells were collected and suspended in lysis BCA for protein quantitation after 48 h. A total of 25 μg of protein was applied to 15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes which were probed with polyclonal antibodies to MDA-7/IL-24 and β-actin. The corresponding fluorescent secondary antibody was hybridized. The second anti-PVDF membrane fluorescence signal was detected with an infrared imaging system.

Fluorescence microscopy evaluation of cell apoptosis

Forty-eight hours after infection, the cells were washed twice with PBS and fixed in 1mL of 4% paraformaldehyde for 10 min at 4℃. After washed twice with PBS, the cells were stained with 100 μL Hoechst33258 in PBS for 15 min at room temperature in dark. Then 1000 cells were counted with their nuclear fragmentation visualized under a fluorescence microscope (TE2000-U, Nikon, Japan). Apoptotic cells were identified by condensation of nuclear chromatin and its fragmentation.

Fluorescence-activated cell sorter analysis

Cells were trypsinized and washed twice with complete media 48 h later. Aliquots of cells (1×10^6) were resuspended in 500 μL binding buffer and stained with 5 μL FITC-labeled Annexin-V according to its manufacturer's instructions. Five microliters of propidium iodide (PI) was added to the samples after stained with Annexin-V to distinguish late apoptotic and necrotic cells, and then put in a dark place for 30 min. Flow cytometry (BD, FACSCalibur, USA) was performed immediately after staining.

Cell-cycle and hypodiploidy analyses

Cells were cultured when they grew about 30%, and then treated with DMEM without FBS for 24 h for synchronization. The cells were divided into DMEM group, Ad.IL-24 group, SG600-EGFP group, and SG600-IL24 group 48 h later, and harvested using trypsin to adjust the cell concentration to 1×10^6 per mL. After fixed in 70% cold ethanol overnight at -20℃, the cells were washed with PBS, and aliquots of 1×10^6 cells were resuspended in 1 mL of PBS containing 1 mg/mL of RNase A and 0.5 mg/mL of PI. After incubated for 30 min, the cells were analyzed by flow cytometry using a FACScan flow cytometer (BD, FACSCalibur, USA). The treated cells were evaluated by FACS analysis for identifying cells at different stages of cell cycle. The percentages of cells at G0/G1, S, and G2/M stages were calculated using multicycle software and the results were analyzed using variance analysis.

Statistical analysis

All experiments were performed at least three times. Data were expressed as mean ± SD. Statistical comparisons were made by analysis of variance. $P < 0.05$ was considered statistically significant. All analyses were performed with SPSS14.0 software.

RESULTS

Expression of SG600-IL24 mediated ectopic MDA-7/IL-24 in cells

The expression of MDA-7/IL-24 mRNA was markedly increased both in normal liver cell line (L02) and in HCC cell lines (HepG2, Hep3B, MHCC97L) with a different p53 state that were infected with SG600-IL24. In contrast, the expression level of MDA-7/IL-24 was very low in cells infected with Ad.IL-24, SG600-EGFP, and DMEM (Figure 1).

Detection of MDA-7/IL-24 in supernatants by ELISA

Secreting MDA-7/IL-24 protein was detected by ELISA after SG600-IL24 infection. The concentrations of MDA-7/IL-24 protein in supernatants of cells infected with SG600-IL24 increased in a time-dependent manner. The expression of endogenous MDA-7/IL-24 was not detected in SG600-EGFP and control groups (Table 1).

Detection of MDA-7/IL-24 protein expression by Western blotting

Mda-7/IL-24 protein was not expressed in control group, Ad.IL-24 and SG600-EGFP groups, while MDA-7/IL-24 was highly expressed in oncolytic adenovirus 48 h after SG600-IL24 infection (Figure 2).

SG600-IL24 inhibited proliferation of HCC cells

To investigate whether SG600-IL24 can inhibit cell proliferation, HCC cell lines (HepG2, Hep3B and MHCC97L) and normal liver cell line L02 were infected with SG600-IL24. The cell proliferation and viability were determined by MTT. No proliferation arrest effect was observed on normal liver cell line L02 (Figure 3). However, the activity of SG600-IL24 in HCC cell lines (HepG2, Hep3B and MHCC97L) was significantly inhibited with an inhibition rate of 75%, 85% and 72%, respectively.

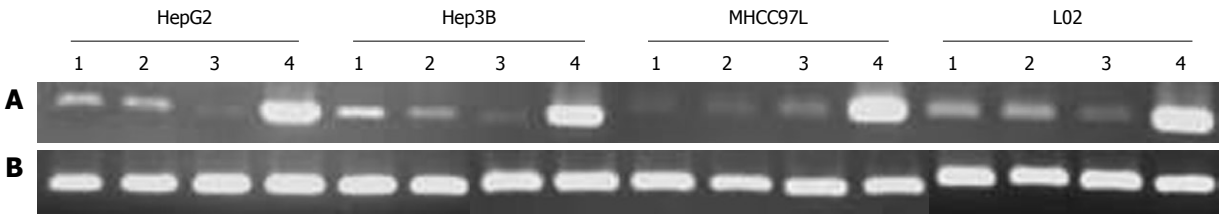

Figure 1 Expression of adenovirus-mediated melanoma differentiation-associated-7/interleukin-24 mRNA in hepatocellular carcinoma cell lines of HepG2, Hep3B and MHCC97L and human normal liver cell line L02. Cells were infected with 10 multiplicity of infection of Ad.IL-24, SG600-EGFP, SG600-IL24,harvested at 24 h, treated as described in "Materials and Methods". A: Lanes 1, 2, 3, 4: Ad.IL-24 group, SG600-EGFP group, control group and SG600-IL24 group; B: β-actin corresponsively.

Table 1 Concentration of melanoma differentiation-associated-7/interleukin-24 protein in different hepatocellular carcinoma cell lines and normal liver cell line (pg/mL)

	Concentration of MDA-7/IL-24 protein			
	HepG2	Hep3B	MHCC97L	L02
Control group	8.0 ± 1.0	9.0 ± 0.5	4.0 ± 0.4	9.0 ± 0.8
Ad.IL-24 group	8.2 ± 0.5	9.0 ± 1.0	4.5 ± 0.5	10.0 ± 1.0
SG600-EGFP group	9.0 ± 0.8	10.0 ± 0.1	3.5 ± 0.5	9.0 ± 0.6
SG600-IL24 group				
24 h	90 ± 10[b]	60 ± 8[a]	56 ± 10[b]	110 ± 12[b]
48 h	160 ± 20[b]	90 ± 15[b]	180 ± 20[b]	180 ± 15[b]
72 h	780 ± 80[b]	800 ± 60[b]	680 ± 50[b]	920 ± 80[b]

[a]$P < 0.05$ *vs* Ad.IL-24 and SG600-EGFP groups; [b]$P < 0.01$ *vs* control group. MDA: Melanoma differentiation-associated; IL: Interleukin.

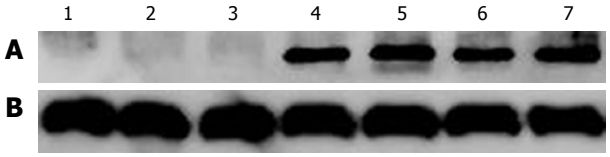

Figure 2 Expression of melanoma differentiation-associated-7/interleukin-24 after infection with SG600-IL24 protein in hepatocellular carcinoma cells and normal liver cells. Cells infected with 10 multiplicity of infection of Ad.IL-24, SG600-EGFP and SG600-IL24 were collected 48 h after infection as described in "Materials and Methods". A: Lane 1: Control group; Lane 2: Ad.IL-24 group; Lane 3: SG600-EGFP group; Lanes 4, 5, 6, 7: HepG2, Hep3B, MHCC97L and L02 SG600-IL24 groups; B: β-actin corresponsively.

SG600-IL24 selectively induced apoptosis of HCC cell lines

Hoechst staining showed that SG600-IL24 induced the apoptosis of human HCC cell lines of HepG2, Hep3B and MHCC97L (Table 2). The apoptosis level of HCC cells was higher in SG600-IL24 group than in other groups (HepG2: $F = 156.6$, Hep3B: $F = 202.4$, MHCC97L: $F = 143.2$, $P < 0.05$), indicating that SG600-IL24 can induce apoptosis of HCC cells. In contrast, no apparent change was observed in normal liver cell line L02, with an apoptosis rate of 1.0%, 1.4%, 1.2% and 2.0%, respectively ($F = 1.78$). Flow cytometry showed the effect of SG600-IL24 on the apoptosis of HCC cell lines of HepG2, Hep3B and MHCC97L and normal liver cell line L02 with Annexin-V and PI staining. The percentage of apoptotic HCC cells was significantly higher in SG600-IL24 group than in control group, SG600-EGFP and Ad.IL-24 groups (HepG2: $F = 203.4$, Hep3B: $F = 313.2$, MHCC97L: $F = 160.6$, $P < 0.05$, Table 2). In contrast, no significantly change was found in normal liver cell line L02 with an apoptosis rate of 0.75%, demonstrating that SG600-IL24 infection can kill HCC cells but not normal liver cells.

SG600-IL24 induced cell cycle block in HCC cells

Cell cycle phase was assayed by flow cytometry after the fixed cells were stained with PI. The accumulation level of HCC cell lines at the G2/M phase was higher in SG600-IL24 group than in control group, Ad.IL-24 and SG600-EGFP groups ($P < 0.05$) with an accumulation rate of 35.4%, 47.3%, 42%, respectively (Figure 4). However, the accumulation rate of normal liver cell line L02 at the G2/M phase was 5.5%, 5.6%, 6.3%, and 6.8%, respectively, suggesting that SG600-IL24 infection can significantly increase the accumulation of HCC cell lines but not of normal liver cell line L02 at the G2/M phase.

DISCUSSION

MDA-7/IL24 is a new member of the IL-10 class-Ⅱ family of cytokines. It has been shown that MDA-7/IL24 not only inhibits the growth of melanoma but also the proliferation and apoptosis of other carcinoma cells, such as ovarian cancer[13,14], lung carcinoma[15,16], breast cancer[17], pancreatic cancer[18], glioma[18,19], prostate[20]and colon cancer[21]. It has been reported that increased expression of IL-24 gene suppresses cell growth and induces cell apoptosis in a variety of cancer cells with single or multiple genetic defects, including alterations in p53, p16/INK4a, and Rb[22,23]. It has been shown that some signal transduction

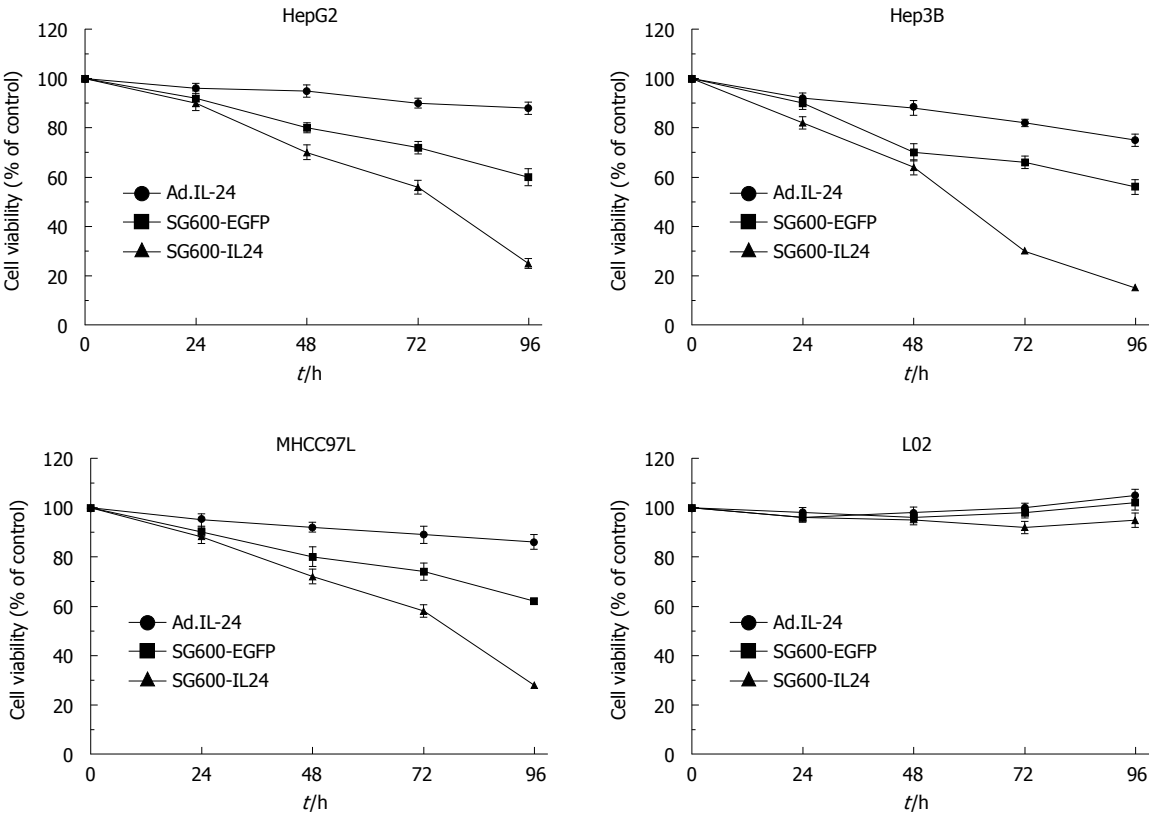

Figure 3 Cell viability of different hepatocellular carcinoma cells and normal liver cells infected with oncolytic adenoviruses SG600-IL24 and replicant replication-deficient adenovirus Ad.IL-24 measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 24, 48, 72 and 96 h after infection. Results are presented as means ± SD (n = 5).

Table 2 Hoechst staining and flow cytometry showing apoptosis of hepatocellular carcinoma cell lines and normal liver cell line (mean ± SD)

Cell line	Apoptotic cells (%)				*F*-value	*P*-value
	Control	Ad.IL-24	SG600-EGFP	SG600-IL24		
Hoechst staining						
HepG2	2.5 ± 0.1	4.0 ± 0.3	5.5 ± 0.3	42.0 ± 4.5	156.6	0.000007
Hep3B	3.0 ± 0.2	3.5 ± 0.3	4.5 ± 0.2	56.0 ± 3.8	202.4	0.000003
MHCC97L	1.6 ± 0.2	3.0 ± 0.4	2.6 ± 0.3	40.5 ± 1.9	143.2	0.000010
L02	1.0 ± 0.1	1.2 ± 0.2	1.4 ± 0.1	2.0 ± 0.1	1.78	0.360
Flow cytometry						
HepG2	2.0 ± 0.1	4.2 ± 0.4	10.0 ± 2.0	56.5 ± 4.0	203.4	0.000003
Hep3B	2.5 ± 0.2	5.0 ± 0.2	13.5 ± 1.5	78.3 ± 3.5	313.2	0.000001
MHCC97L	1.4 ± 0.2	3.2 ± 0.4	10.4 ± 1.0	43.3 ± 2.5	160.6	0.000006
L02	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.62	0.280

pathways and molecules are regulated during IL-24-induced tumor suppression, including activation of caspase cascade, PKR, p38,STAT3, PI3K, GSK-3, ILK-1, BAX, BAK, Fas, DR4,TRAIL, inducible nitric oxide synthase (iNOS), IRF-1, IRF-2 and p53[24].

HCC gene therapy has become a current research focus. However, most methods used are not tumor specific and affect normal cells, thus limiting their clinical application. Gene therapy, a research hot-spot in cancer gene therapy, is able to selectively kill tumor cells without affecting normal cells[25]. In this study, oncolytic adenovirus, which is characterized by antitumor activity and can proliferate and replicate specifically in tumor cells, was used as a carrier. Oncolytic adenovirus can make the exogenous gene copy thousands of times and enhance the effect of anti-cancer gene in a similar manner, ultimately killing tumor cells[26]. Since tumor-specific oncolytic adenovirus can amplify many times in infected cancer cells and dissolve tumor cells, it can be used as a promising anti-tumor gene therapy vector[27]. Adenovirus SG600IL-24, which was constructed in this study, has the E1B 55 kDa defective oncolytic adenovirus (ZD55), replicates in tumor cells and causes significant cytotoxic effect, while normal cells show little or no toxicity[28]. SG600IL-24 was constructed with the oncolytic adenovirus SG600 vector using the telomerase reverse

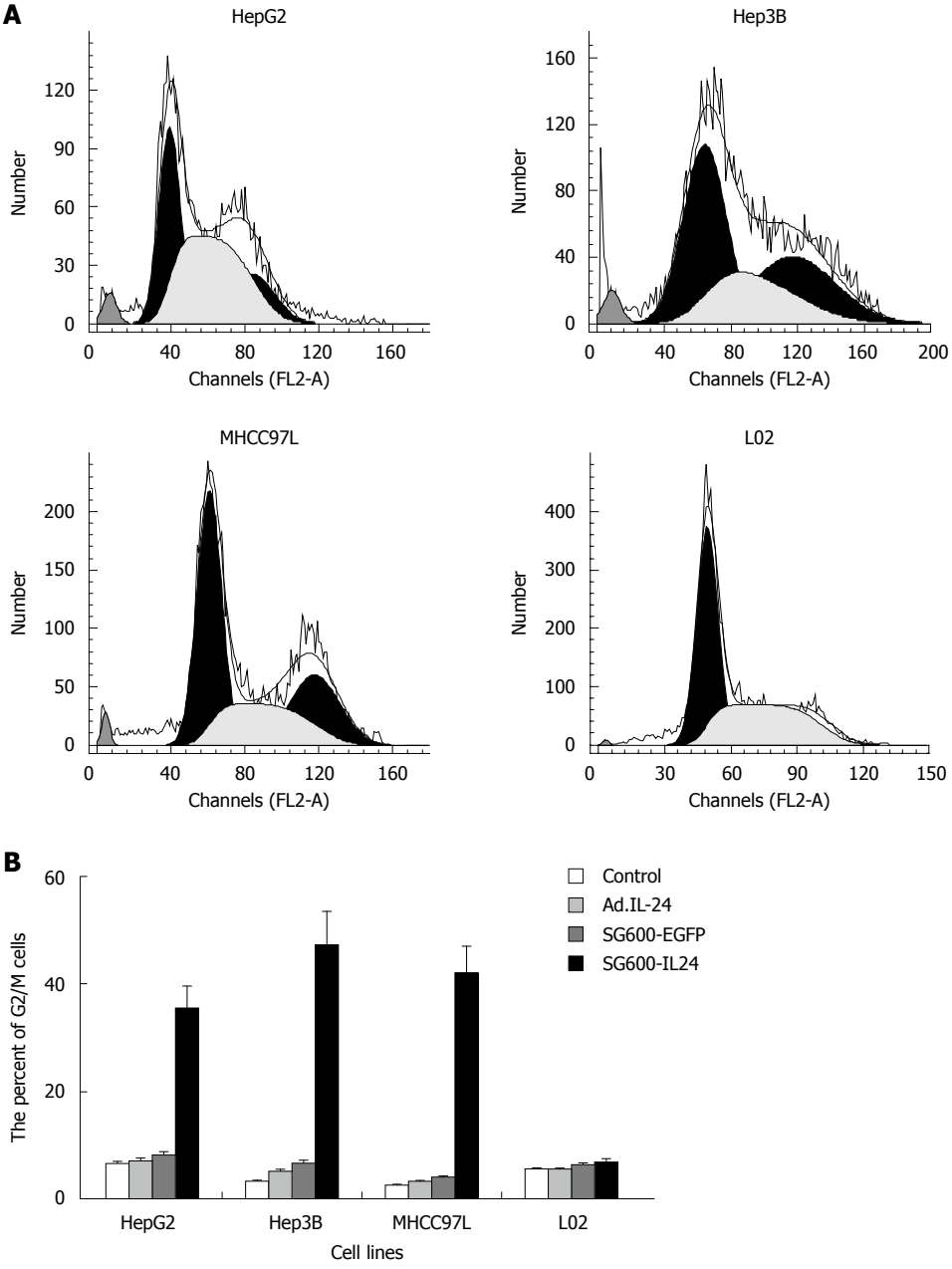

Figure 4 Flow cytometry (A) and histography (B) showing SG600-IL24 induced G2/M arrest in hepatocellular carcinoma cells after SG600-IL24 infection.

transcriptase promoter (TERTp) which is highly active in more than 85% of different human cancers, but inactive in most normal somatic cells, and can thus be applied to a wide range of cancers[29]. Hypoxia regulatory elements can control adenovirus proliferation genes E1a and E1b, and delete the E1a gene, thus promoting virus-specific replication in tumor cells and enhancing the effectiveness and safety of gene therapy[30].

In contrast to the replication-incompetent adenovirus Ad.IL-24, oncolytic adenovirus can replicate in tumor cells exclusively and kill tumor cells, inducing a high expression of MDA-7/IL-24 in these cells. MDA-7/IL-24 protein can also selectively kill cancer cells. Moreover, oncolytic adenovirus can selectively kill tumor cells and MDA-7/IL-24 protein can release into blood and kill distant micrometastatic tumor cells, resulting in a radical treatment of HCC because of the potent "bystander" antitumor activity[31].

Since MDA-7/IL-24 can selectively kill various cancer cells, it has been used in treatment of patients with HCC[32,33] with encouraging results. However, the treatment is restricted only to melanoma, but not to other tumors. HCC has much more cells, bulkier volume, and higher metastatic potential than melanoma. If replication-defective adenovirus is used as a vector, a large number of adenoviruses would be required and lead to mortal immune reaction to human bodies, thus limiting its further clinical application. Oncolytic adenovirus was constructed in this study as a vector carrying the MDA-7/IL-24 gene, which can proliferate in tumor cells exclusively and kill tumor cells, release a large number of adenoviruses which would infect other tumor cells, proliferate and kill tumor cells.

Moreover, MDA-7/IL-24 protein releases and selectively kills tumor cells when tumor cells are dissolved. Therefore, few adenoviruses are required, thus greatly benefiting the clinical application of MDA-7/IL-24. Furthermore, SG600-IL24 has few or no toxic effects on normal cells because it cannot proliferate in normal cells[28].

In this study, RT-PCR, ELISA, and Western-blot demonstrated that MDA-7/IL-24 was successfully transfected into HCC cells and normal liver cells. Mda-7/IL-24 gene and protein were not expressed in control and SG600-EGFP groups. ELISA showed that the expression of secreted and intracellular MDA-7/IL-24 protein in SG600IL-24 group increased in a time-dependent manner. Flow cytometry showed SG600IL-24 significantly inhibited the proliferation of tumor cells, promoted the apoptosis of tumor cells, and blocked tumor cells in the G2/M phase. Normal liver cell line L02 was not affected although it increased by 1.3% in the G2/M phase. Flow cytometry showed that the early and late apoptosis rate of HepG2, Hep3B and MHCC97L was 56.59%, 78.36% and 43.39%, respectively, with Annexin-V and PI staining. MTT showed that SG600IL-24 could promote apoptosis and kill HCC cells but not normal liver cells with Hoechst33258 staining, indicating that the antitumor activity of SG600IL-24 is stronger than that of Ad.IL-24.

HCC cell line HepG2 is a wild type in gene p53 and a mutant type in gene Rb. Hep3B is a line with p53 gene deleted, and MHCC97L is a mutant type in gene p53[34-37]. Although these cell lines have different gene types, they induce apoptosis and growth arrest by infection with SG600IL-24, demonstrating that SG600IL-24 can kill different tumor cells independent of the p53 state.

In conclusion, SG600IL-24 selectively kills HCC cell lines in a p53-independent manner and enhances antitumor activity in HCC cell lines. SG600-IL24 can be used as a HCC gene therapy vector.

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COMMENTS

Background

Gene therapy is able to selectively kill tumor cells without affecting normal cells and has thus become a research hot-spot in cancer gene therapy. However, *in vitro* experimental results of gene therapy for hepatocellular carcinoma (HCC) are imperfect. The aim of this study was to investigate the effect of replication-competent oncolytic adenovirus one HCC cell lines and normal liver cell line.

Research frontiers

In this study, SG600-IL24 could selectively suppress the proliferation and apoptosis of HCC cells *in vitro* in a p53-independent manner. Compared with Ad.IL-24, SG600-IL24 could enhance antitumor activity in HCC cell lines.

Innovations and breakthroughs

Oncolytic adenovirus SG600 was constructed in this study, which has the telomerase reverse transcriptase promoter (TERTp) and hypoxia regulatory elements (HRE). The study showed that SG600 had an excellent antitumor activity *in vitro* on HCC cell lines with a different p53 status.

Applications

The oncolytic adenovirus SG600 vector which has TERTp, hypoxia and HRE, can replicate in tumor cells exclusively and kill HCC cells and other cancer cells. SG600-IL24 displays a better selective replication and antitumor effect than Ad.IL-24 and can thus be used as an efficient agent in anticancer therapies.

Peer review

This is a good paper, although only *in vitro* data are provided.

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BRIEF ARTICLE

ApoB-100, ApoE and CYP7A1 gene polymorphisms in Mexican patients with cholesterol gallstone disease

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Abstract

AIM: To determine the possible association of the ApoB-100 (*Xba* I), ApoE (*Hha* I) and CYP7A1 (*Bsa* I) gene polymorphisms, with the development of cholesterol gallstone disease (GD) in a Mexican population.

METHODS: The polymorphisms were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism, in two groups matched by ethnicity, age and sex: patients with GD (n = 101) and stone-free control subjects (n = 101).

RESULTS: Allelic frequencies in patients and controls were: 34.16% *vs* 41.58% (P = 0.124) for X+ of ApoB-100; 4.46% *vs* 5.94% (P = 0.501) for E2, 85.64% *vs* 78.22% (P = 0.052) for E3, 9.90% *vs* 15.84% (P = 0.075) for E4 of ApoE; and 25.74% *vs* 27.72% (P = 0.653) for C of CYP7A1. Differences in genotypic frequencies between the studied groups were not significant (P < 0.05).

CONCLUSION: These results demonstrated that no association exists between the studied polymorphisms and cholelithiasis in this high prevalent population.

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Key words: Apolipoprotein; CYP7A1; Gallstones; Mexicans; Polymorphisms

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INTRODUCTION

Cholesterol gallstone disease (GD) is the major manifestation of gallbladder disease, and is one of the most common digestive disorders worldwide, especially in Western populations[1]. In México, the prevalence of GD is nearly 14.3%, and is a public health problem with high economic impact[2]. The formation of gallstones is accelerated by impaired gallbladder emptying, hypersecretion of cholesterol into bile, or destabilization of bile by kinetic protein factors[3,4]. The pathogenesis of GD is multifactorial, with

environmental and genetic factors involved[5,6]. Several risk factors, such as obesity, diet, female gender, metabolic syndrome and type-2 diabetes, are usually associated with this pathology[7,8]. On the other hand, the high prevalence of GD in American Indians and Hispanic populations, as well as several twin and family studies, suggested that genetic factors play a key role[9-11].

Epidemiological data have shown that disturbances in serum lipids are associated with GD[12-14], suggesting that proteins involved in transport, synthesis and metabolism of lipids are related to lithogenesis. The candidate lithogenic-genes found in humans are: ABC transporters for phosphatidylcholine (ABCB-4) and bile salts (ABCB-11), hepatocanalicular cholesterol transporter (ABCG5/G8), cholesterol-7α-hydroxylase (CYP7A1), cholecystokinine type-A receptor (CCK1R), cholesteryl ester transfer protein (CETP), and apolipoproteins (Apo) A-I, B and E[4,5,15-18].

Lipoprotein particles transport lipids and cholesterol in the bloodstream. ApoB-100 [the sole protein of low density lipoprotein (LDL)] and ApoE [found in VLDL, high density lipoprotein (HDL) and chylomicron remnants] bind to lipids, and are recognized by hepatic and tissue lipoprotein-receptors[19,20]. These proteins are polymorphic in the population, and are considered as genetic determinants of variations in cholesterol and lipids transport and metabolism[19,21].

Bile formation is essential for the removal of excess dietary cholesterol. The first-step and key regulatory enzyme in bile acid synthesis is CYP7A1, catalyzing the formation of 7-α-hydroxycholesterol. Innate deficiency of this enzyme has been related to hypercholesterolemia[12].

Here, we evaluated the association between ApoB-100 (*Xba* I), ApoE (*Hha* I) and CYP7A1 (*Bsa* I) gene polymorphisms with cholelithiasis, in a population from Sinaloa, México, a country with high prevalence of this disease.

MATERIALS AND METHODS

Subjects

We studied two groups matched by ethnicity (natives from Sinaloa, México), age and sex: consecutive symptomatic patients (n = 101) with cholesterol gallstone disease (GD), and healthy stone-free control subjects (n = 101) confirmed by abdominal ultrasonography (EnVisor Ultrasound System, Philips Medical System, Andover, MA, USA). Patients were cholecystectomized at the Division of Gastroenterology of the Regional Hospital of the "Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado" (ISSSTE, Culiacán, Sinaloa), from May 2008 to April 2009, and only those with cholesterol-stones (≥ 70% of its content) were included in the study. Subjects were also questioned about their past medical history, and their body mass index (BMI) was calculated. In accordance with the World Health Organization's categories, subjects with BMI ≥ 25 kg/m^2 were considered overweight and ≥ 27 as class-I obese. Those with renal or liver malfunction were excluded. The Ethical and Research Committee of ISSSTE approved this study and all subjects signed an informed consent.

Laboratory tests

Fasting serum glucose and lipids, including total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and triglycerides, were determined using the HITACHI 917 automatic biochemical analyzer (Hitachi Koki Co. Ltd, Hitachinaka City, Japan). Individuals with the following values were considered above the normal range: glucose > 120 mg/dL; total lipids > 800 mg/dL; triglycerides > 150 mg/dL; cholesterol > 200 mg/dL; and LDL-C > 130 mg/dL. In the case of HDL-C, values > 40 mg/dL were considered normal.

Recovered gallstones were washed with distilled water and dried at 37℃ to determine the cholesterol content using the Liebermann-Burchard reaction, and classified in accordance with their chemical composition[22,23].

DNA amplification and restriction fragment length polymorphism

Genomic DNA was isolated from whole blood containing EDTA, using the salt precipitation method[24]. The polymorphisms were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Reaction conditions, primers and restriction fragments are summarized in Table 1.

A region from exon 26 of *apo*B-100 (chromosome 2), containing the C-7673T polymorphism, was amplified and digested with *Xba* I (4 U for 2 h at 37℃), and alleles were identified as X- (normal) and X+[25]. PCR products from exon 4 of *apo*E (chromosome 19) were digested with *Hha*I (2 U for 2 h at 37℃) to determine the codominant alleles E2 (residues Cys112 and Cys158), E3 (Cys112 and Arg158) and E4 (Arg112 and Arg158)[26]. In the same way, a region spanning the polymorphic site A-204C in the promoter region of CYP7A1 (chromosome 8), was amplified and cleaved with *Bsa* I (1 U for 2 h at 37℃) to determine A (normal) and C alleles[27].

Five random samples of each polymorphism were sequenced to confirm the results. Abnormal RFLP's bands were also sequenced.

Statistical analysis

Data are presented as mean ± SD. Mean differences in covariates were analyzed by the Student's t-test. A sample size of 96 individuals per group was calculated to detect differences (delta) of 0.14 in polymorphism frequencies between the groups, with 80% power and 5% significance. Allelic frequencies observed in patients and controls were evaluated for differences using the Mantel-Haenzel χ^2 test, or Fisher's exact test when the number of observations in any cell was ≤ 5. The P values were corrected by Bonferroni test for multiple comparisons, taking into account the number of alleles observed, and considered significant when $P < 0.05$[28]. Odds ratios (OR) with 95% confidence intervals (CI) were used as the measure of association between specific genotypes and alleles with GD[29]. Hardy-Weinberg's equilibrium was calculated by χ^2 test. Multiple logistic regression analysis was performed to investigate the independent factors as-

Table 1 Conditions and products of polymerase chain reaction followed by restriction fragment length polymorphism

Gene	Primers	Tm (℃)	RE	bp	Alleles
ApoB	Forward: GGAGACTATTCAGAAGCTAA Reverse: GAAGAGCCTGAAGACTGACT	55	*Xba* I	710	X-: 710 bp X+: 433 and 277 bp
ApoE	Forward: ACAGAATTCGCCCCGGCCTGGTACAC Reverse: TAAGCTTGGCACGGCTGTCCAAGGA	60	*Hha* I	252	E2: 91 and 84 bp E3: 91, 48 and 36 pb E4: 72, 48 and 36 bp
CYP7A1	Forward: CAGAGCATGGACAGGGAGCAG Reverse: GCAACTCCTCATGGCTGAGGTT	55	*Bsa* I	948	A: 581, 367 bp C: 542, 367 and 39 bp

Forward and reverse primers in 5′ to 3′ directions. Tm: Annealing temperature; RE: Restriction enzyme.

Table 2 Clinical characteristics in control subjects and patients with gallstones (mean ± SD)

Variable	Patients (n = 101)	Controls (n = 101)	P
Sex (F/M)	86.10%/13.90%	86.10%/13.90%	1.000
Age (yr)	51.93 ± 11.23	51.74 ± 10.99	0.904
Body mass index (kg/m^2)	29.03 ± 4.19	27.63 ± 4.42	0.024
Serum glucose (mg/dL)	120.24 ± 44.20	100.30 ± 34.64	0.000
Lipids (mg/dL)	622.00 ± 149.77	688.60 ± 160.68	0.003
Cholesterol (mg/dL)	187.32 ± 45.10	208.21 ± 43.14	0.001
LDL cholesterol (mg/dL)	118.58 ± 41.64	130.94 ± 33.80	0.022
Triglycerides (mg/dL)	119.76 ± 87.05	144.45 ± 72.22	0.029
HDL cholesterol (mg/dL)	43.27 ± 13.26	46.56 ± 14.01	0.089
Cholesterol in stones (% by weight)	99.23 ± 2.50		

LDL: Low density lipoprotein; HDL: High density lipoprotein.

sociated with GD. SPSS v16.0 software (SPSS inc., Chicago, IL, USA) was used for data analysis.

RESULTS

Characterization of the population

Patients with cholelithiasis and control subjects with a mean age of 51.93 (± 11.23) years *vs* 51.74 (± 10.99) years (P = 0.904), and a normal distribution according to the Kolmorov-Smirnov test (P = 0.281) were included in this study. In both groups, 86.10% of individuals were female and 13.90% were male (P = 1.000), giving a 6:1 female/male ratio (Table 2). The cholesterol content of recovered gallstones was 99.23% (± 2.50).

There were statistically significant differences in the measured covariates between patients and controls (Table 2), with the exception of HDL-C (P = 0.089). Body mass index (BMI) and serum glucose were higher in patients than in the control group: 29.03 (± 4.19) kg/m^2 *vs* 27.63 (± 4.42) kg/m^2 (P = 0.024) for BMI; and 120.24 (± 44.20) mg/dL *vs* 100.30 (± 34.64) mg/dL (P = 0.0002) for glucose.

In contrast, serum lipids, cholesterol, LDL-C and triglycerides, were lower in patients than in controls: 622.00 (± 149.77) mg/dL *vs* 688.60 (± 160.68) mg/dL for lipids (P = 0.003); 187.32 (± 45.10) mg/dL *vs* 208.21 (± 43.14) mg/dL for cholesterol (P = 0.001); 118.58 (± 41.64) mg/dL *vs* 130.94 (± 33.80) mg/dL for LDL-C (P = 0.022); and 119.76 (± 87.05) mg/dL *vs* 144.45 (± 72.22) mg/dL for triglycerides (P = 0.029).

Table 3 Allelic (af) and genotypic (gf) frequencies of ApoB-100, ApoE and CYP7A1 polymorphisms in patients with gallstones and controls

Polymorphism	Patients (n = 101)		Controls (n = 101)		P value	OR	95% CI
ApoB-100 *Xba* I							
Alleles	n	af	n	af			
X-	133	65.84%	118	58.42%	0.124	1.37	0.92-2.05
X+	69	34.16%	84	41.58%	0.124	0.73	0.49-1.09
Genotypes	n	gf	n	gf			
X-X-	41	40.59%	34	33.66%	0.308	1.35	0.76-2.39
X+X-	51	50.50%	50	49.50%	0.888	1.04	0.60-1.81
X+X+	9	8.91%	17	16.83%	0.093	0.48	0.20-1.14
ApoE *Hha* I							
Alleles	n	af	n	af			
E2	9	4.46%	12	5.94%	0.501	0.74	0.30-1.79
E3	173	85.64%	158	78.22%	0.052	1.66	0.99-2.78
E4	20	9.90%	32	15.84%	0.075	0.58	0.32-1.06
Genotypes	n	gf	n	gf			
E2E2	0	0.00%	1	0.99%	NC	NC	NC
E3E3	74	73.27%	64	63.37%	0.130	1.58	0.87-2.88
E4E4	1	0.99%	2	1.98%	0.561	0.50	0.04-5.55
E2E3	8	7.92%	6	5.94%	0.580	1.36	0.45-4.08
E2E4	1	0.99%	4	3.96%	0.174	0.24	0.03-2.21
E3E4	17	16.83%	24	23.76%	0.221	0.65	0.32-1.30
CYP7A1 *Bsa* I							
Alleles	n	af	n	af			
A	150	74.26%	146	72.28%	0.653	1.11	0.71-1.72
C	52	25.74%	56	27.72%	0.653	0.90	0.58-1.40
Genotypes	n	gf	n	gf			
AA	59	58.42%	56	55.45%	0.670	1.13	0.65-1.97
CA	32	31.68%	34	33.66%	0.764	0.91	0.51-1.65
CC	10	9.90%	11	10.89%	0.818	0.90	0.36-2.22

OR: Odds ratio; 95% CI: 95% confidence intervals; NC: Not calculated.

ApoB-100, ApoE and CYP7A1 gene polymorphisms analysis

Allelic and genotypic frequencies of GD patients and healthy controls are shown in Table 3. Statistical analysis showed no differences in genotypic frequencies of ApoB-100 gene *Xba* I polymorphism between patients and controls (P = 0.210): 40.59% *vs* 33.66% (P = 0.308, OR = 1.35) for X-X-; 50.50% *vs* 49.50% (P = 0.888, OR = 1.04) for X+X-; and 8.91% *vs* 16.83% (P = 0.093, OR = 0.48) for X+X+. Frequencies of X+ allele were 34.16% *vs* 41.58% (P = 0.124, OR = 0.73).

In the case of ApoE gene *Hha* I polymorphism, there were no significant differences in the distribution

of alleles between patients and controls (P = 0.075). Allelic frequencies were 4.46% *vs* 5.94% (P = 0.501, OR = 0.74) for E2; 85.64% *vs* 78.22% (P = 0.052, OR = 1.66) for E3; and 9.90% *vs* 15.84% (P = 0.075, OR = 0.58) for E4. Genotypic frequencies were 0.00% *vs* 0.99% for E2E2; 73.27% *vs* 63.37% (P = 0.130, OR = 1.58) for E3E3; 0.99% *vs* 1.98% (P = 0.561, OR = 0.50) for E4E4; 7.92% *vs* 5.94% (P = 0.580, OR = 1.36) for E2E3; 0.99% *vs* 3.96% (P = 0.174, OR = 0.24) for E2E4; and 16.83% *vs* 23.76% (P = 0.221, OR = 0.65) for E3E4.

Frequencies of C allele (CYP7A1 gene *Bsa* I polymorphism) in patients and controls were 25.74% *vs* 27.72% (P = 0.653, OR = 0.90). Genotypic frequencies between the groups were similar (P = 0.911) with the following distribution in patients and controls: 58.42% *vs* 55.45% (P = 0.670, OR = 1.13) for AA; 31.68% *vs* 33.66% (P = 0.764, OR = 0.91) for CA; and 9.90% *vs* 10.89% (P = 0.818, OR = 0.90) for CC.

The distributions of ApoB-100, ApoE and CYP7A1 gene polymorphisms in both groups were in Hardy-Weinberg equilibrium (all $P \leqslant 0.05$). Multiple logistic regression analysis showed no significant association between the gene polymorphism frequencies and the covariates.

DISCUSSION

In this work, we report on the association of ApoB-100, ApoE and CYP7A1 gene polymorphisms with cholelithiasis in México.

Patients and controls were Mexican Mestizos, natives of the northern state of Sinaloa, who were matched for both age and sex. The female/male ratio (6:1) and mean age (51.93 ± 11.23 year) of the patients were in accordance with the governmental data of the disease in the country, supporting the notion that female gender and age are risk factors.

There were statistically significant differences ($P \leqslant 0.05$) in the covariates between the groups, with the exception of HDL-C (Table 2). BMI and sanguineous glucose were higher in patients than in controls, while serum levels of cholesterol, LDL-C, total lipids, and triglycerides were greater in controls. However, these mean differences were not considered clinically relevant, since both groups were in the class-I obese category, and had normal or borderline levels of serum glucose, cholesterol, LDL-C, total lipids, and triglycerides. In contrast to these results, several studies have demonstrated a clear correlation between BMI and hyperglycemia with GD[30], while other studies found that dyslipidemias, such as decreased HDL-C and increased triglycerides and LDL-C levels, correlated with augmented risk for cholelithiasis[13,14,31].

We found that both genotypic and allelic frequencies of ApoB-100 gene *Xba* I polymorphism did not show significant differences between patients and controls (Table 3). The frequency of the X+ allele in patients was 34.16%, similar to that observed in a Finnish population[18]. A case-control study performed in México, suggested a relationship between the serum concentration of apolipoproteins (B and A-I) and gallbladder disease, however, gene polymorphisms were not analyzed[32]. The *Xba* I polymorphism does not alter the threonine residue at position 2488, however, it may be a marker in strong linkage disequilibrium with several other unknown but functional mutations. It has been reported that the X+ allele is characterized by higher serum levels of cholesterol and LDL-C, and may be a marker for increased risk of GD in the Chinese population[33]. On the contrary, other studies with Polish and India populations did not observe any significant differences between *Xba* I polymorphism and GD[34,35].

In this study, phenotypic and allelic frequencies of ApoE gene *Hba* I polymorphism were similar between the groups (Table 3). E3E3 and E3E4 were the most common phenotypes found in patients (73.27% and 16.83%). The frequencies of E3E3 genotype and the common E3 allele (85.64%) were similar to other reports in Mexican Mestizos[36]. Each of the six possible ApoE phenotypes have particular receptor binding affinities and catabolic rates, as reflected by serum levels and clearance rates of circulating lipoproteins[21]. Previous studies suggested an association between the apoE4 isoform and increased gallstone cholesterol content in cholecystectomized patients from Finland[37], and with a higher risk for gallstones in a case-control study from Spain[38]. In contrast, other studies reported that the E4 polymorphism was not associated with susceptibility to cholesterol GD in Chile and Germany, which have high-risk populations[39]. The E2 allele has been reported as one possible factor in the lithogenesis of cholecystolithiasis[40], but other studies have not yielded consistent findings on this association[41-44].

Statistical analysis showed that CYP7A1 gene *Bsa* I polymorphism frequencies between patients and controls were not different (Table 3). The frequency of C allele in patients was 25.74%, slightly higher than the 37.20% observed in a Chinese population[27]. Other studies have confirmed the relationship between CYP7A1 polymorphism with increased LDL-C levels and gallstone formation in a Chinese population[27]. In addition, CYP7A1 deficiency has been correlated with hypercholesterolemic phenotype[12].

For the three studied genes in this study, the distribution of their genotypes in the groups was not significantly different from the expected distribution for a population in Hardy-Weinberg equilibrium. In addition, there was no correlation of these polymorphisms with BMI, glucose or lipid profile. In this study, we did not take into account type-2 diabetes in the inclusion criteria. However, the stratified analysis of the data (hyperglycemic individuals, with > 140 mg/dL fasting glucose) showed no significant differences (data not shown).

The reason for the inconsistent results obtained from several association studies in different countries, may be due to differences in populations. Mexican populations have a high degree of genetic heterogeneity. HLA analyses have demonstrated that in general, Mexican Mestizos have many Amerindian and few European and African

haplotypes. However, the study population in this work (from Sinaloa state), actually have a particular genetic background, since half of the most common haplotypes found in this population have a proposed European origin[45].

The results of this study showed that no association exists between ApoB-100, ApoE and CYP7A1 gene polymorphisms and cholelithiasis in Mexicans, a population with a high prevalence of this digestive disorder.

COMMENTS

Background

Gallbladder stone disease (GD) is commonly associated with several environmental risk factors such as obesity, diet, gender, metabolic syndrome and type-2 diabetes. However, an increasing number of studies point to genetics factors in the pathogenesis of this important digestive disorder.

Research frontiers

Apolipoproteins (Apo) B and E, as well as cholesterol-7α-hydroxylase (CYP7A1), play a key role in transport, synthesis and metabolism of lipids. Many studies have been performed on the association between these polymorphic genes and lithogenesis, however, the results often differ in populations from different origins.

Innovations and breakthroughs

In México, the prevalence of GD is more than 14% and is a public health problem with a very high economic impact; however, there are very few genetic studies on this disease. Therefore, in this report, the influence of ApoB-100, ApoE and CYP7A1 gene polymorphisms on GD was evaluated in a Mexican population. Results showed that allelic and genotypic distributions were different to those observed in other populations, indicating a particular genetic background in this population. Allelic frequencies in patients and controls were: 34.16% *vs* 41.58% (P = 0.124) for X+ of ApoB-100; 4.46% *vs* 5.94% (P = 0.501) for E2, 85.64% *vs* 78.22% (P = 0.052) for E3, 9.90% *vs* 15.84% (P = 0.075) for E4 of ApoE; and 25.74% *vs* 27.72% (P = 0.653) for C of CYP7A1. Differences in genotypic frequencies between patient and control groups were not significant (P < 0.05).

Applications

These results showed that no association exists between ApoB-100, ApoE and CYP7A1 gene polymorphisms and cholelithiasis in Mexicans. Therefore, the study of more genes involved in disturbances of serum lipids is needed to explain the high prevalence of gallstones in this country.

Peer review

This study reports negative data regarding an association between a number of polymorphisms of proteins involved in transport, synthesis or metabolism of lipids and cholesterol gallstone disease.

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BRIEF ARTICLE

Alanine aminotransferase is an inadequate surrogate marker for detecting lamivudine resistance

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Abstract

AIM: To investigate the accuracy of serum alanine aminotransferase (ALT) in diagnosing lamivudine resistance and factors that contributed to abnormal serum ALT.

METHODS: This was a retrospective study of chronic hepatitis B patients on lamivudine therapy who were followed for 3-mo with liver function tests and hepatitis B virus (HBV) DNA measurement. Lamivudine resistance was defined as HBV DNA $\geqslant$ 1 log from nadir on at least 2 occasions, confirmed by genotyping. Serum ALT levels in patients with lamivudine resistance were compared to serum ALT levels in those without lamivudine resistance.

RESULTS: There were 111 patients with and 117 without lamivudine resistance. The area under the receiver operating characteristic of serum ALT to diagnose lamivudine resistance was 0.645 ± 0.037. Serum ALT > 42.5 U/L gave the best diagnostic accuracy with sensitivity = 61%, specificity = 60%, positive predictive value = 60%, negative predictive value = 61%, positive likelihood ratio = 1.53 and negative likelihood ratio = 0.65 for predicting lamivudine resistance, missing 39% of resistant patients. Using other serum ALT cutoffs, diagnostic accuracy was lower. By multivariate analysis, baseline abnormal serum ALT was associated with abnormal ALT during resistance (OR = 5.98, P = 0.003), and males were associated with serum ALT flares during resistance (OR = 8.9, P = 0.016).

CONCLUSION: Serum ALT is inadequate for diagnosing lamivudine resistance and has implications where viral resistance testing is suboptimal and for reimbursement of rescue therapy.

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Key words: Antiviral therapy; Chronic hepatitis B; Sensitivity; Specificity; YMDD mutants

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INTRODUCTION

Chronic hepatitis B (CHB) is a global public health problem, being the most common cause of chronic viral hepatitis, and the major cause of hepatocellular carcinoma worldwide[1]. There are now five licensed oral antiviral agents for CHB: lamivudine, adefovir, entecavir, telbivudine and tenofovir. Although lamivudine is not recommended as first line therapy for CHB in the American Association for Study of Liver Disease (AASLD) CHB guidelines[2,3], and the European Association for Study of Liver (EASL) CHB guidelines[4], which recommend oral nucleosides with high genetic barrier (entecavir and tenofovir) as first line monotherapy, lamivudine is still widely used in Asia due to cost constraints. Lamivudine is an effective treatment for hepatitis B "e" antigen (HBeAg) positive patients, with HBeAg seroconversion in 16%-18% of patients compared with 4%-6% of untreated controls[5,6]. Lamivudine is also used to treat HBeAg negative Hepatitis B virus (HBV) infection, with serum HBV DNA suppressed to undetectable levels using polymerase chain reaction (PCR) assays in 60%-70% of patients after 1 year of treatment[7,8]. However, lamivudine resistance is a major concern, with genotypic resistance increasing from 14% in year 1 to 38%, 49%, 66% and 69% after 2, 3, 4 and 5 years of treatment, respectively[9]. Detection of genotypic resistance is the first event to occur in the development of viral resistance, followed by the occurrence of viral breakthrough, described as a 1 log rise in HBV DNA above the nadir (in a compliant patient), and finally clinical resistance occurs when alanine aminotransferase (ALT) is abnormal[10]. However, genotypic lamivudine resistance tests are not readily available in developing countries and are expensive. Once resistance develops, rescue therapy with add-on adefovir is usually recommended early before the onset of clinical resistance[4] as the efficacy of rescue therapy appears to be better with early rescue compared to delayed rescue, but it is not clear whether all patients will have clinical resistance if left untreated. Consequently, the utility of ALT to diagnose lamivudine resistance is unclear. Once resistance occurs, strategies for the management of viral resistance are likely to be varied depending on the availability of resources, reimbursement policies, expertise of the physicians, and available generic medications. In addition, monitoring for viral resistance with either genotypic tests or HBV DNA may be prohibitively expensive in developing countries and some patients may only be able to afford to have frequent ALT testing as a surrogate for lamivudine resistance. Thus, we aimed to determine the accuracy of ALT in diagnosing lamivudine resistance, and to determine factors associated with abnormal ALT upon development of viral resistance.

MATERIALS AND METHODS

Study design and patient population

This study was a retrospective analysis of a prospectively collected database. All patients started on lamivudine for clinical indications at the Hepatology Clinic in the National University Hospital, Singapore were enrolled into a clinical database and followed every 3 mo with liver function tests and HBV DNA measurement from December 1999 to January 2007. Patients were excluded if they had prior lamivudine therapy or had organ transplantation. Patient demographic data, baseline biochemical parameters, HBV DNA viral load and lamivudine resistance mutations were entered into the database. This study was approved by the National Healthcare Group Institutional Review Board. Waiver of consent was approved by the Institutional Review Board as patient identifiers were removed (anonymised) during data collection.

Liver function tests (LFTs) were performed using Advia Chemistry, (Advia Centaur Systems, Siemens Medical Solutions Diagnostics Pty Ltd, Bayswater, Australia). HBsAg, HBeAg and anti-HBe were tested using Roche Diagnostic kits (Roche Diagnostics GmbH, Mannheim, Germany). Up to April 2006, serum HBV DNA was measured with the Hybrid Capture Ⅱ HBV DNA Test (Digene Corporation, Gaithersburg, MD, USA) with a detection range of 1.4×10^5 copies/mL (6.1×10^4 IU/mL) to 1.7×10^9 copies/mL (7.4×10^8 IU/mL). Since April 2006, HBV DNA levels were measured with the Artus HBV RG (real time) PCR kit (Qiagen Diagnostics, Hamburg, Germany), with a detection range of 100 copies/mL (1.7×10^1 IU/mL) to 1×10^9 copies/mL (1.7×10^8 IU/mL). HBV DNA results were standardized by converting to WHO IU/mL[11]. The conversion factors for the Hybrid Capture Ⅱ HBV DNA Test was 2.3 copies/mL[12], and for the Artus HBV RG (real time) PCR kit was 5.8 copies/mL equivalent to 1 IU/mL[13]. Lamivudine resistance was tested at the time of virological breakthrough, and was not tested at baseline. To test for lamivudine resistance, the HBV DNA polymerase gene RT domain was amplified by PCR followed by the INNO-LiPA HBV DR v2 detection kit (INNOGENETICS N.V. Belgium). Screened mutations for lamivudine resistance included rtL80V/I, rtV/G173L, rtL180M and rtM204V/I/S.

Definition of terms

Patients with lamivudine resistance were defined as those who had virological breakthrough and the presence of mutations conferring resistance confirmed by genotyping, including rtL80V/I, rtV/G173L, rtL180M and rtM204V/I/S.

Virological breakthrough was defined as a rise in HBV DNA of 1 log from nadir on at least 2 occasions after achieving virologic response during continuous treatment. Persistent abnormal ALT was defined as ALT above the upper limit of normal (ULN) for more than 1 mo during the treatment period, regardless of prior ALT normalization. Patients with persistently elevated ALT during treatment (absence of biochemical response) and with

Table 1 Baseline characteristics of lamivudine treated patients

	Lamivudine resistance (n = 111)	Lamivudine no resistance (n = 117)	P value
Male, n (%)	81 (73.0)	86 (73.5)	0.928
Chinese, n (%)	107 (96.4)	108 (92.3)	0.183
Age, yr [mean (95%CI)]	46.5 (44-49)	49.6 (47-52)	0.080
Cirrhosis, n (%)	36 (32.4)	35 (29.9)	0.682
Baseline bilirubin, μmol/L [mean (95% CI)]	19 (15-23)	30 (17-43)	0.124
Baseline albumin, g/L [mean (95% CI)]	36 (34-37)	37 (36-38)	0.326
Baseline ALT, U/L [mean (95% CI)]	175 (131-219)	269 (191-348)	0.038
Baseline AST, U/L [mean (95% CI)]	132 (88-176)	197 (132-262)	0.100
HBeAg positive, n (%)	77 (70.0)	42 (35.9)	< 0.001
Baseline log HBV DNA, IU/mL [mean (95% CI)]	6.5 (6.3-6.8)	5.7 (5.4-6.1)	< 0.001
Baseline abnormal ALT, n (%)	59 (53.15)	65 (55.55)	0.716

Patients with (n = 111) and without lamivudine resistance (n = 117). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B "e" antigen.

viral resistance were also considered to have persistently abnormal ALT. ALT flare was defined as ALT more than 5 times the ULN[14]. ALT during lamivudine resistance (taken at the time point of virological breakthrough) was compared to ALT levels in those without lamivudine resistance (using the mean ALT value of all time points during treatment). The diagnosis of cirrhosis was based on liver biopsy, when available, or clinical, biochemical and ultrasonography findings.

Statistical analysis

All data was analyzed using the statistical package SPSS (version 12.0: SPSS Inc., Chicago, IL, USA). Categorical data were described in number and percentage, and tested by Fisher's exact test for univariate analysis. Continuous data were tested for normality, described in mean (95% CI), and analyzed by independent sample t-test. Statistically significant differences between analyzed groups were defined when the P value < 0.05. All clinically important variables were included in multivariate analysis using multiple logistic regression. Variables with multi-collinearity were excluded from the model. Analyzed results were shown as point estimates and 95% CI. The sensitivity and specificity of ALT in diagnosing lamivudine resistance was tested using the area under the receiver operating characteristic (AUROC) curve. ALT levels used for this analysis were those measured at the same time point during treatment in which viral rebound was detected.

RESULTS

A total of 228 subjects were included in the analysis, 111 of whom had lamivudine resistance, and 117 had no lamivudine resistance. The majority were Chinese [n = 215 (94.3%)] males [n = 167 (73.2%)], with a median age of 48.2 years. Seventy-one (31.1%) had cirrhosis, and 119 (52.2%) had HBeAg positive CHB. At baseline, the 2 groups with and without lamivudine resistance were similar in gender (P = 0.928), race (P = 0.183), age (P = 0.13), baseline bilirubin (P = 0.899), albumin (P = 0.541), ALT (P = 0.650), aspartate aminotransferase (AST) (P = 0.891) and cirrhosis (P = 0.682). However, patients who developed lamivudine resistance were more likely to be HBeAg positive and have higher HBV DNA at baseline (Table 1), and these parameters were significant after multivariate analysis (Table 2). Of these 111 patients, 74 had abnormal ALT after the development of lamivudine resistance, with a mean duration between viral breakthrough and abnormal ALT of 11.1 + 15.1 mo.

Table 2 Multivariate analysis of baseline characteristics between patients with and without lamivudine resistance

	Adjusted P value	Adjusted OR (95% CI)
Male	0.842	1.080 (0.506-2.307)
Chinese	0.343	2.099 (0.454-9.707)
Mean age, yr	0.448	0.989 (0.960-1.018)
Cirrhosis	0.977	1.011 (0.479-2.133)
Baseline bilirubin, μmol/L	0.457	0.996 (0.984-1.007)
Baseline albumin, g/L	0.108	0.950 (0.893-1.011)
Baseline ALT, U/L	0.099	0.997 (0.994-1.000)
Baseline AST, U/L	0.521	1.001 (0.998-1.005)
HBeAg positive	0.005	2.857 (1.383-5.903)
Baseline log HBV DNA, IU/mL	0.014	1.415 (1.073-1.865)
Baseline abnormal ALT	0.923	0.959 (0.405-2.27)

Baseline hepatitis B virus (HBV) DNA and hepatitis B "e" antigen (HBeAg) status were independent predictors of lamivudine resistance. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

HBV DNA and lamivudine resistance

Out of the 228 patients' baseline HBV DNA results, only 13 were measured with the Artus HBV RG PCR kit. The rest were measured with the Hybrid Capture Ⅱ HBV DNA Test. Among the 111 patients with lamivudine resistance, viral breakthrough in 86 patients was detected with the Hybrid Capture Ⅱ HBV DNA Test. The other 25 patients were detected with the Artus HBV RG PCR kit. For the 117 patients with no lamivudine resistance, these patients continued to have undetectable HBV DNA which was confirmed on subsequent tests with the Artus HBV RG PCR kit during follow-up.

Table 3 Diagnostic characteristics of various alanine aminotransferase cut-off levels for detecting lamivudine resistance

ALT (U/L)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio	Negative likelihood ratio
> 42.5	61	60	60	61	1.53	0.65
> 70	39	81	67	58	2.05	0.75
> 30 (males)	85	28	53	66	1.18	0.56
> 19 (females)	90	7	50	40	0.96	1.40

The alanine aminotransferase (ALT) value of 42.5 U/L was found to be the best curve to fit receiver operating characteristics, while the ALT value of 70 U/L was the upper cutoff limit of the normal range in our laboratory, and ALT 30 U/L and 19 U/L were the cutoff values for males and females, respectively, based on the new Association for Study of Liver Disease guidelines.

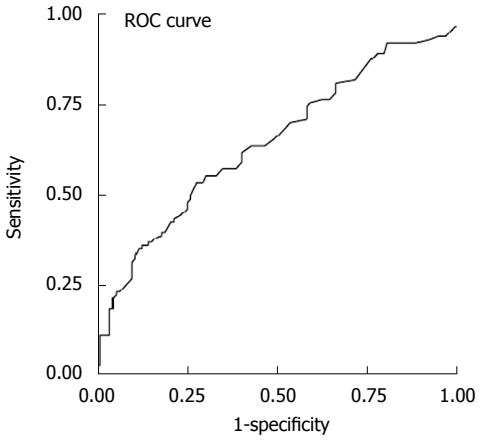

Figure 1 Receiver operating characteristics curve using different alanine aminotransferase levels as cutoffs. Alanine aminotransferase (ALT) cutoff of 42.5 gives the best area under the receiver operating characteristic (ROC) curve 0.645.

Accuracy of serum ALT as a diagnostic marker for lamivudine resistance

When the entire group of lamivudine resistant patients was analyzed, the AUROC was 0.645 (95% CI: 0.569-0.715, standard error: 0.037) for ALT in the diagnosis of lamivudine resistance, with ALT > 42.5 U/L giving the best diagnostic accuracy with a sensitivity of 61%, specificity of 60%, positive predictive value of 60%, negative predictive value of 61%, positive likelihood ratio of 1.53, and negative likelihood ratio of 0.65 for predicting lamivudine resistance (Figure 1 and Table 3). Using this cutoff, lamivudine resistance would be missed in 39% of resistant patients. Using other ALT cutoffs, the diagnostic accuracy for lamivudine resistance was poorer (Table 3). Based on the normal range in our hospital laboratory (ULN ⩽ 70 U/L), only 39% of patients would be diagnosed with lamivudine resistance and 61% would be missed. Based on the new AASLD guidelines (2), which suggested that the ULN for ALT should be decreased to 30 U/L for men and 19 U/L for women, 85% of males and 90% of females would be diagnosed with lamivudine resistance, but a high false positive resistance rate would be observed (72% of males and 93% of females).

A possible confounding factor is that early adefovir rescue (arbitrarily defined as adefovir treatment within 3 mo of lamivudine resistance) would result in resolution of virological breakthrough and arrest the rise in ALT. Consequently, we examined patients who had started adefovir 3 mo after the diagnosis of lamivudine resistance. Of 111 patients, 68 fulfilled this criterion, and we reanalyzed the utility of ALT in the diagnosis of lamivudine resistance using this subgroup. The results were virtually identical to those of the entire cohort of lamivudine resistant patients (n = 111), with the AUROC curve being 0.653, and ALT > 42.5 U/L giving the best sensitivity and specificity for predicting lamivudine resistance.

Abnormal ALT and the duration of lamivudine resistance

Of the 74 patients with abnormal ALT post-resistance, 27 (36%) belonged to the group that started adefovir within 3 mo of developing genotypic lamivudine resistance, indicating that abnormal ALT can occur soon after viral breakthrough.

Patients with persistently normal ALT

For the 37 patients (33.3%) with normal ALT (ULN < 70 U/L) after lamivudine resistance, the mean (SD) follow-up was 11 + 22 mo from the development of lamivudine resistance until adefovir rescue, 24% had at least 6 mo of follow-up from the development of lamivudine resistance until adefovir rescue. However, if the new AASLD guidelines were utilized, only 13 males (ALT < 30 U/L) and 5 females (ALT < 19 U/L) would have fulfilled the criteria for persistently normal ALT.

Patients with abnormal ALT

Of the patients with abnormal ALT (ULN < 70 U/L) [n = 74 (66.7%)] after the development of lamivudine resistance, 24 (21.6%) had ALT flares during lamivudine resistance. Of these 74 patients, 42 (57%) had persistently abnormal ALT, while 32 (43%) had transiently abnormal ALT during resistance. By univariate analysis, only baseline abnormal ALT was associated with abnormal ALT during resistance (P = 0.009). Gender (P = 0.111), race (P = 0.107), cirrhosis (P = 0.673), baseline HBeAg status (P = 1.00) and duration of lamivudine treatment (P = 0.42) were not associated with abnormal ALT during resistance by univariate analysis. Multivariate analysis showed that only abnormal ALT at baseline was associated with abnormal ALT during resistance (OR = 5.98, P = 0.003, 95% CI: 1.8-19.7). Gender, baseline HBV DNA, and baseline HBeAg status were not associated with abnormal ALT during resistance, by multivariate analysis. By univari-

ate analysis, male gender was associated with ALT flares during resistance ($P = 0.02$). Race, age, baseline cirrhosis, albumin, ALT, AST, bilirubin, ALT flare at baseline and HBV DNA level were not associated with ALT flares during resistance. By multivariate analysis, only male gender was associated with ALT flares during resistance (OR = 8.9, $P = 0.016$, 95% CI: 1.5-53.3). Baseline ALT, abnormal ALT, and HBeAg status were not associated with ALT flares during resistance by multivariate analysis.

DISCUSSION

Development of viral resistance is a major concern in the treatment of CHB. This is particularly true for lamivudine, the first licensed nucleoside analogue for therapy of CHB. Lamivudine is highly efficacious but this efficacy is blunted by the rapid development of viral resistance[15]. Since the first descriptions of lamivudine resistance were published, much has been learned about the development of resistance. The first appearance of HBV resistance is the detection of genotypic mutations that confer resistance. Subsequently, viral breakthrough occurs and finally biochemical resistance (defined as the development of abnormal ALT due to viral resistance) appears[10]. However, the question of whether all patients develop biochemical resistance[16] has not been addressed. We have shown that not all patients with viral resistance develop abnormal ALT, with 33% having persistently normal ALT (ULN < 70 U/L) after the development of lamivudine resistance. This finding, however, may be confounded by the insufficient length of follow-up before adefovir rescue. Consequently, when we excluded patients who had early adefovir rescue (arbitrarily defined as adefovir rescue within 3 mo of lamivudine resistance), there were still 31% of patients with persistently normal ALT (ULN < 70 U/L) of which 71% of patients were followed for > 6 mo before adefovir treatment, indicating that length of follow-up was unlikely to be a confounder. Our study showed ALT to be an inadequate diagnostic test for viral resistance, with an AUROC of 0.645 in the best case scenario using an ALT cutoff of 42.5 U/L, which showed a sensitivity of 61%, specificity of 60%, positive predictive value of 60%, negative predictive value of 61%, positive likelihood ratio of 1.53 and negative likelihood ratio of 0.65, and with 39% of patients with lamivudine resistance being missed. Using other ALT cutoff levels, such as the normal range for our laboratory (ULN = 70 U/L) or the AASLD guidelines (males ULN = 30 U/L, females ULN = 19 U/L), did not improve diagnostic accuracy.

Although two different assays were used in our study to measure HBV DNA levels, all patients who were initially assessed to have no viral breakthrough based on the less sensitive Hybrid Capture Ⅱ HBV DNA Test, were subsequently found to have undetectable HBV DNA when tested with the sensitive Artus HBV RG real time PCR kit during follow-up, with a lower limit of detection of 100 copies/mL (1.7×10^1 IU/mL). The majority of HBV DNA quantifications were carried out using a relatively insensitive method (lower limit of detection of 140 000 copies/mL), which might have led to an overestimation of the predictive value of ALT for diagnosing viral resistance, as "late" viral rebounds could result in more cases of viral resistance with abnormal ALT, thus making the poor performance of ALT even more striking.

Our study also showed that not all patients develop clinical resistance. Hence, the postulated evolution of HBV resistance starting with the development of genotypic resistance mutations, followed by viral breakthrough, then clinical resistance may not be applicable to all patients. This is particularly pertinent since the EASL guidelines for the management of CHB[4] state that rescue therapy should be instituted before the advent of clinical resistance, however, this latter finding may never be seen if ALT remains persistently normal. The most important predictor of the development of abnormal ALT upon development of lamivudine resistance was the baseline ALT value and abnormal ALT at baseline. While the ALT value may not be a very useful diagnostic marker for lamivudine resistance, there are additional implications of having a normal ALT despite the presence of viral resistance. Normal ALT values can be associated with histological damage[17] and disease progression in patients with CHB, but does this apply in cases of viral resistance? The evidence is mixed. We previously reported that patients with cirrhosis and lamivudine resistance have a high risk of mortality when untreated. In this group, ALT was normal in a substantial proportion of patients (40%)[18]. In the presence of lamivudine resistance, liver histology may still show improvement. In a pathological study of liver biopsies in patients with and without lamivudine resistance, improvement in histology was highest in those who had no evidence of genotypic resistance, and those who developed lamivudine resistance still had improvement in histology, albeit in a smaller proportion of patients[19]. Other than the implications for histological damage and disease progression, in some Asia-Pacific countries such as South Korea[20], Japan[20], Australia[20,21], and Taiwan (China)[22], an abnormal ALT during viral resistance is a requirement for reimbursement from the Government for rescue therapy with adefovir. This would mean that a significant proportion of patients with lamivudine resistance would not be able to receive rescue therapy and may run the risk of disease progression.

In conclusion, abnormal ALT does not occur in a significant proportion of patients with lamivudine resistance, consequently the predictive value of ALT in diagnosing lamivudine resistance is low, and cannot be used as a surrogate for lamivudine resistance. Although lamivudine is the cheapest oral antiviral agent available in Asia and is globally still the most widely used oral antiviral agent for CHB[23], the added cost of monitoring with expensive HBV DNA assays and viral resistance tests such as InnoLIPA, makes it economically attractive to consider cheaper options to evaluate lamivudine resistance. Unfortunately serum ALT cannot fulfill this role.

COMMENTS

Background

Chronic hepatitis B (CHB) is a global public health problem, being the most

common cause of chronic viral hepatitis, and the major cause of hepatocellular carcinoma worldwide. Lamivudine is still used widely to treat CHB in Asia. However, lamivudine resistance is a major concern.

Research frontiers

The authors determined that alanine aminotransferase (ALT) is an inadequate surrogate marker for detecting lamivudine resistance.

Applications

Other than the implications for histological damage and disease progression, in some Asia-Pacific countries such as South Korea, Japan, Australia, and Taiwan, an abnormal ALT during viral resistance is a requirement for reimbursement from the Government for rescue therapy with adefovir. This would mean that a significant proportion of patients with lamivudine resistance would not be able to receive rescue therapy and may run the risk of disease progression.

Peer review

The manuscript is interesting, since lamivudine is still used in several countries and considering that hepatitis B virus (HBV) DNA quantification and detection of HBV genotypic resistance are not easily available in many of these countries.

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BRIEF ARTICLE

Prospective study of MMP7 serum levels in the diagnosis of cholangiocarcinoma

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Abstract

AIM: To determine whether the serum level of matrix metalloproteinase-7 (MMP7) has the potential to diagnosis cholangiocarcinoma from benign biliary tract diseases.

METHODS: This study was performed according to the PRoBE (a prospective-specimen-collection, retrospective-blinded-evaluation) design. A total of 187 patients with obstructive jaundice were consecutively enrolled. After the diagnostic status of these patients was ascertained, their levels of serum MMP7 were assayed and compared with serum carbohydrate antigen 19-9 (CA19-9). This was conducted in a blinded case (cholangiocarcinoma)-control (benign biliary tract disease) setup.

RESULTS: MMP7 and CA19-9 serum levels were significantly elevated in cholangiocarcinoma patients ($P < 0.001$). The area under the curve (AUC) from a receiver operating characteristic (ROC) curve analysis for the diagnosis of cholangiocarcinoma, using MMP7 was more accurate than CA19-9 (AUC = 0.84, 95% CI: 0.778-0.903 for MMP7 and AUC = 0.79, 95% CI: 0.708-0.868 for CA19-9). The sensitivity and specificity of serum MMP7 (cut-off value of 5.5 ng/mL) was 75% and 78%, respectively, while the sensitivity and specificity of serum CA19-9 (cut-off value of 100 U/mL) was 68% and 87%, respectively.

CONCLUSION: Serum values of MMP7 and CA19-9 appear to be useful biomarkers for differentiating cholangiocarcinoma from benign biliary tract obstructive diseases.

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Key words: Carbohydrate antigen 19-9; Cholangiocarcinoma; Matrix metalloproteinase-7; Sensitivity; Specificity; Tumor marker

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INTRODUCTION

Cholangiocarcinoma is one of the most common causes of cancer-related mortality in Thailand[1]. The high mortality rate of cholangiocarcinoma is due to the aggressiveness of tumors that are often discovered at a late-stage

of disease progression[2]. To improve the survival rate, the diagnosis and treatment of these patients should be performed as soon as possible. Bile duct obstruction is the focal symptom that the vast majority of patients with cholangiocarcinoma present with at hospital. However, there are many cases of benign biliary tract diseases including common bile duct stone and bile duct stricture. These other conditions often present with clinical symptoms similar to those of patients with cholangiocarcinoma. In addition, it is very difficult to obtain pathological tissue for the diagnosis of cholangiocarcinoma due to both the desmoplastic reaction and the tumor location[3,4]. Brush cytology has a sensitivity of only 62.5% for detecting cholangiocarcinoma[5]. Owing to the differences in treatment and prognosis between cholangiocarcinoma and benign biliary tract diseases, the most important issue is to obtain a reliable method to differentially diagnosis patients with cholangiocarcinoma from those with benign biliary tract diseases. Identification of tumor markers in the serum would aid in the accurate diagnosis of cholangiocarcinoma.

To date, carbohydrate antigen 19-9 (CA19-9) is used as a tumor marker for detecting cholangiocarcinoma. The sensitivity and specificity of CA19-9 in diagnosing cholangiocarcinoma were shown to be 53%-89% and 80.5%-86%, respectively[6-9]. Unfortunately, elevated serum levels of CA19-9 have also been found in patients with benign obstructive jaundice[10,11]. Due to this, CA19-9 is a not a reliable marker for differentiating cholangiocarcinoma from benign obstructive jaundice.

Previous studies demonstrated that high expression of matrix metalloproteinase-7 (MMP7) could be readily detected in cholangiocarcinoma specimens[12-14]. We previously performed research to study the serum levels of CA19-9, CEA, MMP9 and MMP7 in patients with obstructive jaundice[6]. This previous study was performed using a case-control design and serum collected from a serum bank. The results showed that only the level of MMP7 was significantly higher in patients with cholangiocarcinoma compared to patients suffering from benign biliary tract disease. In addition, when comparing the areas under the curve of the receiver operating characteristic (ROC) for CEA, CA19-9 and MMP9, a ROC curve analysis demonstrated that the detection of MMP7 in serum was the most accurate for differentiating cholangiocarcinoma from benign biliary tract disease. This finding indicated that serum MMP7 should be used as a tumor marker for the diagnosis of cholangiocarcinoma in patients with obstructive jaundice.

According to the study of biomarker use, it is now widely appreciated that the evaluation of biomarker performance must be separated from biomarker discovery. In discovery research, its performance in samples may be biased in an overoptimistic direction. To estimate performance without bias, an independent dataset should be investigated[15-18]. Therefore, the aim of the present study was to evaluate the performance of serum MMP7 and CA19-9 for their potential in the diagnosis of cholangiocarcinoma. We used a new and independent dataset of prospective consecutive cases with evidence of bile duct obstruction due to various etiologies. This study was performed according to the PRoBE (a prospective-specimen-collection, retrospective-blinded-evaluation) design[15]. We collected serum from a cohort that was representative of the target population (consecutive cases of obstructive jaundice who had undergone ERCP, PTBD or bile duct surgery). After the diagnostic status of these patients was ascertained, the levels of serum MMP7 and CA19-9 were assayed in a fashion that blinded the analysis to a case-control status. In addition, we implemented STARD statements (STAndards for the Reporting of Diagnostic accuracy studies)[16-18] to ensure standardization and transparency of our study.

MATERIALS AND METHODS

Patients and study design

This study was conducted within the Rajavithi Hospital Surgery Department located in Bangkok, Thailand. The local ethics committee approved the study protocol. Sample size was calculated on the basis of an expected area under the ROC curve of MMP7 serum levels (= 0.70) for the diagnosis of cholangiocarcinoma[6]. Using a significance level of 0.05 (two-sided) and a power of 0.95, a sample of 50 cholangiocarcinoma patients was required for the study[19]. From previous data, the prevalence of cholangiocarcinoma detection in patients with obstructive jaundice treated at our department was shown to be in the range of 27%-30%. Therefore, we prospectively included consecutive patients with symptoms of obstructive jaundice who had undergone ERCP, PTBD or bile duct surgery during the period from June of 2008 to July of 2009. Exclusion criteria included presence of other cancers, age less than 20 years and the presence of severe pulmonary fibrosis[20]. All patients gave written informed consent. The diagnosis of cholangiocarcinoma was carried out using one of the following tests[6]: (1) tissue biopsy; and (2) cytology plus radiological (helical CT scan or MRI) and clinical observation to identify tumor progression at a follow-up of at least two months. Patients with an inconclusive diagnosis were excluded from this study.

Serum collection and the measurement of serum biochemistry

Five-milliliter samples of fasting peripheral venous blood were collected from the patients before ERCP, PTBD, or bile duct surgery were carried out, and their serum was separated and stored at -78℃ within 2 h. Serum biochemical tests including albumin, globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, alkaline phosphatase (ALP) and CA19-9 were measured using routine automated methods in Rajavithi Hospital Pathological Laboratory.

Measurement of serum MMP7 levels

The serum levels of MMP7 were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Sys-

tems, Minneapolis, MN, USA), as previously described[6]. Briefly, the diluted serum samples were added in duplicate to 96-well plates coated with MMP7 antibody and then incubated at room temperature for 2 h. After washing, the conjugated secondary antibody was added, and the plate was further incubated for another 2 h. Plates were washed again prior to incubation with the substrate solution for 1 h. Following termination of the reaction with the stop solution (1 N sulfuric acid), the optical density was measured at 490 nm using a spectrophotometric microplate reader. The concentration of MMP7 in each sample was calculated from a standard curve. The scientist examining these serum samples was unaware of the patient's diagnosis. In addition, the MMP7 test results had no influence on the clinical diagnosis of the patients in the study.

Statistical analysis

Data are presented as the mean ± SD, unless otherwise mentioned. Comparisons between the quantitative variables were performed using Mann-Whitney *U* or Student's *t*-test, as appropriate. One-way analysis of variance (ANOVA) with multiple comparisons by the Post HOC Scheffe method or Kruskal Wallis test was used to compare each value (MMP7, CA19-9) to the control early and late stage cholangiocarcinoma groups. Qualitative variables were reported as counts, and comparisons between independent groups were performed using Pearson Chi-square tests. Correlations between MMP7 levels and other parameters were examined using the Pearson correlation coefficient. A ROC curve was generated by plotting the sensitivity against 1-specificity, and the area under the curve with 95% confidence intervals (CI) was calculated. The optimal cutoff points for MMP7 were selected based on the ROC curve analysis. Sensitivity, specificity, positive predictive value and negative predictive values were calculated using a 2 × 2 table of the collected data. The data on various blood chemistries and levels of CA19-9 and MMP7 that were significantly different between the control and cholangiocarcinoma groups were analyzed by multiple logistic regression analysis.

RESULTS

Patient characteristics

A total of 230 patients with obstructive jaundice were consecutively enrolled. Twenty-four cases were excluded according to their diagnosis of ampullary cancer (7 cases), pancreatic cancer (9 cases), gall bladder cancer (3 cases), duodenum cancer (2 cases), metastatic cancer from ovarian cancer (1 case) and hepatocellular carcinoma (2 cases). In addition, nineteen cases were excluded according to their uncertain diagnosis. The 187 subjects studied included 128 patients with benign biliary tract diseases (control group) which included intra-hepatic duct stones, common bile duct stones, and benign bile duct strictures, and a total of 59 patients with cholangiocarcinoma. For cholangiocarcinoma, 40 cases were diagnosed as perihilar-cholangiocarcinoma, 16 cases were diagnosed as intrahepatic cholangiocarcinoma and 3 cases were diagnosed as distal common bile duct cholangiocarcinoma (Figure 1).

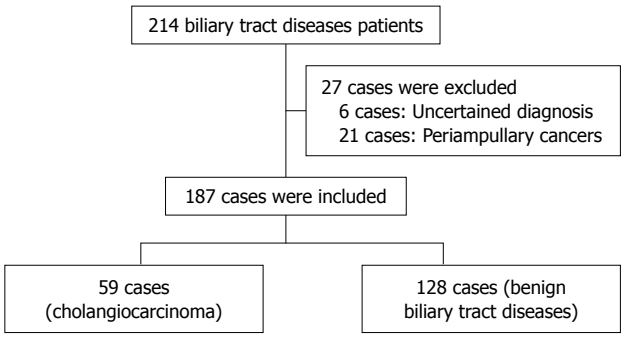

Figure 1 A flow diagram of 187 obstructive jaundice patients who were consecutively enrolled in this study.

Table 1 Clinical characteristics of patients with benign biliary tract diseases (control) and cholangiocarcinoma

	Control *n* = 128	Cholangiocarcinoma *n* = 59	*P*
Age (yr)	61 ± 7	67 ± 5	0.451
Sex (M:F)	62:66	36:23	0.118
Albumin (mg/dL)	3.9 ± 0.67	3.1 ± 0.59	< 0.001
Globulin (mg/dL)	3.9 ± 0.72	4.1 ± 0.91	0.073
Total bilirubin (mg/dL)	3.3 ± 3.71	12.0 ± 11.35	< 0.001
AST (U/L)	73.4 ± 78.14	91.2 ± 75.91	0.003
ALT (U/L)	76.3 ± 83.32	52.2 ± 43.58	0.884
ALP (U/L)	320.3 ± 230.03	380.5 ± 314.52	< 0.001

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

As shown in Table 1, no statistically significant differences in gender, age, serum globulin and ALT levels were identified among the data from the control patients when compared to the cholangiocarcinoma patients. However, the level of serum albumin, AST, bilirubin and ALP were significantly higher in cholangiocarcinoma patients than in the control patients (Mann-Whitney *U* test, $P < 0.05$).

Serum levels of CA19-9 and MMP7

The serum CA19-9 and MMP7 levels were compared among disease groups. The median values of serum CA19-9 levels were 20.43 U/mL (range: 0.6-71000 U/mL) in the control group and 571.2 U/mL (range: 0.6-71000 U/mL) in the cholangiocarcinoma group. The mean values of serum MMP7 levels were 3.7 ± 2.81 ng/mL in the control group and 8.7 ± 4.56 ng/mL in the cholangiocarcinoma group. As shown in Figure 2A and B, serum CA19-9 and MMP7 values were significantly higher in cholangiocarcinoma cases when compared to the control patients (CA19-9: Mann-Whitney *U* test, $P < 0.001$ and MMP7: Student's *t*-test, $P < 0.001$).

Moreover, we also classified cholangiocarcinoma patients into two groups: early (TNM stage I and Ⅱ; 11 patients) and advanced (TNM stage Ⅲ and Ⅳ; 48 patients) stages. Although the serum CA19-9 values in the early and late stages of cholangiocarcinoma were significantly higher than in the controls (Kruskal Wallis test, $P < 0.001$), the values were not significantly different between the early

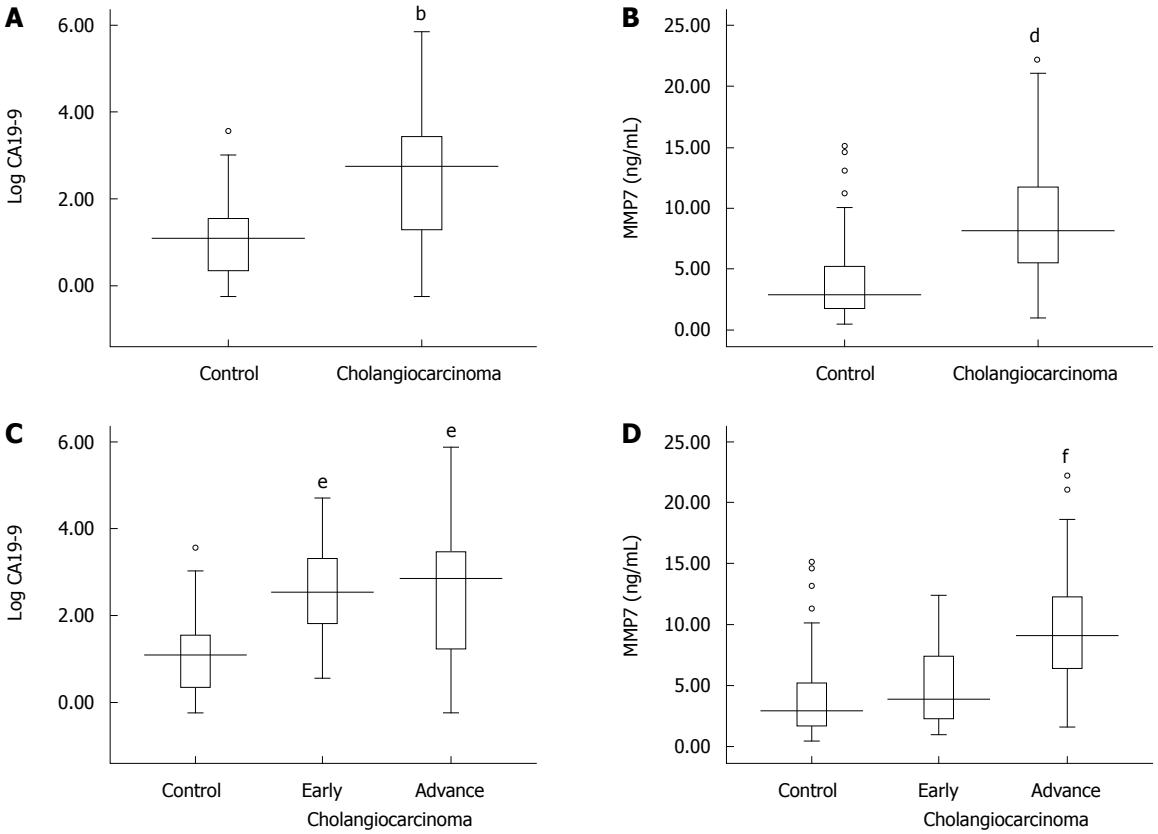

Figure 2 Serum levels of carbohydrate antigen 19-9 and matrix metalloproteinase-7 in cholangiocarcinoma and control (benign biliary tract disease) patients. A: Box plots comparing levels of carbohydrate antigen 19-9 (CA19-9); B: Matrix metalloproteinase-7 (MMP7) between cholangiocarcinoma and control patients are illustrated; C: Box plots comparing levels of CA19-9; D: MMP7 between early and advanced stages of cholangiocarcinoma and controls are illustrated. Levels of MMP7 are presented as ng/mL, while CA19-9 is presented with the log data to accommodate the wide range. ([b]Mann-Whitney *U*, $P < 0.001$ *vs* control; [d]Student's *t*-test, $P < 0.001$ *vs* control; [e]Kruskal Wallis test, $P < 0.001$ *vs* control; [f]ANOVA, $P < 0.001$ *vs* control).

and late stages of cholangiocarcinoma (Figure 2C). The data shown in Figure 2D demonstrates that the MMP7 levels tended to increase according to the progression of cholangiocarcinoma. The serum MMP7 levels were significantly different between early and late stages of cholangiocarcinoma (ANOVA, $P < 0.001$). However, the serum MMP7 levels in early stage cholangiocarcinoma were not significantly different from the serum MMP7 levels in benign control patients (ANOVA, $P = 0.47$).

Serum levels of CA19-9 and MMP7 for the diagnosis cholangiocarcinoma

To determine the diagnostic accuracy of serum CA19-9 and MMP7 levels for differentiating cholangiocarcinoma from benign bile duct diseases, a ROC curve analysis was applied to calculate the area under the curve (AUC). These levels were determined to be 0.79 (95% CI: 0.708-0.868) and 0.84 (95% CI: 0.778-0.903) for CA19-9 and MMP7, respectively (Figure 3). The sensitivity, specificity, positive and negative predictive values for selected cut-off points of CA19-9 and MMP7 are presented in Table 2.

When the cut-off value of serum MMP7 was set at 5.5 ng/mL and serum CA19-9 values were set at 100 U/mL, the predictive probabilities for the diagnosis of cholangiocarcinoma could then be calculated from logistic regression analysis. As shown in Table 3, if the patients had a serum MMP7 and CA19-9 level higher than the cut-off values, the probability of a diagnosis of cholangiocarcinoma was equal to 86.12%. In addition, if the patients had a serum MMP7 and serum CA19-9 level less than the cut-off values, the probability of a positive diagnosis of cholangiocarcinoma was very low (< 6.4%).

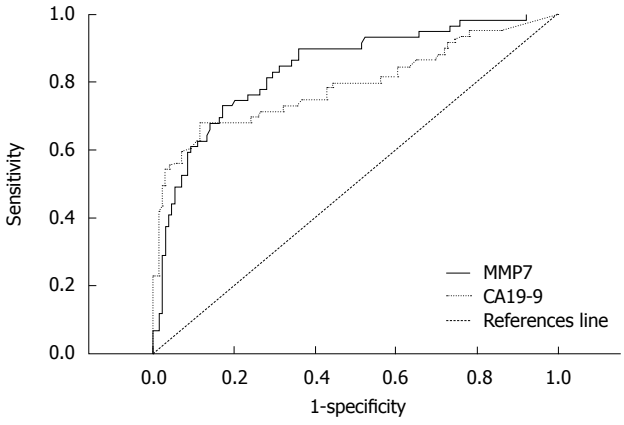

Figure 3 Receiver operating characteristic curve analyses of carbohydrate antigen 19-9 and matrix metalloproteinase-7 for the diagnosis of cholangiocarcinoma. The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, are presented by receiver operating characteristic curve analysis. MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Table 2 Performance of the biomarkers for the diagnosis of cholangiocarcinoma (%) (95% CI)

Tumor markers (cut-off value)	Sensitivity	Specificity	PPV	NPV	LR+	LR-
MMP7 (5.5 ng/mL)	75 (63-86)	78 (71-85)	61 (50-72)	87 (81-93)	3.41 (2.38-4.89)	0.33 (0.21-0.51)
MMP7 (6.5 ng/mL)	63 (50-75)	87 (81-93)	69 (56-81)	83 (77-90)	4.72 (2.91-7.66)	0.43 (0.31-0.60)
MMP7 (7.5 ng/mL)	53 (40-65)	92 (88-97)	76 (62-89)	81 (74-87)	6.73 (3.54-12.70)	0.51 (0.39-0.68)
CA19-9 (35 U/mL)	71 (60-83)	73 (66-81)	55 (44-66)	85 (78-91)	2.68 (1.93-3.73)	0.39 (0.26-0.59)
CA19-9 (100 U/mL)	68 (56-80)	87 (81-93)	70 (58-82)	85 (79-91)	5.1 (3.17-8.22)	0.37 (0.25-0.54)
CA19-9 (200 U/mL)	59 (47-72)	93 (89-97)	80 (68-91)	83 (77-89)	8.44 (4.34-16.40)	0.44 (0.32-0.60)

PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; CI: Confidence interval; MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Table 3 Predicted probability of the combination of serum carbohydrate antigen 19-9 and matrix metalloproteinase-7 for diagnosis of cholangiocarcinoma

CA19-9 (> 100 U/mL)	MMP7 (> 5.5 ng/mL)	Predicted probability (%)
-	-	6.40
-	+	36.10
+	-	42.84
+	+	86.12

MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Table 4 Pearson's correlation coefficients of matrix metalloproteinase-7, carbohydrate antigen 19-9, albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase

Pearson correlation	CA19-9	Albumin	Total bilirubin	AST	ALT	ALP
MMP7	0.415[a]	-0.577[a]	0.328[a]	0.154[a]	-0.055	0.268[a]
CA19-9	0.415[a]	-0.370[a]	0.356[a]	0.064	-0.022	0.139

[a]Statistically significant, $P < 0.05$. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Correlation between MMP7, CA19-9 and other blood chemistry

The correlations between the levels of serum albumin, AST, ALT, ALP, total bilirubin, CA19-9, and MMP7 were investigated. As presented in Table 4, the level of serum MMP7 was significantly correlated with serum albumin, AST, ALP, total bilirubin and CA19-9, although none of these parameters had a high Pearson correlation coefficient value (> 0.7). We suggest that the significant correlation of these blood chemistries with serum MMP7 was caused by the high number of samples analyzed in this study.

Evaluation of serum CA19-9 and MMP7 levels for the diagnosis of cholangiocarcinoma: Multiple logistic regression analysis

To determine whether the levels of serum CA19-9 and MMP7 were predictive of cholangiocarcinoma independent of the other blood chemistry levels that were significantly different between control and cholangiocarcinoma patients, we carried out a logistical regression analysis. In a multivariable model using CA19-9 (cut-off value = 100 ng/mL), MMP7 (cut-off value = 5.5 ng/mL), total bilirubin (cut-off value = 5 mg/dL), albumin (cut-off value = 4 mg/dL), AST (cut-off value = 100 U/L) and ALP (cut-off value = 200 U/L), CA19-9, MMP7 and albumin were shown to be independent predictors for cholangiocarcinoma. None of the other parameters (total bilirubin, AST and ALP) reached statistical significance (Table 5).

Table 5 Odd Ratios estimates for diagnosis of cholangiocarcinoma

Variables	OR (95% CI)	*P*
CA19-9	15.2 (5.20-44.56)	< 0.001
MMP7	5.5 (1.87-16.03)	0.002
Albumin	0.015 (0.01-0.15)	< 0.001
Total bilirubin	2.4 (0.81-7.20)	0.115
AST	1.2 (0.37-4.12)	0.738
ALP	0.3 (0.09-1.05)	0.060

The significant parameters ($P < 0.05$) selected by the model are shown. AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

DISCUSSION

Our study demonstrates that serum MMP7 levels are significantly elevated in patients with a diagnosis of cholangiocarcinoma when compared to patients suffering from benign bile duct diseases. When we compared MMP7 to CA19-9, which is a common clinically-used biomarker of cholangiocarcinoma, the value of AUC from the ROC curve demonstrated that serum levels of MMP7 are better than CA19-9 for the diagnosis of cholangiocarcinoma. These results are consistent with our previous study, in which serum MMP7 was higher in cholangiocarcinoma than in benign obstructive jaundice patients[6]. This suggested that serum MMP7 has the potential to be a tumor marker for cholangiocarcinoma in patients with obstructive jaundice.

Previous studies have demonstrated that MMP7 plays a key role in the mechanism of cancer invasion *via* proteolytic cleavage of the extracellular matrix tissues. It has also been shown to activate other MMPs, such as proMMP-2 and proMMP-9[21], and inhibit E-cadherin function by ectodomain shedding of E-cadherin[22]. The results of several recent studies indicate that MMP7 is over-expressed in a

variety of epithelial tumors including those of the esophagus[23], colon[24,25], pancreas[26], and cholangiocarcinoma tumors[12]. In addition, several studies have shown that MMP7 could be detected in the serum of cancer patients, including patients with ovarian[27], colorectal[28] and gastric cancer[29]. This finding suggests that high levels of serum MMP7 are not specific to cholangiocarcinoma. It can be detected in many types of cancer. Therefore, it should be used with other diagnostic modalities (clinical presentation and imaging study) before making a diagnosis.

In this study, the levels of blood chemistry markers were shown to be significantly different between control and cholangiocarcinoma groups. Although several differences were observed, serum CA19-9 and MMP7 levels were shown to be predictors of cholangiocarcinoma, independent of other blood chemistry values. In addition, the present study is the first to demonstrate the probability of a diagnosis of cholangiocarcinoma using the combination of serum MMP7 and CA19-9 levels (Table 3). We suggest that the combination of these markers will aid the physician to identify cholangiocarcinoma from benign obstructive jaundice.

The values of AUC from the ROC curve for MMP7 and CA19-9 in this study were shown to be much higher than those observed in our previous study[6]. The differences in the designs of these studies should be considered. Our previous study was designed as a retrospective case-control study for diagnostic accuracy. Therefore, some bias from the selection of samples may have occurred. A strength of the present study was the implementation of the strategies of the PRoBE designs to avoid the problems of bias that may affect the studies of the diagnostic test[15]. We collected serum from all obstructive jaundice patients before the diagnosis of cholangiocarcinoma or benign biliary tract diseases was determined. This procedure assured that biases related to differences in sample collection and handling would be avoided[30]. Limitations of this design include the fact that the majority of the study participants were in advanced stages of cholangiocarcinoma. The number of patients with early-stage cholangiocarcinoma was small (n = 11), and this number of patients would not have had the statistical power to detect a difference in mean value between these early stages of cholangiocarcinoma and the control group. Further studies, which should include an increased number of early-stage cholangiocarcinoma cases, need to be carried out before using MMP7 as a screening test for the detection of early stage cholangiocarcinoma. In addition, this study was performed in a referral center, which has a high prevalence of cholangiocarcinoma. As a result, the findings may not be broadly applicable to other hospitals that typically have a low volume of cholangiocarcinoma.

In conclusion, this study demonstrated that serum MMP7 levels are significantly elevated in cholangiocarcinoma patients. This marker has the potential to be used as a new tumor marker for discriminating cholangiocarcinoma patients from benign biliary tract disease patients.

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COMMENTS

Background

To date, carbohydrate antigen 19-9 (CA19-9) is used as a tumor marker for detecting cholangiocarcinoma. Unfortunately, elevated serum levels of CA19-9 have also been found in patients with benign obstructive jaundice. Previous studies demonstrated that cholangiocarcinoma cells express a high level of matrix metalloproteinase (MMP)-7.

Research frontiers

High expression of MMP7 was detected in cholangiocarcinoma specimens. In addition, the authors' previous nonconsecutive case-control study demonstrated that the serum level of MMP7 is higher in cholangiocarcinoma than in benign biliary tract disease patients. However, a prospective consecutive study of the evaluation of serum MMP7 as a diagnostic marker for cholangiocarcinoma has not been established. In this study, the authors collected a new and independent dataset of prospective consecutive cases with evidence of bile duct obstruction due to various etiologies, and demonstrated that the serum level of MMP7 could be a potential tumor marker for differentiating cholangiocarcinoma from benign biliary tract obstruction.

Innovations and breakthroughs

This is the first consecutive prospective study to report that the serum level of MMP7 was significantly higher in patients with cholangiocarcinoma than in those with benign biliary tract obstruction. The authors suggest that the serum level of MMP7 may be a potential tumor marker for differentiating cholangiocarcinoma from benign biliary tract obstruction.

Applications

This study may represent a future strategy for diagnosing patients with cholangiocarcinoma by the detection of serum level of MMP7 and CA19-9.

Peer review

This is a prospective-specimen-collection and retrospective-blinded-evaluation study of 187 patients with obstructive jaundice where a novel serum marker, MMP7, for the diagnosis of cholangiocarcinoma was investigated. In general, it's a nicely designed and accomplished study with sound conclusion, hopefully of interest for a wide range of readers and researchers.

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BRIEF ARTICLE

Usefulness of Y-shaped sheaths in CT angiography for examination of liver tumors

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Abstract

AIM: To conduct a single-stage, combined computed tomography (CT) arterial portography (CTAP) and CT arteriography (CTA) imaging operation, we used Y-shaped sheaths with 2 valves, which allowed the insertion of 2 catheters simultaneously.

METHODS: Of 1254 patients who underwent abdominal angiography for transarterial embolization and/or intraarterial chemotherapy in our department from May 2002 to November 2009, 664 patients in whom Y-shaped sheaths with 2 valves were used underwent CT angiography using a combination of CTA and CTAP. The Seldinger method was used to insert a 10 cm Y-shaped short sheath with 2 valves into the femoral artery. Under radiographic guidance, a 3.2 French (Fr) catheter was placed in the celiac artery or proper hepatic artery, and a second 3.2 Fr catheter was then placed distal to the inferior pancreaticoduodenal artery of the superior mesenteric artery. CTAP was then performed followed by CTA 10 min later. Photographs were taken during the early and late phases of the procedure.

RESULTS: Insertion of 3.2 Fr catheters was not possible in 6 of 664 (0.9%) patients with strong curvature of the femoral artery and 4 of 664 (0.6%) patients with strong curvature of the abdominal aorta. In addition, performing CTAP and CTA as a single-stage combined intervention was not possible in 14 of 664 (2.1%) patients whose right hepatic artery originated from the superior mesenteric artery and in 8 of 664 (1.2%) patients whose left hepatic artery branched from the left gastric artery. There were no sheath-related complications such as those related to arterial dissection or hemostasis.

CONCLUSION: Although transfers to and from the CT room were necessary for anatomically variant patients, CT angiography using the Y-shaped sheath for combined CTAP and CTA was considered useful.

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Key words: Computed tomography angiography; Interventional radiology; Liver tumors

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INTRODUCTION

Computed tomography (CT) arterial portography (CTAP)

has an extremely high detectability of liver tumors such as hepatocellular carcinoma (HCC) and metastatic liver carcinoma[1-3]. However, the disadvantage of using CTAP alone is that it does not allow for evaluation of arterial blood flow or the existence of non-neoplastic low-concentration regions[3]. Therefore, the combination of CTAP with CT arteriography (CTA) would make it possible to evaluate arterial blood flow, improve the detectability of liver tumors, and reduce the occurrence of false positives[4,5]. Murakami *et al*[3] revealed that there was no significant difference between the sensitivity of CTAP (85%) and CTA (87%) for HCC nodules. However, they concluded that the combination of CTAP and CTA showed significantly higher sensitivity than either CTAP or CTA alone.

In facilities where interventional radiology CT systems are not available, performing the 2 procedures can be complicated and may include having to transfer patients between the angiography and CT rooms. To conduct a single-stage, combined CTAP and CTA imaging operation, we used Y-shaped sheaths with 2 valves, which allowed the insertion of 2 catheters simultaneously. In the current paper, we report on the usefulness of this approach and the problems associated with it.

MATERIALS AND METHODS

Subjects and methods

CT angiography has been used in our department since May 2002. Of the 1254 patients who underwent abdominal angiography in our department from May 2002 to November 2009, 664 patients (421 men and 243 women) were selected to undergo CT angiography with a combination of CTA and CTAP using Y-shaped sheaths with 2 valves. Exclusion criteria included patients older than 80 years and patients with advanced liver disease (Child-Pugh class C), hepatic encephalopathy, refractory ascites, coagulation abnormality, and portal branch occlusion. Excluded patients in this study underwent abdominal angiography with/without CTAP only through a 5 French (Fr) single sheath. In addition, these patients were excluded from the study because the tumor lesion showed no hypervascularity and CTA was not needed.

A 10 cm Y-shaped short sheath (6 Fr) with 2 valves (S1 Sheath, Terumo Clinical Supply Co., Ltd, Japan) was inserted into the right or left femoral artery, using the Seldinger method (Figure 1). Angiography then was performed. The Y-shaped sheaths with 2 valves allowed for simultaneous insertion of 2 catheters (3.2 Fr; Selecon PA catheters, Terumo Clinical Supply Co., Ltd, Japan) from either the linear directional valve or the 30 degree angle valve (Figure 1).

Shepherd hook or cobra-type 3.2 Fr catheters (Terumo Clinical Supply Co., Ltd., Japan) were used (Figure 1). The first was placed under radiographic guidance into the proper hepatic artery, and the second was placed distal to the inferior pancreaticoduodenal artery of the superior mesenteric artery. Patients then were moved to the CT room, where CTAP was performed followed by CTA 10 min later. For CTAP, 40 mL of a nonionic contrast medium 140 mg I/mL (Omnipaque 140; Daiichi Sankyo, Tokyo, Japan) was injected into the superior mesenteric artery at a speed of 2.0 mL/s. CT imaging of the entire liver was performed 30 s after the beginning of the injection of the contrast medium. For CTA, 40 mL of a 140 mg I/mL nonionic contrast medium was injected at a speed of 2.0 mL/s after insertion of a catheter into the proper hepatic or common hepatic artery. CT imaging of the entire liver was performed 10 s after the beginning of the injection of the contrast medium for phase I (early arterial phase) and 40 s later for phase Ⅱ (delayed phase). CT imaging was performed at a voltage of 130 kV, a tube current of 200 mA, an X-ray beam width of 7 mm, and a table speed of 7 mm/s (Figure 2).

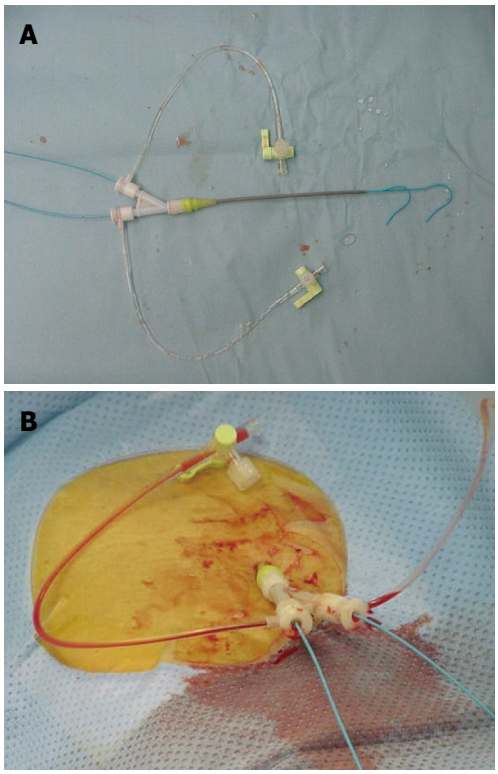

Figure 1 Y-shaped sheath and catheter systems. A: Y-shaped sheath with 2 valves and 2 catheters (3.2 French in size) through the angiographic sheath. B: Placing Y-shaped sheath introducers through the right femoral artery.

RESULTS

A total of 552 patients had HCC and 112 patients had metastatic liver cancer. The primary tumor was colon cancer in 61 cases, gastric cancer in 31 cases, and pancreatic cancer in 20 cases. Average age (± SD) was 69 ± 6 years.

Although 3.2 Fr catheters lack torque ability, we encountered no resistance to their insertion into the 2 Y-shaped valves when a 0.025 inch guidewire was inserted into the catheters. We were able to select the superior mesenteric artery and the common hepatic artery easily. A representative case revealed that classical HCC was diagnosed simultaneously by CTAP and CTA using 3.2 Fr catheters in the Y-shaped sheath (Figure 3). Insertion presented difficulties in only 26 (4.0%) of the 664 patients;

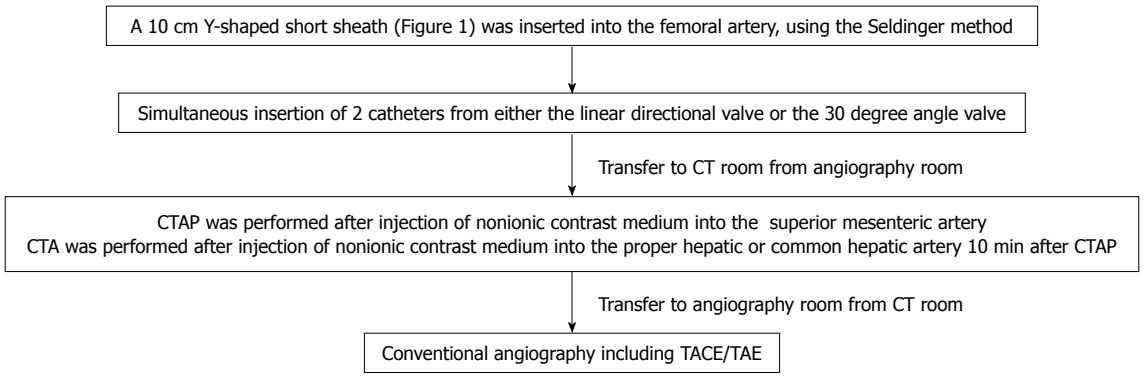

Figure 2 The whole diagnostic process including insertion of the catheters, angiography, computed tomography arterial portography and computed tomography arteriography. CT: Computed tomography; CTAP: CT arterial portography; CTA: CT arteriography; TACE: Transcatheter arterial chemoembolization; TAE: Transarterial embolization.

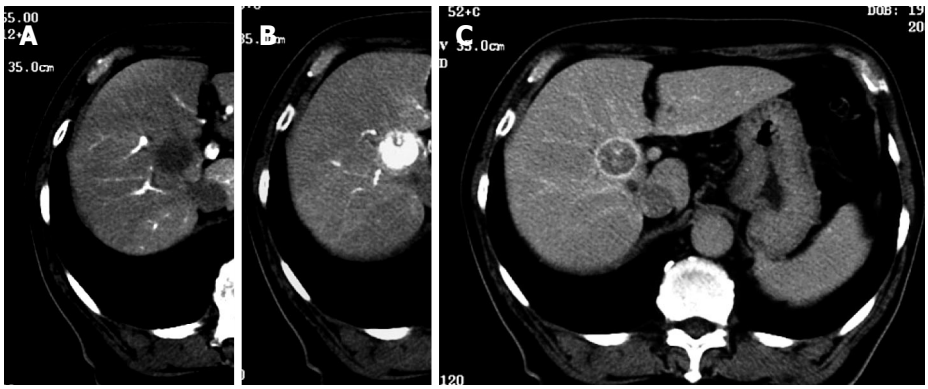

Figure 3 Representative case of classical hepatocellular carcinoma diagnosed using 3.2 Fr catheters in the Y-shaped sheath. A: Computed tomography (CT) arterial portography; B: CT arteriography early phase; C: CT arteriography late phase.

in these cases, CTAP and CTA had to be performed separately. Of the 26 patients, 21 (3.8% of 552 cases) had HCC, and 5 (4.46% of 112 cases) had metastatic liver cancer (Table 1). There was one patient in whom the presence of the first catheter impeded smooth sliding of the second catheter, rendering insertion impossible. In this case, manipulation of the second catheter caused the first catheter to move. As a result, it was impossible to perform CTAP and CTA as a single-stage combined operation. In 6 patients with strong curvature of the femoral artery and 3 patients with strong curvature of the abdominal aorta, sheath insertion was difficult and insertion of the 3.2 Fr catheter impossible (Figure 4).

In addition, it was impossible to perform CTAP and CTA in 2 separate rounds in 12 of 18 patients with an anatomical anomaly (replaced right hepatic artery), in which the right hepatic artery branched from the superior mesenteric artery. In addition, CTAP and CTA as a single-stage combined operation was not possible in 6 (0.9%) of 664 patients whose left hepatic artery branched from the left gastric artery.

No arterial spasm or intimal damage was found in any of the patients, and catheter placement was feasible. Time to hemostasis was 15 min or less in all patients and was not different from time to hemostasis using 5 Fr sheaths. However, a marked subcutaneous hematoma was found in one patient with advanced hepatic cirrhosis and a marked decrease of coagulation factors.

Table 1 Insertion difficulty cases

	n (%)
Overall	26/664 (3.91)
Hepatocellular carcinoma	21/552 (3.80)
Metastatic liver tumor	5/112 (4.46)
Reason for insertion difficulty	
Sliding by friction of 2 catheters: 1 case	
Strong curvature of femoral arteries: 6 cases	
Strong curvature of abdominal aorta: 3 cases	
Anatomical anatomy	
Replaced right hepatic artery: 12 cases	
Left hepatic artery from the left gastric artery: 4 cases	

DISCUSSION

CTA and CTAP are useful for understanding the dynamics of blood flow in the diagnosis of liver tumors[1-3]. They are particularly useful as diagnostic imaging methods for evaluating the degree of progression of HCCs. These liver tumor imaging techniques provide transcatheter angiography technology, allowing separate evaluation of hepatic arterial and portal blood flow, in combination

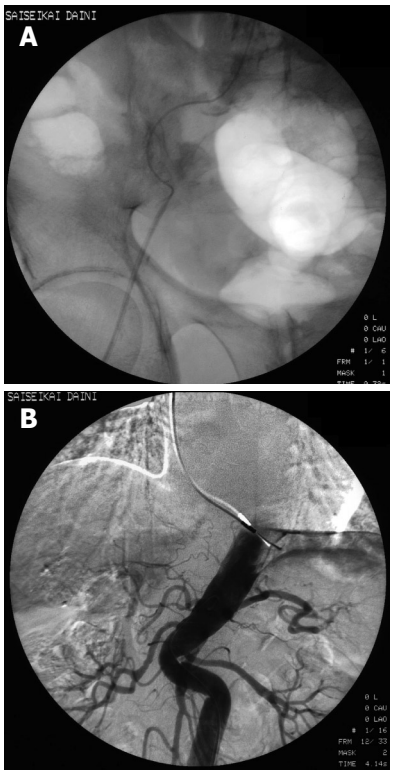

Figure 4 Sheath insertion was difficult and insertion of the 3.2 Fr catheter was impossible. A: Representative case with strong curvature of the femoral artery; B: Representative case with strong curvature of the abdominal aorta.

with CT technology, resulting in an exhaustive volume of data[6].

In Japan, CT angiography has become the gold standard for the diagnosis of liver tumors since its development by Matsui *et al*[7,8]. CTA/CTAP have made early diagnosis of early HCC possible. In addition, these techniques have made it possible to detect microscopic HCC that are difficult to detect with conventional CT. Results provide information that is important in determining treatment selection for patients with HCC.

CTA/CTAP selectively enhances the contrast of the portal vein and the hepatic artery through the superior mesenteric artery, and the resulting CT liver images are used for evaluation. During angiography, it is necessary to insert a catheter into the superior mesenteric and hepatic arteries, or, in certain circumstances, into other blood vessels in order to perform a contrast-enhanced CT. For this reason, it is necessary to move between the angiography device and the CT device. When there is a need to perform CTA/CTAP, a catheter is placed into the superior mesenteric artery under radiographic guidance, with the patient positioned on the angiography equipment; the patient then is transferred to a stretcher for transfer *via* a long corridor to the CT room, where he or she is moved again, this time to the CT machine. During this laborious process, patients sometimes experience dislodgement of the catheter, which may occur after CT images are taken. Therefore, in many facilities without interventional radiology systems, the transfer from the angiography room to the CT room can pose problems when CTAP and CTA are performed together. Consequently, revising the process to include only one transfer is desirable to minimize examination time, the burden on patients, and any associated complications. Thus, various innovations, such as puncture in both inguinal regions[9], and the use of balloon catheters and coaxial systems, have been devised.

Irie *et al*[10] devised a process for combining CTAP and CTA by performing a single puncture of the femoral artery using balloon catheters. However, the procedure was complicated because of the possibility of inflow of contrast medium into the hepatic artery during CTAP. Therefore, expectations shifted to sheaths allowing insertion of 2 catheters, ideally through a puncture at a single location. In our hospital, the use of Y-shaped sheaths with 2 valves is used in combination with CT angiography, thereby reducing the coming and going from the CT room to the angiography room to a minimum.

To our knowledge, there have no reports on the efficacy and safety of these procedures. CTAP images are not obtained by this method because of the possibility of inflow of the contrast medium to the hepatic artery through the inferior pancreaticoduodenal artery due to the introduction of the contrast medium at the superior mesenteric artery. In addition, collateral circulation, such as a splenorenal shunt accompanying portal hypertension, can reduce the hepatopetal flow from the portal vein and attenuate contrast images of the liver. However, pure CTAP images have been obtained by placing a catheter in the superior mesenteric artery distal to the inferior pancreaticoduodenal artery, thereby avoiding inflow to the hepatic artery. Additionally, because 3.2 Fr catheters have no torque ability, it is necessary to replace the catheters in patients with strong curvature or arteriosclerosis of the abdominal aorta. Murakami *et al*[11] reported that the triple-lumen balloon catheter technique is useful and convenient in the serial performance of CTAP and CTA.

Although movement to and from the CT room is necessary for some patients with anatomical anomalies, CT angiography using the Y-shaped sheath with 2 valves is still considered useful. These sheaths allow for the simultaneous insertion of 2 catheters (3.2 Fr) from either the linear directional valve or the 30 degree angle valve. In addition, even though the 6 Fr catheter has a bigger diameter than the 5 Fr catheter, no sheath-related complications relating to arterial dissection or hemostasis have been observed. Because 3.2 Fr catheters have low visibility under radioscopy, we selected the celiac artery and the superior mesenteric artery by inserting a 0.025 inch guidewire into the catheter. However, because 3.2 Fr catheters also have no torque ability, some patients with strong curvature or arteriosclerosis of the abdominal aorta also need catheter replacement. In some of these patients, the issue has been resolved by the use of 25 cm long sheaths. There were no sheath-related complications such as those related to arterial dissection or hemostasis. No arterial spasm or intimal damage was found in any of the patients, and catheter placement was feasible.

There have been reports that treatment outcomes

have been improved by the use of CTAP and CTA in transcatheter arterial chemoembolization[12]. Determining how to deal with anatomical anomalies of the hepatic artery during CTA remains an ongoing challenge. Of the various modalities for the diagnostic imaging of liver tumors, CTA is a complex and invasive procedure; however, it is a unique and also the most sensitive method for the assessment of blood flow in hepatocellular nodules, and it allows excellent, separate analysis of the blood supply in the hepatic artery and portal vein.

In conclusion, we report that the complicated process involved in the transfer of patients between angiography and CT rooms could be improved by the use of the Y-shaped sheath with 2 valves.

COMMENTS

Background

Computed tomography (CT) arterial portography (CTAP) and CT arteriography (CTA) has been widely used for the high detectability of liver tumors. However, in facilities where interventional radiology CT systems are not available, it is necessary to move twice between the angiography room and the CT room to perform CTAP and CTA. Y-shaped sheaths with 2 valves are useful to conduct a single-stage, combined CTAP and CTA imaging operation.

Research frontiers

In this study, the usefulness of CTAP and CTA by Y-shaped sheath and the problems associated with the procedure are reported.

Innovations and breakthroughs

This study has confirmed that a Y-shaped sheath with 2 valves could avoid the complicated process involved in the transfer of patients between angiography and CT rooms.

Applications

Y-shaped sheaths in CT angiography for examination of liver tumors is useful to conduct a single-stage, combined CTAP and CTA imaging operation.

Peer review

This is an interesting study. The authors present a series of 664 patients with hepatic tumors who underwent CT arteriography and arterial portography, applying a new approach using Y-shaped sheaths. The authors concluded that using this new device, the complicated process involving the transfer from the angiography CT suite could be improved.

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BRIEF ARTICLE

Usefulness of magnifying endoscopy for iodine-unstained lesions in a high-risk esophageal cancer population

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Abstract

AIM: To investigate the usefulness of magnified observations of iodine-unstained esophageal lesions in the histological diagnosis of esophageal mucosa abnormalities, in high-risk esophageal cancer groups.

METHODS: The subjects included 38 patients who had at least one of the four criteria known to be high-risk factors for esophageal cancer. Following endoscopic observation, magnified observations were performed on iodine-unstained lesions of the esophagus. The total number of lesions was 43. These lesions were classified as type A (clear papilla), type B (fused papilla), and type C (non-visible papilla) according to the findings. Tissue biopsy was then carried out. Finally the histological findings were graded in terms of histological factors, and their relationships were compared.

RESULTS: Of the 43 lesions, 11 were type A, 17 were type B, and 15 were type C under magnifying endoscopy. Histological findings such as inflammatory cell infiltration and basal cell hyperplasia were significantly increased in type B and type C lesions compared with type A lesions ($P < 0.05$). Low-grade esophageal dysplasia was apparent in 1 (9%) of 11 type A lesions, in 3 (18%) of 17 type B lesions, and in 6 (40%) of 15 type C lesions, with the highest rate in type C.

CONCLUSION: Magnified observations of the esophagus, classified by papillary aspects using magnifying endoscopy of iodine-unstained lesions in high-risk esophageal cancer groups, are considered useful in estimating dysplasia and inflammation of esophageal mucosa.

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Key words: Esophageal cancer; Iodine; Magnifying endoscopy

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INTRODUCTION

Although early diagnosis of esophageal cancer is known to be an important determinant of clinical outcome, it is not easy to diagnose early-stage esophageal cancer with conventional endoscopy[1]. The best complementary measure for this is the iodine staining technique, which is used for the diagnosis of early-stage esophageal cancer, especially in high-risk esophageal cancer groups.

However, the iodine staining technique has low specificity in diagnosing esophageal cancer[2], and therefore, the development of other diagnostic measures are needed to complement this technique. The recent development of magnifying endoscopy has enabled more detailed observations of various gastrointestinal disorders. In addition, magnified observations of the aspects of small blood vessels and microscopic surface structure has been proved to be clinically useful, however, the clinical application of magnifying endoscopy is at an early stage. The present study was performed to determine the usefulness of magnified observations of iodine-unstained esophageal lesions in the histological diagnosis of esophageal mucosa abnormalities.

MATERIALS AND METHODS

Inclusion and exclusion criteria

The subjects included in this study had at least one of the four criteria known as high-risk factors for esophageal cancer, which were older age, smokers, alcoholics or those with a history of non-esophageal primary malignant tumor. The cut-off points for old age, smoking and drinking were 55 years and 5 d/wk, respectively. Patients were excluded if they had dysphagia, recent upper gastrointestinal hemorrhage, known liver cirrhosis or cardiac or coagulation disorders (Table 1).

Table 1 Patient inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Old age (> 55 yr old)	Dysphagia
Smoking (> 5 d/wk)	Recent upper GI hemorrhage
Alcohol intake (> 5 d/wk)	Liver cirrhosis
History of non-esophageal primary cancer	Cardiac or coagulation disorder

Table 2 Characteristics of the patients

Age (yr)	61.4 (43-86)
Sex (M:F)	24:4
Smoking amount (packs/yr)	29.4 (14 patients)
Alcohol intake dose (g/wk)	367 (6 patients)
History of non-esophageal carcinoma	Stomach cancer: 10, Breast cancer: 1

Baseline characteristics of subjects

Thirty eight subjects were included. Multiple iodine-unstained lesions were observed in 7 patients, and 3 patients had no iodine-unstained lesions. The total number of lesions was 43. The average age of these patients was 61.4 years, the male to female ratio was 24:14, 14 of 38 patients smoked, with an average smoking history of 29.4 packs/year, and 10 of 38 patients consumed alcohol, with an average consumption of 367 g/wk. In addition, 11 patients had a history of non-esophageal primary malignant tumors, including gastric cancer in 10 patients, and breast cancer in 1 patient (Table 2).

Screening chromoscopy

Following conventional endoscopic inspection in patients who were classified in the high-risk esophageal cancer group, mucus was removed by spraying water onto the entire esophagus. A polyethylene catheter was passed down through the biopsy channel and 20 mL of 1.5% iodine solution was sprayed on the mucosal surface, followed by identification of the presence of iodine-unstained lesions. The size of the iodine-unstained lesions was measured at the time of detection, to include lesions over 3 mm and less than 30 mm. Magnified observations were then performed (Figure 1).

Magnified observations of the iodine-unstained areas

The magnifying endoscope used in this study was a GIF-Q240Z (Olympus Co., Ltd, Tokyo, Japan) with a maximum magnification power of 80 ×. When the tip of the endoscope approached the target area, the zoom lever was pulled inferiorly as shown in Figure 2A, B, and 80 × magnification observations were performed. Following magnified observations on the iodine-unstained lesions, tissue biopsy was performed in the same area (Figure 2).

Magnifying endoscopic classification of iodine-unstained lesions

The magnified observations on each of the iodine-unstained lesions were classified into the following three categories: clear papilla with well-maintained and regularly arranged papillae was classified as type A; fused papilla in which papillae could be seen but were not regular and were either merged or partially seen was classified as type B; and non-visible papilla in which papillae were not observed at all was classified as type C. This reflected the classification of non-iodine stained lesions outlined by Arima *et al*[3] in Japan. In addition, photographs and video were taken of these lesions in order to reduce the interobserver variation, and were determined by two specialists in endoscopy who did not participate in the examination. The views of at least 2 of the 3 specialists were compared to that of the examiner, and all were found to be consistent in the classification of each mucosal form (Figure 3).

Histological grading of iodine-unstained esophageal lesions

Following magnified observations of the iodine-unstained lesions, and their classification which was followed by tissue biopsy in the same area, the tissue was graded according to the following histological factors: inflammatory cell infiltration, basal cell hyperplasia, vascular lake, balloon cell, acanthosis, dysplasia, carcinoma grade from 0 to 3 according to their extent (0: normal, 1: mild, 2: moderate, 3: high) and analysis of their correlation with the types found on magnifying endoscopy.

Statistical analysis

Statistical data are described as the mean ± SD, and one-

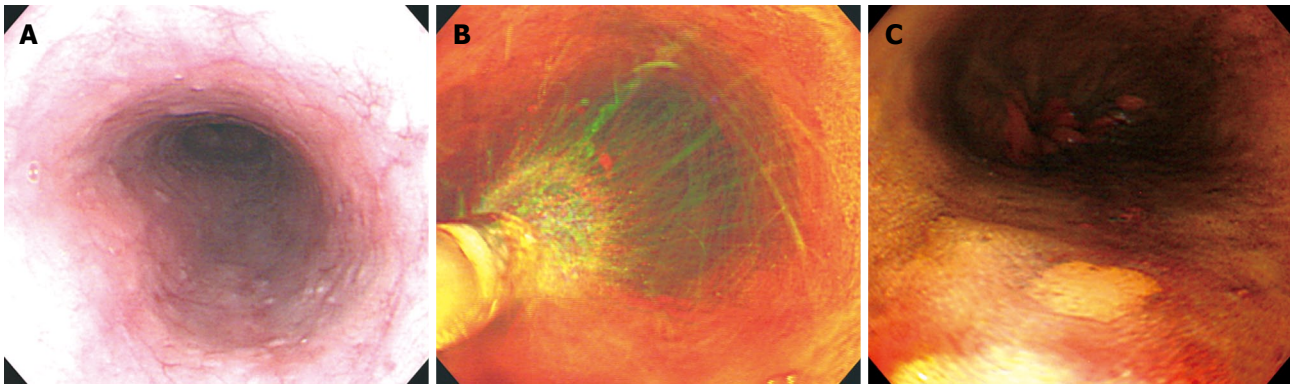

Figure 1 Screening chromoendoscopy. A: After conventional examination using magnifying endoscope; B: 1.5% Lugol's solution was sprayed onto the entire esophagus; C: Iodine-unstained lesions were identified.

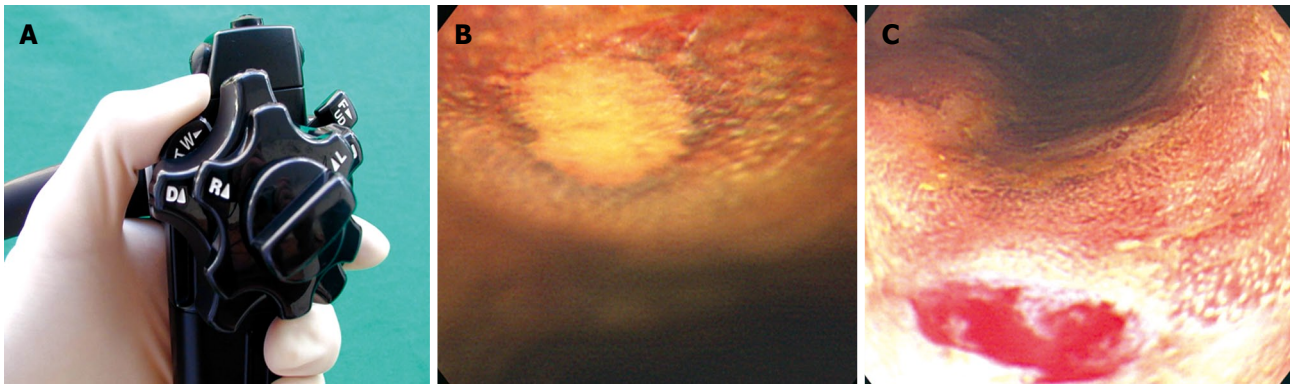

Figure 2 Magnifying endoscopy. A and B: Magnified observations were performed on iodine-unstained lesions by pulling the zoom lever in a downward direction; C: After magnified observations, biopsy of iodine-unstained lesions was performed.

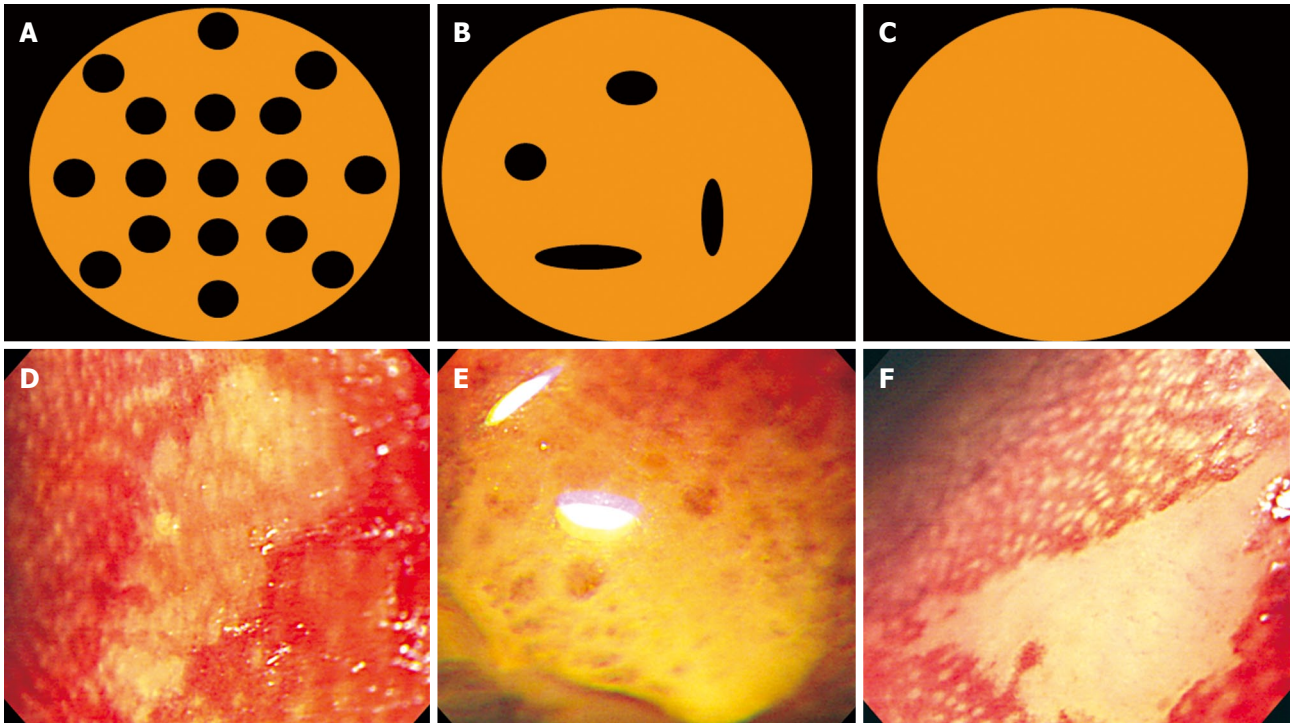

Figure 3 Type of papilla pattern. Magnifying endoscopic findings in iodine unstained lesions were classified into the following three papilla patterns: Clear papilla pattern that showed regularly arranged white spots (Type A: A and D), fused papilla pattern that had a tendency to be fused but still had a distinguishable outline (Type B: B and E), non-visible papilla pattern that was amorphous, irregular and not stained at all (Type C: C and F).

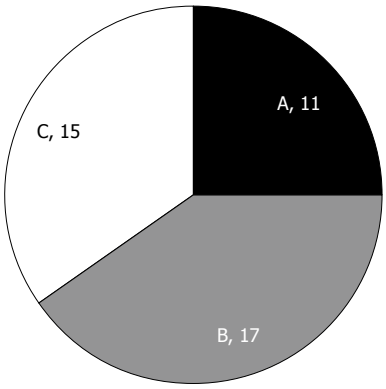

Figure 4 Distribution of types of papilla pattern on magnifying endoscopy. Total number: 43; A: Clear papilla; B: Fused papilla; C: Non-visible papilla.

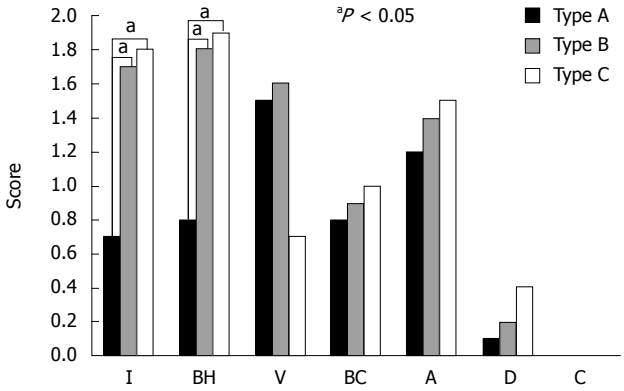

Figure 5 Relationship between types of papilla pattern and histological findings. I: Inflammatory cell infiltration; BH: Basal cell hyperplasia; V: Vascular lake; BC: Balloon cell; A: Acanthosis; D: Dysplasia; C: Carcinoma. (All dysplasias were low-grade).

way ANOVA and Chi-square were used for the analysis. Data were significant when $P < 0.05$.

RESULTS

Distribution of mucosal forms in iodine-unstained lesions on magnifying endoscopy

According to papillary form, magnified observations of iodine-unstained lesions showed 11 lesions of type A, 17 lesions of type B and 15 lesions of type C, with type B being the most frequent (Figure 4). When examining age, smoking history, alcohol consumption, presence of non-esophageal tumor, and the size of the iodine-unstained area based on the type of papillary form, the average age was 59.8 ± 5.1 years for type A, 65.8 ± 11.6 years for type B, and 55.8 ± 8.8 years for type C; alcohol consumption was 106.9 ± 222.1 g/wk for type A, 67.7 ± 123.3 g/wk for type B, and 162.5 ± 251.9 g/wk for type C; smoking history was 12.8 ± 17.5 packs/year for type A, 13.8 ± 19.6 packs/year for type B, and 7.5 ± 10.6 packs/year for type C; non-esophageal tumor was found in 2/11 cases with type A, 4/17 cases with type B, and 5/15 cases with type C; and the size of the iodine-unstained area was 7.0 ± 4.8 mm for type A, 9.4 ± 7.5 mm for type B, and 5.7 ± 1.8 mm for type C, and thus did not show any significant difference between the different types.

Relationship of mucosal types with magnifying endoscopy and histological findings

The findings on magnifying endoscopy i.e., the histological findings based on the papillary form showed that the total score for each histological factor increased as it moved from type A to type B, and type C (5.1 ± 2.4 for type A, 7.4 ± 2.7 for type B, and 7.4 ± 2.9 for type C). In particular, inflammatory cell infiltration and basal cell hyperplasia associated with the degree of inflammation in the histological findings was significantly increased in type B and C compared with type A ($P < 0.05$) (Figure 5). Low-grade esophageal dysplasia was apparent in 1 of 11 type A lesions, in 3 (21%) of 17 type B lesions, and in 6 (44%) of 15 type C lesions, with type C showing the highest rate with no statistical significance, however, dysplasia showed a tendency to increase from type A to type B and type C (Table 3). There was no high-grade dysplasia or carcinoma in any of the lesion types. When examining age, smoking history, alcohol consumption, presence of non-esophageal tumor, and the size of the iodine-unstained area based on the presence of dysplasia, the average age was 63.0 ± 9.7 years in the non-dysplasia group, and 56.1 ± 10.8 years in the dysplasia group; alcohol consumption was 135.9 ± 207.0 g/wk in the non-dysplasia group, and 122.5 ± 245.0 g/wk in the dysplasia group; smoking history was 14.1 ± 18.0 packs/year in the non-dysplasia group, and 3.8 ± 7.4 packs/year in the dysplasia group; non-esophageal tumors were found in 10/33 cases in the non-dysplasia group, and in 1/10 cases in the dysplasia group; the size of the iodine-unstained lesion was 6.8 ± 4.0 mm in the non-dysplasia group, and 9.5 ± 8.6 mm in the dysplasia group. No significant differences were found between patients with and without dysplasia.

Table 3 Relationship between types of papilla pattern and dysplasia

	Type A	Type B	Type C
Dysplasia (-)	10	14	9
Dysplasia (+)	1	3	6
Dysplasia/total	1/11 (9%)	3/17 (18%)	6/15 (40%)

All patients with dysplasia had low-grade dysplasia.

DISCUSSION

While most patients with symptoms caused by squamous cell carcinoma of the esophagus have a poor prognosis, due to an advanced stage at the time of diagnosis, the 5-year survival rate of superficial esophageal cancer, in which the area of invasion does not include the submucosal layer, is very high at 70%-80%. In particular, esophageal cancers limited to the epithelium or lamina propria have a low risk of lymph node metastasis, and thus successful treatment is possible with endoscopic

mucosal resection. As such, the 5-year survival rate is known to be over 95%. Therefore, early diagnosis of esophageal cancer is an important determinant of clinical outcome. However, it is very difficult to diagnose early-stage esophageal cancer with conventional endoscopic and radiologic examinations[1]. Lugol's solution, which is used for chromoscopy of the esophagus, is an absorbent dye based on iodine which has an affinity for glycogen in non-keratinized squamous epithelium. This solution turns a dark greenish-brown color, and gradually becomes lighter with time. It is diluted to 1%-5% when used, and is applied using a catheter. There is no glycogen present in inflammatory squamous epithelia such as erosive esophagitis, neoplastic tissue, dysplasia or non-squamous epithelium such as columnar epithelium. Therefore, these lesions remain unstained, while glycogenic acanthosis is stained darker. Such iodine staining of the esophagus allows the early diagnosis of esophageal cancer compared with conventional endoscopy. This technique also enables a more definite preoperative diagnosis of the range of esophageal cancers[4,5]. However, while chromoscopy of the esophagus using iodine has few false-negative results and has high sensitivity, staining cannot be performed in diseases other than esophageal cancer. Therefore, it is limited in that it is not specific to early-stage esophageal cancer[2]. Consequently, the development of more effective methods of examination is needed in addition to histological diagnosis. Squamous cell carcinoma of the esophagus has wide regional differences and has a very high prevalence in China and Iran, but is rare in Japan, America and Europe. Older age, smoking, alcohol consumption and insufficient intake of fresh fruit and vegetables are known to be risk factors, and it has recently been reported that the prevalence of multiple primary tumors is increasing[1]. Kodama *et al*[6] reported that non-esophageal primary carcinoma was present in 20.6% of 2418 superficial esophageal cancer patients, and Shimizu *et al*[1] reported that non-esophageal primary carcinoma was present in 29.2% of 233 patients who received treatment with either surgery or endoscopic mucosal resection and were followed up. In particular, head and neck tumors are known to be high risk factors for causing esophageal cancer. Although there are no definite standards for age, smoking and alcohol consumption, advanced age, smoking, and alcohol consumption are high risk factors for esophageal cancer[1,7]. Considering the cost-effectiveness of iodine staining and patient discomfort, the preselection of high-risk patients seems to be a more cost-effective procedure during screening examinations. Therefore, the present study selected subjects based on the above-mentioned standards. Magnifying endoscopy of the digestive tract observes the mucosal forms of the digestive tract in detail, using an endoscope with a magnifying power of over 30 ×[8]. The origins of endoscopy was based on the observations of foveola in the stomach by Gutzeit *et al*[9] in 1954 and on observations by Takemoto *et al*[10] in 1966 in Japan. Sakaki *et al*[11] presented the first classification of gastric mucosa on magnifying endoscopy, especially atrophic gastritis, by dividing mucosa into 5 different types. Focal adjustment is difficult in the esophagus due to peristaltic movement, respiration, and heart beat, whereas the advantages in the large intestines are, that it is histologically uniform, there is no chronic inflammation, and there is almost no difference in the normal pit patterns between different individuals. Thanks to these advantages, magnified observations are more useful in the lower digestive tract than in the upper digestive tract, and although its use in the lower digestive tract was started later, it has advanced rapidly. It is no exaggeration to say that the work of Kudo *et al*[12] on magnifying endoscopy of the large intestine has resulted in an increase in current magnified observations. However, as the high-pixel electronic endoscope has recently been used more generally, various studies are being carried out not only on the large intestine, but also in the esophagus and the stomach. Magnifying endoscopy is useful in the differential diagnosis of non-tumor and tumor through the observation of papillary blood vessels in the esophagus, and its range of use is expanding to the stomach to determine the range of mucosal resection for early-stage stomach cancer or the presence of recurrence after endoscopic therapy[13]. The present study employed magnifying endoscopy to complement the low specificity of iodine staining in the esophagus. In 1997, Arima *et al*[3] performed magnifying endoscopy on esophageal mucosa from resected specimens of esophageal cancer. A total of 55 unstained lesions less than 3 cm in diameter from 22 patients were studied. Similarly, 114 unstained lesions were studied *in vivo* using magnifying endoscopy and were classified according to papillary pattern. The findings in both groups were compared with histological findings and showed a favorable co-relationship. The size of unstained lesions was limited to between 3 mm and 30 mm, because carcinoma is known to be extremely rare in lesions less than 3 mm, whereas most carcinomas occur in lesions over 30 mm. The classification of types and size used in the current study was also identical to these authors, and although their results were different from those observed in this study, the findings showed an increase in the frequency of dysplasia and an increase in inflammatory cell infiltration from type A to type C similar to our findings. Antonioli[14] reported that approximately 30% of patients with severe esophageal dysplasia progressed to invasive carcinoma. This frequency was 15% for mild dysplasia. Thus, dysplasia seems to be a precursor of squamous cell carcinoma in the upper digestive mucosa and has a direct correlation with severity of cellular abnormalities and progression to invasive carcinoma. Rubio *et al*[15] classified mild and moderate dysplasia as low-grade intraepithelial carcinoma and severe dysplasia as high-grade carcinoma. Magnifying endoscopic observations of the esophagus did not have a significant diagnostic role relative to the pit pattern of gastric or colonic mucosa, as the former was covered with squamous epithelium and thus had a smooth amor-

phous surface[16].

Methylene blue, indigo carmine, or acetic acid chromoendoscopy combined with magnification endoscopy also allows identification of specific mucosal patterns (tubular, ridged, or villous) which are highly associated with the presence of specialized intestinal metaplasia in patients with Barrett's esophagus in directed biopsy examinations[17]. Two prospective Japanese cohort studies[18,19] reported high specificity (92%-100%), but sensitivity was variable (53%-85%).

However, as a result of the leading studies on magnification observations of esophageal mucosa by Arima *et al*[3,20] and Inoue *et al*[21-23], diagnostic standards have been established to some extent. Arima *et al*[20] classified 4 different types through the enhanced findings of papillary blood vessels, other than the 3 types mentioned above, in a study on the magnifying endoscopic diagnosis of superficial esophageal cancer. These authors reported that the risk of esophageal cancer was high and the depth of cancer invasion was also high since these papillary blood vessels showed irregular shapes or were dilated. In addition, in a study on magnifying endoscopic observations with the highest magnification power, Inoue *et al*[21-23] reported that the intrapapillary capillary network, or the thickness of neoplastic blood vessels, increased with progression of the depth of cancer invasion in addition to the shape becoming irregular. By doing so, they stated that magnifying endoscopic diagnosis of the depth of invasion of esophageal cancer was possible and reported that ultrasonic endoscopy was outstanding in the diagnosis of elevated lesions. However, magnifying endoscopic diagnosis was also useful for the diagnosis of depressed lesions. As shown in our study, an increase in the frequency of dysplasia and an increase in inflammation from type A to type C were observed in the high-risk esophageal cancer group. Enhanced findings of the esophagus, through classification of the papillary forms, using magnifying endoscopy on iodine-unstained lesions, seems useful in the diagnosis of dysplasia and inflammation of esophageal mucosa. More case studies on magnifying endoscopy should be performed in the future.

COMMENTS

Background

Various chromoendoscopy techniques combined with magnifying endoscopy are useful in assessing the presence of specialized intestinal metaplasia in patients with Barrett's esophagus. However, little data is currently available regarding the clinical usefulness of magnifying endoscopy using Lugol's solution in a high-risk esophageal cancer population.

Research frontiers

In this study, the authors demonstrate the clinical usefulness of magnifying endoscopy for iodine-unstained lesions in a high-risk esophageal cancer population.

Innovations and breakthroughs

This study demonstrates that magnified observations of the esophagus classified by papillary aspects using magnifying endoscopy on iodine-unstained lesions in a high-risk esophageal cancer group are useful in estimating dysplasia and inflammation of esophageal mucosa.

Applications

By providing an understanding of the relationships between magnified observations on iodine unstained lesions and esophageal mucosal dysplasia and inflammation, the results of this study may represent a future strategy in the management of patients with a high risk of esophageal cancer.

Terminology

The magnified observations on each of the iodine-unstained lesions were classified into the following three categories: type A, clear papilla with well maintained and regularly arranged papillae; type B, fused papilla in which papillae could be seen but were not regular and were either merged or partially seen; type C, non-visible papilla in which papillae were not observed at all.

Peer review

This is a useful study on the subject of dye spray to facilitate detection of dysplasia. In the West adenocarcinoma is much more common and although not the main focus of this study, some reference should be made to dye spray in Barretts.

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BRIEF ARTICLE

Loss of chromosome 9p21 and decreased p16 expression correlate with malignant gastrointestinal stromal tumor

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Abstract

AIM: To investigate loss of heterozygosity (LOH) of chromosome 9p21 and the prognostic relevance of p16 expression in gastrointestinal stromal tumor (GIST).

METHODS: Fifty-one GIST patients (30 men and 21 women; median age 59 years; range 29-80 years) treated surgically within a 10-year period were grouped by aggressive behavior risk (17 with very low and low, 14 intermediate, and 20 high risk). GISTs were characterized immunohistochemically and evaluated for LOH of 9p21 by microsatellite analysis at D9S1751, D9S1846, D9S942, and D9S1748. LOH of 9p21 and immunohistochemical expression of p16 protein encoded at 9p21 were correlated with clinicopathological parameters, and the prognostic significance of p16 alterations was evaluated.

RESULTS: Thirty-one (63.3%) cases showed LOH with at least one microsatellite marker. LOH frequency was 37.0% at D9S1751, 37.5% at D9S1846, 42.1% at D9S942, and 24.2% at D9S1748. There was a higher LOH frequency of D9S942 in high-risk than in non-high-risk tumors ($P < 0.05$, $\chi^2 = 4.47$). Gender, age, tumor size and site were not correlated with allelic loss. Ninety percent (18/20) of the GIST patients in the high risk group showed LOH with at least one of the 9p21 markers, while 57.1% (8/14) in the intermediate risk group and 33.3% (5/15) in the very low and low risk groups, respectively ($P < 0.05$, $\chi^2 = 12.16$). Eight (28.5%) of 31 patients with LOH and 1 (5.6%) of 18 patients without LOH died of the disease during the follow-up period. Loss of p16 protein expression occurred in 41.2%, but in 60% of the high risk group and 23.5% of the very low and low risk groups ($P < 0.05$, $\chi^2 = 4.98$). p16 loss was associated with poor prognosis ($P < 0.05$, $\chi^2 = 4.18$): the 3- and 5-year overall survival rates were 84.8% and 70.8% for p16-negative and 100% and 92.0% for p16-positive patients, respectively.

CONCLUSION: LOH at 9p21 appears to play an important role in GIST progression; decreased p16 expression in GIST is highly predictive of poor outcome.

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Key words: Gastrointestinal stromal tumor; Loss of heterozygosity; p16; Prognosis; Tumor suppressor gene

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) comprise the largest subset of mesenchymal tumors of the digestive tract. Most GISTs have activating mutations of the c-kit proto-oncogene that have been implicated in their tumorigenesis. They are characterized by the expression of the KIT (CD117, stem cell factor receptor) protein. More recently, activating mutations of platelet-derived growth factor receptor α (PDGFRA) have been identified. Clinically and pathologically, GISTs represent a spectrum of tumors, including benign, malignant, and borderline variants. It is generally recognized that a final consensus on the grading of GISTs has not yet been reached, and their biologic behavior often remains unclear. Most clinicopathological studies have suggested that the tumor site and size and the mitotic index are the most important prognostic indicators of GISTs. However, they do not always reliably predict patient outcomes. The lack of a reliable method for prognosis prediction hampers the selection of patients eligible for imatinib mesylate (Gleevec) therapy. Imatinib was the first targeted therapy approved for the treatment of GIST. The development of imatinib in the treatment of metastatic GIST represents a therapeutic breakthrough in molecularly targeted strategies, while its usefulness in adjuvant setting is under study. Obtaining genetic information of each patient may be critical in tailoring individualized treatment strategies.

Although mutational activation of c-kit or PDGFRA plays an important role in GIST pathogenesis, other cytogenetic alterations, mostly losses of genetic material, have been found, with evidence that losses at chromosome 9p are highly specific for malignant and metastatic GISTs[1]. P16, a cyclin-dependent kinase (CDK) inhibitor, encoded by CDKN2A gene at 9p21 has been shown to be inactivated in a variety of tumors by loss of heterozygosity (LOH), homozygous deletions, or point mutations[2]. P16 as a product of tumor suppressor gene that arrests cells in the G_1 phase through reducing the kinase activity of CDKs 4 and 6, thus leaving the retinoblastoma tumor suppressor protein (Rb) in its unphosphorylated active form, which blocks the E2F transcription factor 1 (E2F1), free E2F1 accumulates in the nucleus and initiates S-phase entry *via* transcription of several genes necessary for DNA synthesis and cell cycle progression. Because this situation leads to the management of these tumors, it is helpful to add new molecular markers that may play a role in the diagnosis and treatment of the disease. The aim of this study was to evaluate the status of the LOH at 9p21 and the expression of p16, which controls cell cycle progression in a series of GISTs with diverse biologic aggressiveness, to determine whether alterations in cell cycle regulatory protein can be used as prognostic markers. The identification of an additional criterion for the selection of high-risk cases for treatment with imatinib mesylate was also attempted.

MATERIALS AND METHODS

Patients and pathological analysis

A total of 51 cases of GIST, consecutively resected between 1999 and 2007, were retrieved from the archives of our hospital. None of the patients received imatinib therapy. There were 30 males (58.8%) and 21 females (41.2%), aged from 29 to 80 years (median, 59 years). Primary tumors originated from the stomach (n = 30), small intestine (n = 18), and mesentery (n = 3). The tumors were diagnosed as GISTs using previously established histological, immunohistochemical, and molecular genetic criteria[3]. Fifty-one samples of formalin-fixed paraffin-embedded (FFPE) tumor material were examined, and 4-μm-thick sections were initially cut and stained with hematoxylin and eosin. All tumors were positive for CD117. For the purpose of clinicopathological comparison, the GISTs were classified as very low and low (n = 17), intermediate (n = 14), and high risk (n = 20) according to the consensus approach of Fletcher *et al*[4].

Microsatellite analysis

All cases were positive for KIT, supporting the diagnosis of GIST. Tumor and normal tissue samples were dissected from FFPE tissue blocks. DNA was extracted from FFPE tumor material using a standard extraction protocol (Qiagen, Hilden, Germany). LOH was evaluated by PCR amplification of four microsatellite markers at chromosome 9p21. Primer sequences (provided by Shanghai GeneCore BioTechnologies Co., Ltd. Shanghai, China) were obtained from human genome microsatellite marker databases linked to the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and are shown in Table 1. PCR amplifications were performed in a final volume of 50 μL containing 50 ng sample DNA, GeneAmp 10 × PCR reaction buffer, 25 mmol/L $MgCl_2$, 5 pmol/L of each primer, 2.5 mmol/L each of dATP, dCTP, dGTP, and dTTP, and 5 U of AmpliTaq DNA Polymerase (Applied BioSystems, Foster City, USA). After denaturation at 95℃ for 10 min, DNA amplification was performed for 40 cycles, consisting of denaturation at 94℃ for 15 s, primer annealing at 50℃ for 15 s, and elongation at 72℃ for 30 s. A final extension step at 72℃ for 30 min completed the reactions. Amplification products were analyzed using the ABI Prism Genetic Analyzer 3730 (Applied Biosystems, Foster City, USA). Data were processed using Genemapper software (Applied BioSystems, Foster City, USA). LOH was defined based on the recommendations by previous studies[5]. The ratio of the

Table 1 Primer sequences and product size used in polymerase chain reaction for each primer on chromosome 9p21

Marker	Forward primer (5'→3')	Reverse primer (5'→3')	PCR product size (bp)
D9S1751	TTGTTGATTCTGCCTTCAAAGTCTTTTAAC	CGTTAAGTCCTCTATTACACAGAG	150-170
D9S1846	AATGGCTGGTTCTAGGACTG	AAACTGGTCTGGTGTTTGC	183-197
D9S942	GCAAGATTCCAAACAGTA	CTCATCCTGCGGAAACCATT	100-130
D9S1748	CACCTCAGAAGTCAGTGAGT	GTGCTTGAAATACACCTTTCC	130-150

PCR: Polymerase chain reaction.

peak high values between longer and shorter alleles was calculated for the normal and tumor tissues. To obtain the LOH value, the allele ratio from the normal tissue was divided by the allele ratio from the tumor tissue [allele ratio = $(T_1:T_2)/(N_1:N_2)$]. Values $\leqslant 0.5$ and $\geqslant 1.5$ were considered to represent LOH. In questionable cases, the PCR amplification and LOH analysis were repeated to ensure the consistency in the results.

Immunohistochemistry

KIT protein: All GISTs were immunohistochemically positive for the KIT protein (antibody CD117, GA450202, DAKO, Carpinteria, CA, USA).

P16 protein: Briefly, FFPE tissue sections were dewaxed with xylene, dehydrated with an ethanol series (100%, 90%, 70%), and then microwave retrieved in 10-mmol/L citrate buffer, pH 6.0, for 10-20 min. Endogenous peroxidase was blocked with 0.5% H_2O_2 for 15 min. The sections were pretreated with blocking serum and washed in Tris-buffered saline (TBS). Each section was incubated with the anti-p16 (M0425, 1:50; Antibody, California, USA) overnight at room temperature. After TBS washing, the sections were incubated with Envision™ (Mouse) (K4001; Dako, Carpinteria, CA, USA) for 30 min at room temperature. Finally, the sections were developed in DAB (Amresco, Ohio, USA) for 5-15 min. Ten high-power fields (HPF) were estimated, and a section was considered to be immunohistochemically positive for p16 if tumor nuclei were stained (with or without cytoplasmic staining), according to a 4-point semiquantitative scale, as follows: negative (-), less than 5% of cells stained; positive (+), 5%-10% stained; positive (++), 11%-50% stained; positive (+++), 51%-75% stained; positive (++++), greater than 75% stained. A cutoff at 10% positivity in at least 10 HPF was used for prognostic analysis. Nontumorous stromal cells showing nuclear reactivity served as an internal control.

Statistical analysis

All statistical analyses were carried out using the χ^2 test or Fisher's exact test in cross tables to assess the relationships between p16 loss and clinicopathological factors. All statistical tests were two-sided. Overall survival curves were drawn according to the Kaplan-Meier method and compared using the log-rank test. $P < 0.05$ was considered statistically significant. Calculations were carried out using the SPSS version 13.0 software package (SPSS, Chicago, USA).

RESULTS

The data for the 51 GISTs are summarized in Table 2. Tumor size ranged from 1.4 to 19 cm in the greatest dimension (mean 6.7 cm). The tumors were histologically classified as predominantly spindled ($n = 32$), epithelioid ($n = 15$), or mixed-spindled epithelioid ($n = 4$). As noted in the methodology, all cases were KIT-positive and showed diffuse strong cytoplasmic and/or membranous staining.

Genetic studies

A total of four microsatellite markers were used to screen 51 tumors for LOH on chromosome 9p21. Two patients (3.9%) had constitutional homozygosity (noninformative loci) with 4 markers. Overall, 63.3% (31/49) of the tumors showed LOH with at least one locus on chromosome 9p21. The highest frequency of LOH was seen at D9S942 (42.1%, 16/38). The other markers showed the following deletions: D9S1751, 37.0% (10/27); D9S1846, 37.5% (12/32); and D9S1748, 24.2% (8/33). The frequencies of LOH on chromosome 9p21 in the 51 GISTs are shown in Table 3. Representative examples of LOH analysis are shown in Figure 1.

LOH on chromosome 9p21 and clinicopathological features of GISTs

LOH of 9p21 was compared with the clinical features of the GIST patients. There was no significant difference in LOH frequency by age (< 50 years, $\geqslant 50$ years), sex, and tumor site and size (< 5cm, $\geqslant 5$ cm). There were also no substantial differences in LOH frequencies among epithelioid and spindle cell tumors. The LOH frequency increased in accordance with the tumor's risk of aggressive behavior (Table 4). Moreover, GISTs assigned to the high risk group had a higher LOH frequency than the other groups on D9S942 ($P < 0.05$, $\chi^2 = 4.47$). Ninety percent (18/20) of the GIST patients in the high risk group was found to show LOH with at least one of the 9p21 markers, while 57.1% (8/14) in the intermediate risk group and 33.3% (5/15) in the very low and low risk groups, respectively ($P < 0.05$, $\chi^2 = 12.16$). Eight (28.5%) of 31 patients with LOH and 1 (5.6%) of 18 patients without LOH died of the disease during the follow-up period.

P16 protein expression

In our series, 14 cases were (-), 7 cases were (+), 14 cases were (++), 13 cases were (+++), and 3 cases were (++++) (Figure 2) for p16 immunoreactivity. Adopting a threshold of 10% cells with low to absent p16 immunostaining, Of

Table 2 Clinicopathological data and p16 expression in gastrointestinal stromal tumors

Case	Sex	Age (yr)	Risk[1]	Site	OS (mo)	1751	1846	942	1748	p16 expression
1	Male	41	Low	SI	Alive (50)	2	2	2	3	N
2	Male	73	Low	S	Alive (47)	3	2	4	4	N
3	Male	33	Very low	SI	Alive (21)	4	3	2	4	N
4	Female	66	Low	S	Alive (18)	4	4	3	3	P
5	Female	71	Low	SI	Alive (17)	4	4	4	4	P
6	Male	46	Low	SI	Alive (15)	4	4	4	3	P
7	Female	59	Low	SI	Alive (5)	3	4	4	3	P
8	Female	45	Low	SI	Alive (13)	4	3	3	4	P
9	Male	65	Low	SI	Alive (11)	3	3	3	4	P
10	Male	66	Low	S	Alive (11)	4	4	4	4	P
11	Male	55	Low	S	Alive (5)	3	2	2	4	N
12	Male	72	Low	SI	Alive (102)	2	3	4	2	P
13	Female	54	Low	S	Alive (50)	3	3	3	3	P
14	Male	56	Low	S	NA	4	4	4	4	P
15	Female	60	Low	S	NA	3	3	3	3	P
16	Male	70	Very low	S	Alive (45)	3	3	4	4	P
17	Female	75	Low	S	Alive (18)	3	3	4	3	P
18	Male	70	Intermediate	S	Alive (100)	4	2	2	3	N
19	Female	50	Intermediate	SI	Alive (81)	4	4	4	4	P
20	Female	70	Intermediate	S	Alive (88)	4	4	2	2	N
21	Male	72	Intermediate	SI	Alive (78)	2	4	4	3	P
22	Male	56	Intermediate	SI	Dead (60)	3	2	2	3	P
23	Male	47	Intermediate	S	Alive (53)	2	3	4	4	P
24	Male	50	Intermediate	M	Dead (27)	3	3	4	4	P
25	Female	73	Intermediate	S	Alive (21)	3	2	2	4	N
26	Male	79	Intermediate	S	Alive (16)	4	3	4	4	P
27	Male	63	Intermediate	S	Alive (12)	3	4	4	4	P
28	Male	68	Intermediate	S	Alive (12)	3	2	4	4	P
29	Female	56	Intermediate	S	Alive (11)	3	4	4	3	P
30	Female	57	Intermediate	SI	Alive (9)	4	4	3	4	P
31	Male	59	Intermediate	S	Alive (35)	3	4	3	2	N
32	Female	29	High	SI	Dead (38)	3	4	4	2	N
33	Female	77	High	S	Dead (35)	4	4	2	4	N
34	Male	64	High	M	Alive (71)	2	4	2	3	N
35	Male	47	High	S	Dead (56)	2	4	2	4	N
36	Female	50	High	S	Alive (62)	3	2	4	2	N
37	Female	36	High	SI	Dead (11)	2	4	4	4	N
38	Female	62	High	S	Alive (55)	4	2	3	2	P
39	Male	60	High	S	Alive (54)	3	4	3	4	P
40	Female	61	High	S	Alive (63)	2	3	3	4	N
41	Male	50	High	S	Alive (51)	3	3	3	2	P
42	Male	75	High	S	Dead (18)	2	4	2	3	N
43	Male	58	High	S	Alive (46)	3	3	3	4	P
44	Male	52	High	S	Alive (31)	4	3	2	3	N
45	Male	56	High	SI	Alive (31)	3	2	4	3	P
46	Male	57	High	SI	Alive (19)	2	3	2	4	P
47	Male	67	High	M	Alive (18)	3	2	3	2	P
48	Female	47	High	SI	Dead (5)	4	3	2	3	N
49	Female	80	High	SI	Alive (12)	3	3	2	4	N
50	Female	48	High	S	Alive (12)	3	3	2	3	P
51	Male	44	High	S	Dead (86)	4	2	4	3	N

[1]According to the consensus approach by Fletcher *et al*[4]; [2]Indicates loss of heterozygosity (LOH); [3]Uninformative (homozygosity); [4]Indicates no LOH. S: Stomach; SI: Small intestine; M: Mesentery; NA: Not available; OS: Overall survival; N: Negative expression; P: Positive expression.

Table 3 Results of loss of heterozygosity analyzed with four microsatellite markers in 51 gastrointestinal stromal tumors

Marker	LOH (*n*)	Heterozygosity (*n*)	Frequency of LOH (%)
D9S1751	10	17	37.0
D9S1846	12	20	37.5
D9S942	16	22	42.1
D9S1748	8	25	24.2

LOH: Loss of heterozygosity.

the 51 cases of GISTs, p16 protein-negative expression was detected in 21 (41.2%) samples, and p16 protein-positive expression was detected in 30 (58.8%) samples using a threshold of 10% cells with low to absent p16 immunostaining.

Correlation of p16 protein expression and clinicopathological factors

Loss of p16 protein expression was compared with the clinicopathological features of the GIST patients (Table 5).

Table 4 Rresults of loss of heterozygosity in 51 gastrointestinal stromal tumors according to Fletcher's classification

Risk classification	1751			1846			942[1]			1748		
	LOH (*n*)	Hetero-zygosity (*n*)	Rate (%)	LOH (*n*)	Hetero-zygosity (*n*)	Rate (%)	LOH (*n*)	Hetero-zygosity (*n*)	Rate (%)	LOH (*n*)	Hetero-zygosity (*n*)	Rate (%)
Very low and low	2	7	22.2	3	6	33.3	3	9	25.0	1	9	10.0
Intermediate	2	5	28.6	4	7	36.4	4	8	33.3	2	8	20.0
High	6	5	54.5	5	7	41.7	9	5	64.3	5	8	38.5

[1]There was a higher loss of heterozygosity (LOH) frequency of D9S942 in the high-risk than in non-high-risk tumors ($P < 0.05$, $\chi^2 = 4.47$). There were no substantial differences in LOH frequencies among three groups of 4 markers.

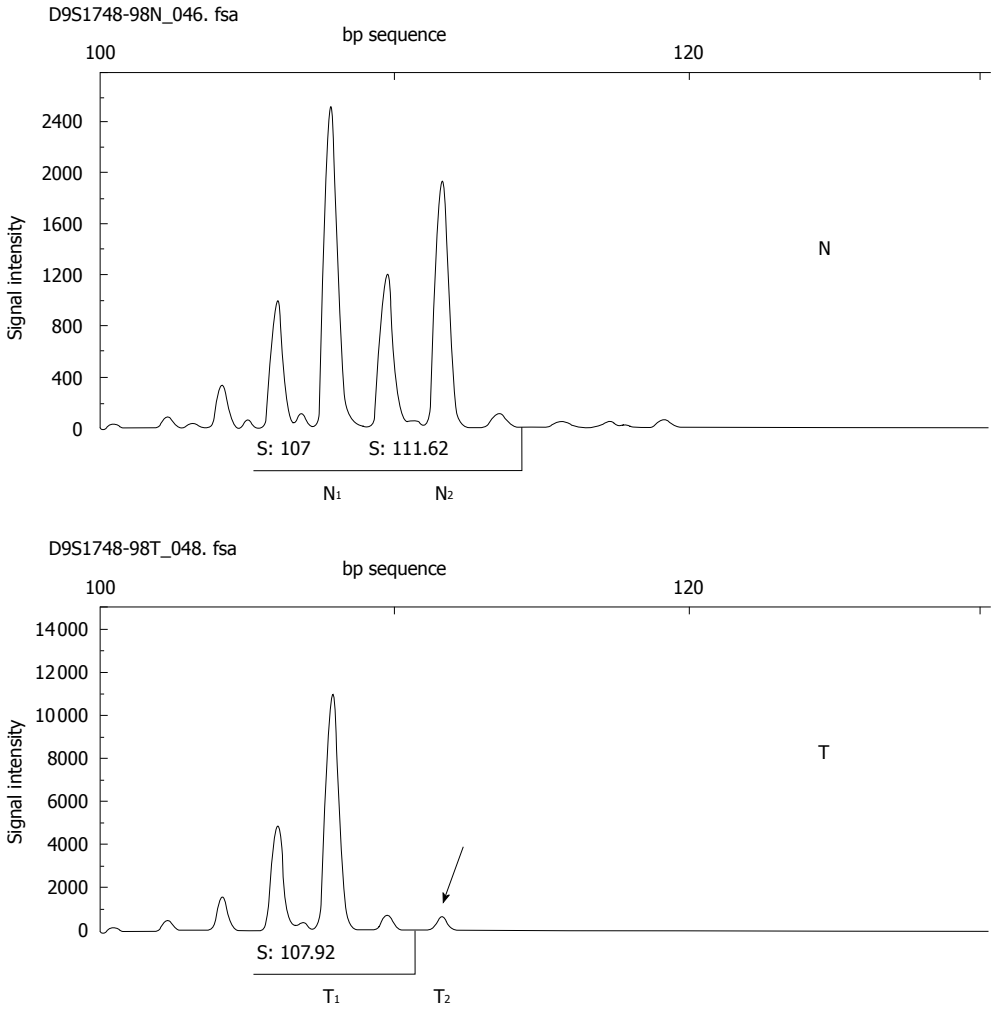

Figure 1 Representative image of loss of heterozygosity. Black arrow indicates the lost allele. Allele ratio = (T_1:T_2)/(N_1:N_2). N: Normal; T: Tumor.

Patient age, sex, tumor size and site did not correlate with p16 protein expression. But p16 protein-negative expression had a high mitotic index ($P < 0.05$, $\chi^2 = 5.13$). The rate of p16 protein-negative expression was 60% (12/20) in the high risk group, whereas the rate was 23.5% (4/17) in the very low and low risk group and 35.7% (5/14) in the intermediate risk group. There was a significant difference in p16 down-regulation between the high risk and the very low and low risk groups ($P < 0.05$, $\chi^2 = 4.98$).

P16 expression and survival analysis

Until April 30, 2008, 49 (96.1%) patients had been followed up. The median follow-up period was 31 mo (range, 5-102 mo). Forty (81.6%) patients were still alive, whereas nine (18.4%) patients died of the disease. Patients who had tumors with p16 protein loss had a worse prognosis than those having tumors without p16 protein loss. Eight (38.1%) of 21 patients with p16-negative expression tumors, but only one (3.6%) of 28 patients with p16-positive expression tumors, died of GIST. The 1-, 3-, and 5-year overall survival rates were 100%, 84.8% and 70.8%, respectively, in the p16 protein-negative expression group. The 1-, 3-, and 5-year overall survival rates were 100%, 100% and 92.0%, respectively, in the p16 protein-positive

Table 5 Statistical analysis of p16 expression and clinicopathologic factors

	p16 (-)	p16 (+)	*P* value (χ^2)
Sex			
Male	12	18	
Female	9	12	0.838 (0.042)
Age (yr)			
< 50	5	4	
⩾ 50	16	26	0.334 (0.933)
Size (cm)			
< 5	4	13	
⩾ 5	17	17	0.070 (3.279)
Mitotic index			
⩽ 5/50 HPF	8	21	
> 5/50 HPF	13	9	0.024 (5.126)
Risk			
Very low and low	4	13	
High	12	8	0.045 (4.98)
Site			
Stomach	13	17	
Intestine	7	11	
Other	1	2	0.917 (0.173)

HPF: High-power fields.

expression group. There was a strong correlation between p16 alterations and overall survival using the Kaplan-Meier method followed by comparison with the log-rank test ($P < 0.05$, $\chi^2 = 4.18$, Figure 3).

Correlation of p16 protein expression and LOH results

Twenty-one (67.7%) of the 31 patients with 9p21 LOH showed p16 protein-negative expression. The coincident rate between p16 expression and 9p21 LOH was 60% (6/10) on D9S1751, 66.7% (8/12) on D9S1846, 87.5% (14/16) on D9S942, and 70.8% (4/8) on D9S1748.

DISCUSSION

GISTs comprise the largest subset of mesenchymal tumors of the digestive tract, although they account for < 2% of all gastrointestinal tumors. Before the advent of imatinib (imatinib; Gleevec, Novartis, Switzerland), surgery was the only therapeutic approach for GISTs. However, even after complete resection of a GIST, most patients with advanced disease relapsed, and the prognosis of patients with metastatic and/or recurrent GISTs was extremely poor[6]. Clinically and pathologically, GISTs represent a spectrum of tumors that include benign, malignant, and borderline variants. It is often difficult to predict the malignant behavior of GISTs. Prognostic features indicative of malignancy or high aggressive clinical behavior risk are generally identified by increased tumor size and mitotic activity[4], but this lacks predictive accuracy. Although mutational activation of c-kit or PDGFRA plays an important role in GIST pathogenesis, other changes, mostly losses of genetic material, have been documented in primary tumors[7]. Total or partial loss of chromosome 9 has been found in benign and malignant GISTs, indicating that this change might play a role in GIST tumorigenesis[8]. A CDK

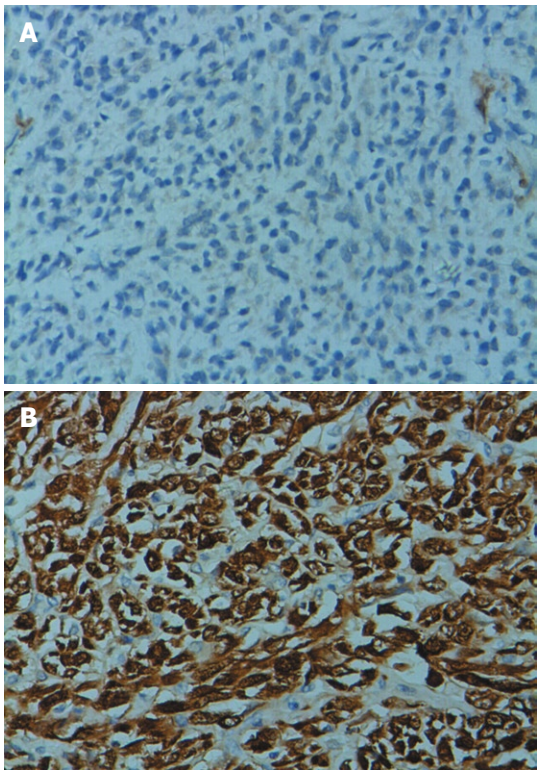

Figure 2 p16 immunostaining in gastrointestinal stromal tumor. A: Negative p16 immunostaining in gastrointestinal stromal tumor (GIST) (200 ×); B: p16 immunostaining ++++ in GIST (200 ×).

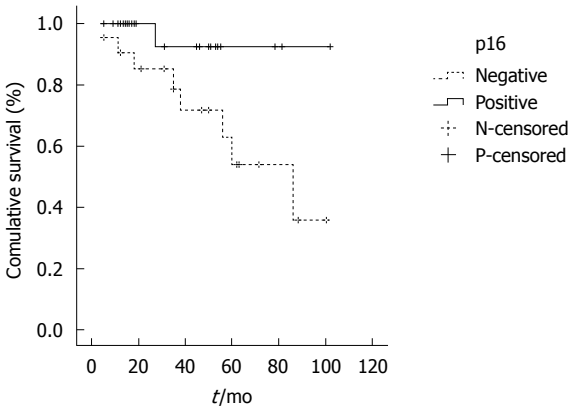

Figure 3 Kaplan-Meier plot for overall survival of gastrointestinal stromal tumor patients with p16-negative and p16-positive alterations ($P < 0.05$).

4 inhibitor (p16) gene located at 9p21 has been shown to be inactivated in a variety of tumors. However, the relationship between p16 expression and GIST prognosis is still under debate. For instance, Schneider-Stock *et al*[9] found that aberrant loss of p16 expression was predictive of poor patient survival, but Nakamura *et al*[10] failed to validate its prognostic value in Japanese patients. These discrepant data raised a practical concern about whether p16 can be indiscriminately used as a surrogate marker for various inactivating mechanisms of the p16 gene for prognosis. In this context, p16, as an early G1 phase negative cell-cycle regulator, represents a likely candidate. The aim

of this study was to address the issue of whether alterations in cell cycle regulatory protein can be used as prognostic markers.

Most human cancers are characterized by genomic instability, in addition to oncogene activation, the inactivation of tumor suppressor genes has been shown to play an important role in tumorigenesis. Oncogenes obviously play an important role in cell proliferation. Tumor suppressor genes may play important roles in tissue differentiation. LOH is a common form of allelic imbalance, and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages, and progression. In 1987, Hansen *et al*[11] suggested that when there is one gene deletion of both alleles, the other gene appears to be insufficient to carry out its normal functions, i.e. transcriptional transactivation of downstream target genes that regulate the cell cycle and apoptosis. Thus, a tumor may develop. The frequency of LOH always exceeds 20% at some chromosomes where the tumor suppressor gene exists, which means the allele is related to tumorigenesis[12]. Microsatellites are reliable genetic markers for studying LOH. When LOH occurs, microsatellite markers near the allele will be lost. Therefore, microsatellite analysis can be used to score for LOH.

In this study, LOH on chromosome 9p21 was evaluated in 51 well-characterized GISTs using 4 PCR-based microsatellite markers and gel electrophoresis. The results showed that 31 cases (63.3%, two were uninformative cases) had LOH on chromosome 9p21. These results suggest that LOH on chromosome 9p21 is a common phenomenon. With respect to the correlation between clinicopathological features and LOH, Sabah *et al*[1] found no correlation between loss of chromosome 9p and patient age and sex, and site and histological features of the tumor. However, Pylkkänen *et al*[13] validated that loss of chromosome 22 was found more often in the intestine than in the stomach, though a statistically relevant level was not reached. Our results confirmed that the frequency of LOH on chromosome 9p21 increased in a manner consistent with the risk of aggressive behavior of the tumor. Moreover, GISTs had a higher LOH frequency in the high risk group than in the other groups on D9S942 ($P < 0.05$). And there was substantial difference in LOH frequencies with at least one of the 9p21 markers in different risk groups ($P < 0.05$). The death rate with LOH is higher than those without LOH. This suggests that LOH on chromosome 9p may represent possible primary events in the development of GIST.

It is helpful to find the correlation between tumor suppressor gene and tumor progression and unfavorable outcome by LOH analysis. P16, a CDK4 inhibitor, has been shown to be inactivated in a variety of tumors[14-17]. And the cyclin D-CDK4/6/p16 /Rb/E2F1 transcription factors have been found to be altered in more than 80% of human neoplasms and implicated in the pathogenesis and progression of sarcomas[18]. Loss of p16 expression in GIST is described as a significant predictive value in some but not all studies. Schneider-Stock *et al*[9] reported that p16 alteration was detected in benign, borderline, and malignant GISTs, but it was not considered an independent, poor prognostic factor. Sabah *et al*[1] reported that inactivation of p16 was detected in almost all malignant GISTs. Romeo *et al*[19] also found that impaired p16 expression was common in advanced GISTs. In our study, four microsatellite markers at 9p21 were selected: two were located at the upstream of the p16 gene, and two at the downstream of the p16 gene. D9S942 is the most proximate marker to p16, a distance of less than 1 centimorgan (cM). The highest frequency of LOH on chromosome 9p21 in GISTs was seen at D9S942 (42.1%). Here, we studied the immunohistochemical results for the proposed biomarkers of p16 in GISTs to evaluate their possible usefulness in clinical prognostic assessment. P16 protein-negative expression was detected in 21 (41.2%) samples. Patients who had tumors with p16 loss showed a poor clinical outcome, and had a nearly 11-fold increased risk of dying of the disease (38.1% *vs* 3.6%). The 5-year overall survival probability was 70.8% in the p16 protein-negative expression group. However, the 5-year overall survival probability was 92.0% in the p16-protein positive expression group. Thus, p16 loss may be an important prognostic factor for GISTs. Our LOH and p16 expression results are in agreement with those of previously published studies[1,8,9,20,21]. However, Schmieder *et al*[22] indicated that the expression of p16 was highly predictive of poor outcome. Steigen *et al*[23] also found a positive relationship between p16 immunohistochemical staining and poor prognosis of GIST. The discrepant results should be clarified through further studies in the future.

Generally, p16 under-expression resulted from promoter methylation, LOH at 9p21, and point mutations. In our study, LOH was found in 31 (60.8%) of the 51 GIST cases, and 21 (66.7%) of them showed p16-negative expression. However, no p16-negative expression was found in a few cases of LOH. Multiple genetic and epigenetic alterations of oncogenes and tumor suppressor genes are implicated in the multistep process of human neoplasms[24-26]. LOH is one cause of multiple genetic alterations involved in the under-expression of tumor suppressor genes. In addition to genetic events, epigenetic alterations are also involved in tumor development[27,28]. According to this study, LOH may be a basic event to p16 loss, but epigenetic alterations, such as promoter methylation, may also influence p16 expression. Ricci *et al*[29] reported that p16 down-regulation, partly due to p16 promoter methylation, was implied in GIST progression.

In summary, LOH on chromosome 9p21 in GISTs could be found in both early and late stages of tumor development in the present study, but the frequency of total gene loss was significantly increased in high-risk GISTs. The p16 protein is encoded by the p16 tumor suppressor gene, which is in the vicinity of the locus with the highest frequency of LOH (D9S942), and its down-regulation is associated with high-risk GISTs. Patients with p16-negative expression had a lower survival rate, therefore ex-

pression of p16 might be a useful prognostic factor. P16 expression in GISTs, combined with Fletcher's aggressive risk scheme, appears to be an accurate evaluation for malignancy risk, particularly in the high-risk recurrent and/or metastatic GISTs. From a clinical perspective, such information can be expected to assist in the selection of cases for adjuvant systemic therapies (i.e. imatinib) after surgery. In addition, other pathogenic mechanisms, besides LOH in the regulation of p16 protein expression, should be the subject of further studies.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract, previously often diagnosed as benign or malignant smooth muscle tumors. The incidence of GISTs is increasing gradually. Because GISTs have a wide clinical spectrum that ranges from benign to malignant behavior, and GISTs often present with nonspecific symptoms including abdominal pain, anorexia, weight loss, or GI hemorrhage, it is hard to give a prompt treatment. Although mutational activation of c-kit or platelet-derived growth factor receptor α proto-oncogene plays an important role in GIST pathogenesis, other changes, mostly losses of genetic material and the alteration of tumor suppressor gene, have remained unknown.

Research frontiers

Total or partial loss of chromosome 9p has been found in benign and malignant GISTs, indicating that this change might play a role in GIST tumorigenesis. Whether the expression of tumor suppressor gene at 9p21 is correlated with pathogenesis of GIST is one of the hotspots in recent researches.

Innovations and breakthroughs

This study has found that loss of chromosome 9p21 and decreased p16 expression are correlated with malignant GIST. The confirmatory result is vital to elucidate the pathogenesis of GIST, and helpful for GIST treatment.

Applications

Loss of chromosome 9p21 and p16 expression analysis, combined with Fletcher's aggressive risk scheme, appears to be an accurate evaluation for malignancy risk of GIST. Such information can be expected to assist in the selection of cases for adjuvant systemic therapies (i.e. target drug-imatinib) after surgery.

Peer review

In this study, the authors provide original data showing that loss of heterozygosity of chromosome 9p21 leading to p16INK4A down-regulation, has a high prognostic value in GIST. The study is interesting, well done, methodologically correct and the conclusion drawn fits in with the results shown.

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BRIEF ARTICLE

Undifferentiated liver embryonal sarcoma in adults: A report of four cases and literature review

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Abstract

AIM: To evaluate the undifferentiated embryonal sarcoma of liver (UESL) in adults in order to improve its diagnosis and treatment.

METHODS: Four primary and one recurrent cases of UESL were clinicopathologically evaluated and immunohistochemically investigated with a panel of antibodies using the EnVision+ system. Relevant literature about UESL in adults was reviewed.

RESULTS: Three males and one female were enrolled in this study. Their chief complaints were abdominal pain, weight loss, or fever. Laboratory tests, imaging and pathological features of UESL in adults were similar to those in children. Immunohistochemistry showed evidence of widely divergent differentiation into mesenchymal and epithelial phenotypes. The survival time of patients who underwent complete tumor resection followed by adjuvant transcatheter arterial chemoembolization (TACE) was significantly longer than that of those who underwent surgical treatment alone.

CONCLUSION: UESL in adults may undergo pluripotential differentiation and its diagnosis should be made based on its morphological and immunohistochemical features. Complete tumor resection after adjuvant TACE may improve the survival time of such patients.

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Key words: Undifferentiated embryonal sarcoma; Liver; Pluripotential differentiation; Transcatheter arterial chemoembolization; Adult

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INTRODUCTION

Undifferentiated embryonal sarcoma of liver (UESL) is a rare and highly malignant spindle cell tumor, usually occurring in children and young adults[1,2]. Although UESL is generally a disease in childhood, middle-aged and elderly patients have rarely been reported[3]. To our knowledge, in the past 50 years, less than 60 adult cases have been reported[3-5]. In fact, previous reports describing its general features do not separate infants from adults, and few studies focusing on adult cases are available. Given that the majority of such patients are under the age of 30 years, adult cases over the age of 30 years are quite exceptional[5-18]. Furthermore, its detailed clinical, radiological or pathological characteristics of adult cases based on

particular immunohistochemistry are not yet clear. Herein, we present 4 adult cases of UESL, and highlight its clinical features, immunohistochemical findings, and treatment.

MATERIALS AND METHODS

From 2001-2008, 4 adult cases of UESL were retrieved from the pathology files of Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. Haematoxylin and eosin (HE) slides and all special stains were reviewed and all pathologic diagnoses were made according to the World Health Organization Histologic Classification of Liver Tumors and Intrahepatic Bile Ducts (2000)[19]. Four primary and one recurrent tumor specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue was cut into 4-μm thick sections, which were stained with HE, periodic acid-Schiff (PAS) with and without diastase, and trichrome, respectively.

Immunohistochemistry was performed on representative blocks using a panel of antibodies (Table 1) and the EnVision+ system. Appropriate positive and negative controls were used throughout the experiment. Staining was considered positive when > 5% of cells showed staining with the appropriate pattern, and positive but focal when the cells showed definite 1%-5% staining or patchy staining.

A MEDLINE search from 1977 to July 2009 was performed with the key words "undifferentiated embryonal sarcoma" and "liver", and the relevant literature was reviewed.

Table 1 Primary antibodies used in this study

Antibody	Clone	Source	Dilution
Vimentin (M)	Vim3B4	Dako (Glostrup, Denmark)	1:100
α-1-antitrypsin (P)		Changdao (Shanghai, China)	1:100
Cytokeratin 18 (M)	DC10	Dako (Glostrup, Denmark)	1:25
Cytokeratin 19 (M)	RCK108	Dako (Glostrup, Denmark)	1:50
CD68 (M)	PG-M1	Changdao (Shanghai, China)	1:50
CD56 (M)	123C3	Dako (Glostrup, Denmark)	1:50
Desmin (M)	D33	Dako (Glostrup, Denmark)	1:50
α-SMA (M)	1A4	Dako (Glostrup, Denmark)	1:50
Myoglobin (M)	MYO18	Dako (Glostrup, Denmark)	1:50
CD117 (P)		Changdao (Shanghai, China)	1:50
S-100 (M)	4C4.9	Changdao (Shanghai, China)	1:200
CD34 (M)	QBEnd10	Dako (Glostrup, Denmark)	1:25
Melanosome (M)	HMB-45	Dako (Glostrup, Denmark)	1:25
Hep Par 1 (M)	OCH1E5	Dako (Glostrup, Denmark)	1:50
AFP (M)	AF04	Changdao (Shanghai, China)	1:100
HBsAg (M)	3E7	Dako (Glostrup, Denmark)	1:50
p53 (M)	DO-7	Dako (Glostrup, Denmark)	1:50
pCEA (P)		Changdao (Shanghai, China)	1:200
mCEA (M)	COL-1	Changdao (Shanghai, China)	1:50
EMA (M)	E29	Dako (Glostrup, Denmark)	1:50
NSE (M)	E27	Changdao (Shanghai, China)	1:50
Chromogranin A (M)	DAK-A3	Dako (Glostrup, Denmark)	1:100
Synaptophysin (M)	SY38	Dako (Glostrup, Denmark)	1:20
Ki67 (M)	MIB-1	Dako (Glostrup, Denmark)	1:100

M: Monoclonal; P: Polyclonal; AFP: α-fetoprotein; HBsAg: Hepatitis B virus surface antigen; pCEA: Polyclonal carcinoembryonic antigen; mCEA: Monoclonal carcinoembryonic antigen; EMA: Epithelial membrane antigen; NSE: Neuron specific enolase; SMA: Smooth muscle actin.

RESULTS

Clinical findings

The patients, including 3 males and 1 female, were at the age of 39, 43, 56 and 63 years, respectively. Lesions involved the right lobe in 2 cases, middle lobe in 1 case and the left lobe in 1 case. Abdominal swelling, with or without a palpable mass, and pain were usually found in the patients. Of the 4 patients, 3 complained of various nonspecific gastrointestinal symptoms, fever and weight loss. Schistosomiasis was found in case 3. The clinical data are summarized in Table 2.

Laboratory findings

The serum alkaline phosphatase (AKP) activity was slightly increased in the 4 patients. Serum α-fetoprotein (AFP), carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) levels were within the normal range. Serum markers of hepatitis B and C virus were negative in 3 patients. However, case 3 was positive for hepatitis B virus surface antigen (HBsAg) and hepatitis B virus core antibody.

Imaging findings

Sonography demonstrated a large confused and disorderly low level echo in the liver (Figure 1A). Computer tomography showed a hypodense and well-circumscribed mass which was multicystic in appearance with a hyperdense septum of variable thickness and dense peripheral rim corresponding to the fibrous pseudocapsule in all patients (Figure 1B and C). Complete tumor resection was performed for each patient.

Gross findings

Grossly, the tumor size ranged 11-16 cm. The tumor was globular and well demarcated, but encapsulation was uncommon or incomplete. The cut surface was polychromatic and variegated tan to grey either soft with fluid and mucoid zones or firm with fleshy areas and necrotico-hemorrhagic changes (Figure 1D). The recurrent mass in one case showed similar gross features as described above.

Microscopic findings

The 4 cases showed the same pathological patterns. Their tumors that were not encapsulated infiltrated the adjacent hepatic parenchyma. Entrapped bile ducts and hepatic cords were often present in areas at the periphery of the tumor (Figure 2A).The tumor cells were spindle or polygonal with small and round or large and bizarre nuclei. Some cells showed marked anisonucleosis with hyperchromasia and sometimes multinucleated giant cells could be observed. The cytoplasm was slightly eosinophilic and contained sharply defined hyaline globules in varying size that were positive for diastase resistant PAS (Figure 2B).

Table 2 Clinical features of undifferentiated liver embryonal sarcoma in adults over the age of 30 years

No.	Ref.	Yr	Sex	Age (yr)	Symptoms and signs	Laboratory findings	Location	Size (cm)	Surgery	Adjuvant treatment	Recurrence	Follow-up
1	Esposito *et al*[6]	1977	M	36	RUQA pain, hepatomegaly, jaundice	↑Bil, AST, ALT	R + L	NA	Liver biopsy	No	NA	DOD 2 mo
2	Tanner *et al*[7]	1978	F	66	RUQA pain, vomiting, diarrhea, weight loss, fever, hepatomegaly	↑ALP	R	NA	HA ligation	No/5-FU	v	AWD 3 yr
3	Chang *et al*[8]	1983	F	55	RUQA pain, weight loss, diarrhea, hepatomegaly	↑ALT	L	10 × 10 × 8	Left lob	No/CAV	No	ANED 1 mo
4	Ellis *et al*[9]	1983	F	86	RUQA pain and mass	Normal	R	18 × 12 × 12	Wedge resec	No	Yes	DOD 8 wk
5	Forbes *et al*[10]	1987	M	69	RUQA pain, nausea, weight loss, hepatomegaly	Normal	NA	NA	No	RT/RT	NA	DOD 10 mo
6	Forbes *et al*[10]	1987	F	49	RUQA pain, nausea, weight loss, hepatomegaly	NA	NA	NA	No	No	NA	NA
7	Zornig *et al*[11]	1992	M	42	RUQA pain	Normal	R	8	Biseg	adm + ifs	No	ANED 30 mo
8	Zaheer *et al*[12]	1994	F	44	RUQA pain and mass, weight loss, fever	↑AFP	R	10	Liver biopsy	adm	NA	DOD 1 wk
9	Grazi *et al*[13]	1996	F	60	Dyspepsia, RUQA mass	Normal	R	22	Rx Hep	No	Yes	DOD 11 mo
10	Nishio *et al*[3]	2003	F	49	RUQA pain	NA	R	14 × 8 × 8	Rx lob	No	Yes	DOD 29 mo
11	Nishio *et al*[3]	2003	M	62	RUQA pain	NA	L	10 × 9 × 7	Left lob	No	No	ANED 10 mo
12	Lepreux *et al*[14]	2005	F	51	RUQA pain	↑AFP	R	NA	No	VAC	NA	Dead 2 mo
13	Lepreux *et al*[14]	2005	F	49	RUQA pain	Normal	L	NA	Left lob	ifs + dtic + adm	No	ANED 6 mo
14	Agaram *et al*[15]	2006	F	33	NA	NA	NA	14.5	NA	NA	No	ANED 6 mo
15	Agaram *et al*[15]	2006	F	50	NA	NA	NA	17	NA	NA	No	ANED 8 mo
16	Scudiere *et al*[4]	2006	F	51	Pneumonia	↑AST, ALT	R	25 × 19 × 8.5	Rx lob	NA	NA	NA
17	Ma *et al*[16]	2008	F	61	RUQA pain	Normal	R	12 × 9 × 8	Rx hep	NA	NA	DOD 8 mo
18	Yang *et al*[17]	2009	M	46	RUQA pain and fever	↑AST, ALT, GGT	R	6.2 × 5.9 × 5.4	Rx hep	NA	NA	NA
19	Yang *et al*[17]	2009	M	54	RUQA pain and fever	↑AST, ALT, GGT	R	13 × 11.7 × 12.4	Rx hep	NA	NA	NA
20	Xu *et al*[18]	2010	F	36	RUQA pain	Normal	L	30 × 25 × 15	Rx hep	NA	NA	NA
21	Present case 1		M	63	RUQA pain and fever	↑AKP	R	15 × 15 × 20	Rx hep	NA	Yes	DOD 18 mo
22	Present case 2		M	39	weight lose and fever	↑AKP	L	16 × 13 × 9	Left lob	TACE	Yes	DOD 32 mo
23	Present case 3		M	56	RUQA pain	↑AKP, ALT, AST, GGT	R	12.5 × 11.5	Rx hep	TACE	Yes	DOD 28 mo
24	Present case 4		F	43	RUQA pain	↑AKP	R	14 × 12 × 10	Rx hep	NA	Yes	DOD 24 mo

R: Right; L: Left; NA: Not available; RUQA: Right upper quadrant abdominal; Rx hep: Right hepatectomy; Rx lob: Right lobectomy; Biseg: Bisegmentectomy; CAV: Cyclophosphamide + doxorubicin + vincristine; VAC: Vincristine + actinomycin D + cyclophosphamide; ifs: Ifosfamide; adm: Adriamycin; dtic: Dacarbazine; DOD: Dead of disease; AWD: Alive with disease; ANED: Alive with no evidence of disease; AKP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein; RT: Radiation therapy; ALP: Alkaline phosphatase; HA: Hepatectomy.

Numerous typical and atypical mitoses were observed. The mucoid areas were consisted of a loose oedematous myxoid matrix and characterized by sparse stellate atypical mesenchymal cells (Figure 2C). Hemorrhage and focal necrosis were present. Fibroblast-like or smooth muscle-like fascicles and bundles were seen in compact areas (Figure 2D). These features are typical of an undifferentiated liver embryonal sarcoma. In a recurrent tumor, the cells showed similar histological features to those described above. However, its cellularity and anaplasia were greater than those of the primary tumor. Focally, different types of mesenchymal differentiation were noted, such as solid spindle cell proliferation, and herringbone or chevron-, angiosarcoma-, liposarcoma-, haemangiopericytoma-, and rhabdomyosarcoma-like components (Figure 2E and F).

Immunohistochemical findings

Immunohistochemical staining of undifferentiated liver embryonal sarcoma in 4 adults is shown in Table 3. Most tumor cells were strongly reactive to vimentin (Figure 3A). Multinucleated giant cells and some spindle cells showed variable granular cytoplasmic positivity for CD68. Diffuse membranous immunostaining for CD56 was shown in all cases (Figure 3B). A diffuse multifocal cytoplasmic immunostaining was observed with a distinct paranuclear dot-like staining using cytokeratins 18 and 19 as a finding not previously described[20] (Figure 3C). Most eosinophilic hyaline globules were positive for a1-antitrypsin (a1-AT). Focal cytoplasmic positivity for desmin was found in some tumor cells (Figure 3D). However, Hep Par 1, HMB-45, CD117, CD34, HBsAg, S100, myoglobin and AFP were immuno-

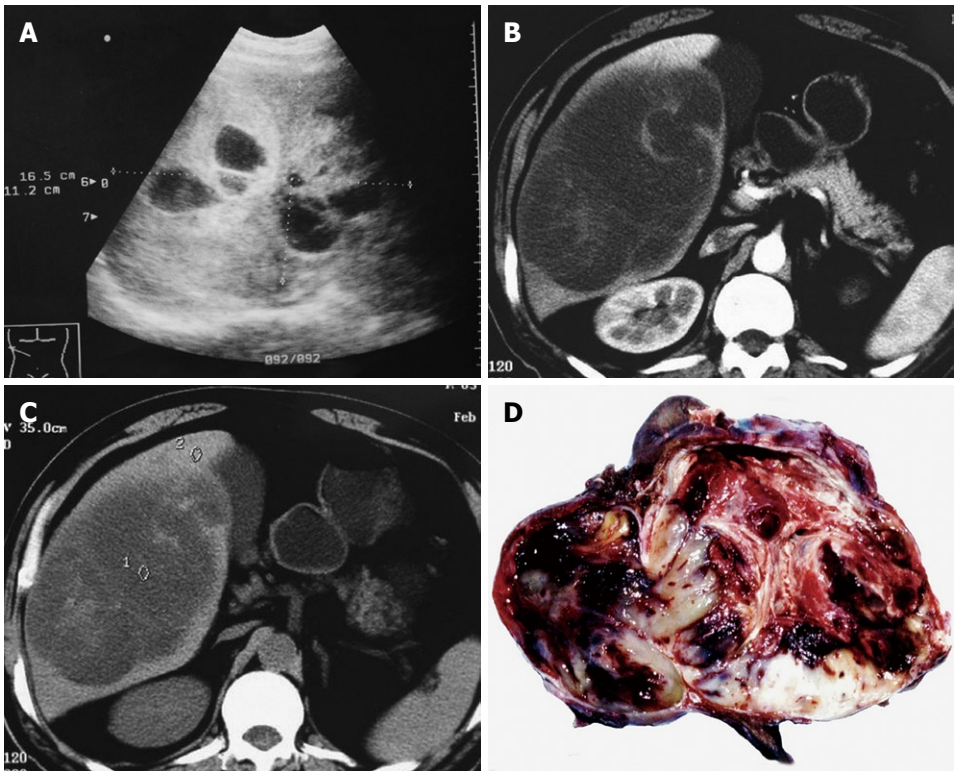

Figure 1 Abdominal ultrasonography showing a 16.5 cm × 11.2 cm multilocular cystic liver mass (A), computed tomography imaging demonstrating a large, hypodense tumor occupying the right lobe of liver with multicystic (B) and solid portions (C), and polychromatic cut surface which is soft with fluid and mucoid zones, firm with fleshy areas and necrotico-hemorrhagic changes (D).

Table 3 Immunohistochemical staining of undifferentiated liver embryonal sarcoma tissue in 4 adults

Antibody	Case 1	Case 2	Case 3	Case 4	
				Primary	Recurrent
Age (yr)/sex	63/M	39/M	56/M	43/F	44/F
Vimentin	+	+	+	+	+
α-1-antitrypsin	+	+	+	+	+
Cytokeratin 18	+F	+F	-	+F	+F
Cytokeratin 19	+F	+F	-	+F	+F
CD68	+	+	+	+	+
CD56	+	+	+	+	+
Desmin	-	+F	+F	-	+F
α-SMA	-	-	-	-	+F
Myoglobin	-	-	-	-	+F
CD117	-	-	-	-	-
S-100	-	-	-	-	+F
CD34	-	-	-	-	+F
HMB45	-	-	-	-	-
Hep Par 1	-	-	-	-	-
AFP	-	-	-	-	-
HBsAg	-	-	-	-	-
p53	-	-	+	+	+
pCEA	-	-	-	-	-
mCEA	-	-	-	-	-
EMA	-	-	-	-	-
NSE	-	-	-	-	-
Chromogranin A	-	-	-	-	-
Synaptophysin	-	-	-	-	-
Ki67	60%	50%	45%	40%	65%

+F: Focal staining; AFP: α-fetoprotein; SMA: Smooth muscle actin; HBsAg: Hepatitis B virus surface antigen; EMA: Epithelial membrane antigen; NSE: Neuron specific enolase; pCEA: Polyclonal carcinoembryonic antigen; mCEA: Monoclonal carcinoembryonic antigen.

negative in all primary cases. Compared with primary tumors, some tumor cells were focally positive for α-smooth muscle actin (α-SMA), CD34, S100 (Figure 3E and F) and myoglobin in one recurrent tumor.

Treatment and follow-up data

Follow-up imaging study with abdominal CT scan showed evidence of recurrent lesions at 9 and 30 mo after initial tumor resection in the 4 patients who died of hepatic failure due to tumor recurrence and thrombosis of intrahepatic veins after 18-32 mo of initial tumor resection. Of the 4 patients, 2 received one additional course of transcatheter arterial chemoembolization (TACE, lipiodol, epirubicin, and hydroxy camptothecin) after liver resection. The recurrent tumor occurred at 30 mo (case 2) and 14 mo (case 3) following resection, suggesting that this tumor is highly chemosensitive to TACE. Permission for an autopsy was not granted.

DISCUSSION

Clinical features

Undifferentiated liver embryonal sarcoma, also known as malignant mesenchymoma, mesenchymal sarcoma, undifferentiated rhabdomyosarcoma, fibromyxosarcoma and lipo fibrosarcoma of the liver, is a rare tumor, most often occurring in late childhood (at the age of 6-10 years) but infrequently in adults. The main features of these patients include a mean age of 51 years (range 33-86 years) and female preponderance. Undifferentiated liver embryonal sar-

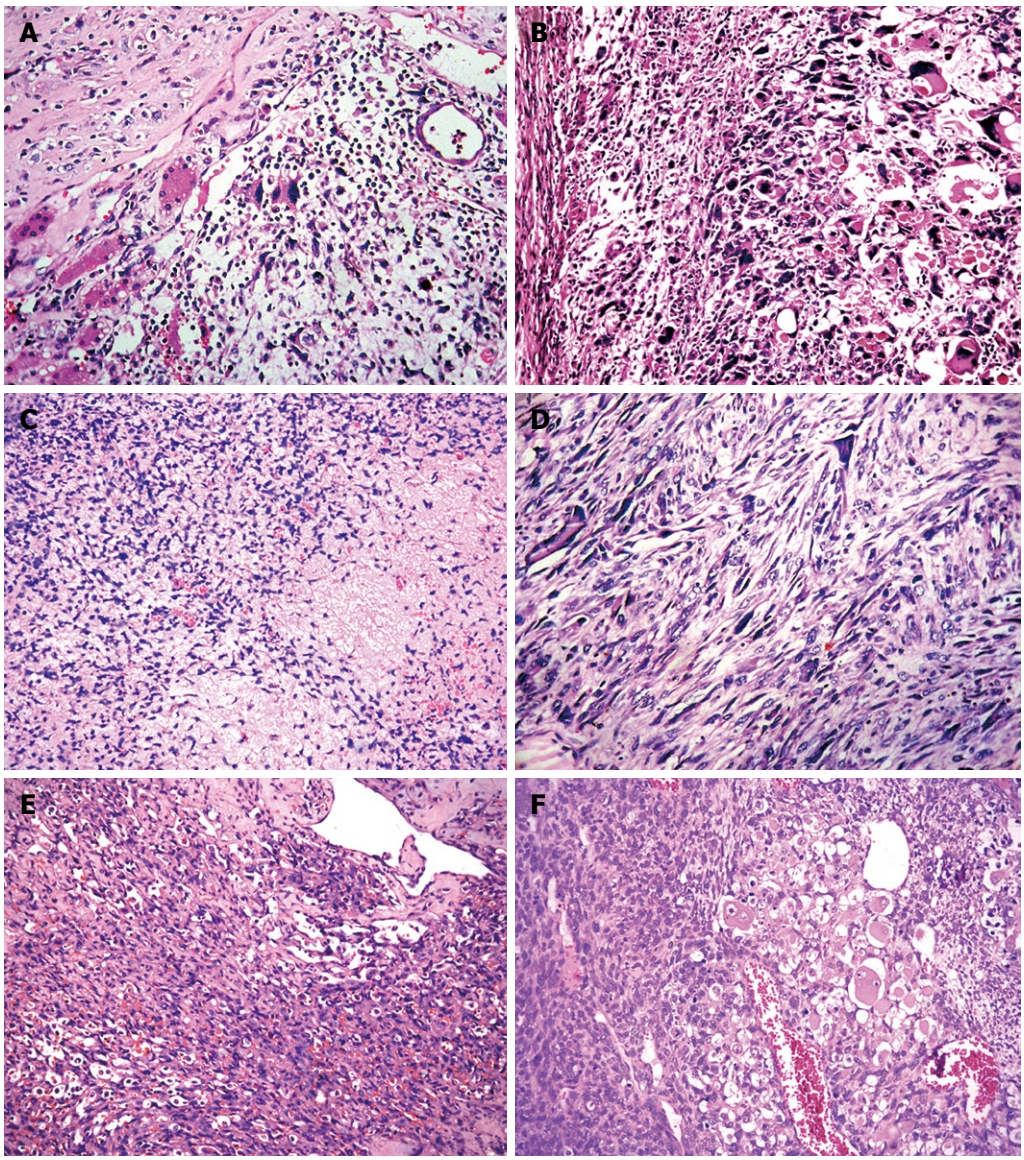

Figure 2 Histology showing residual hepatocytes and bile ducts in the tumor (A), giant cells containing eosinophilic hyaline globules in the cytoplasm (B), loose oedematous myxoid matrix with sparse stellate atypical mesenchymal cells (C), fibroblast-like fascicles (D), angiosarcoma-like cells (E) and pericytoma-like and rhabdomyosarcoma-like cells (F) in compact areas (HE, × 400).

coma has no specific clinical features. It has been shown that children patients usually have the clinical symptoms of a large palpable mass with or without abdominal pain[21]. Some patients complain of various nonspecific gastrointestinal symptoms and signs, such as weight loss, nausea or anorexia, vomiting, jaundice, diarrhea, and fever[5]. However, mass and larger liver were found only in one of our cases, persistent fever and weight loss also presented in our adult cases. According to the literature, UESL is not related to hepatitis and liver cirrhosis, and the liver function and tumor markers such as AFP, CEA and CA19-9 are normal in most cases. In our adult cases, laboratory tests showed mildly elevated levels of alanine aminotransferase and aspartate aminotransferase in one case with positive hepatitis B surface antigen due to hepatic injury. AKP was elevated in all patients. A history of schistosomiasis was first reported. The lesion could be found by ultrasound, CT and MRI. Since tumor presents as a large cystic hepatic mass, it is diagnosed as a benign lesion instead of an UESL in some cases[5]. The features of cystic change shown by CT and MRI are usually different from those of solid-to-cystic change revealed by sonography or pathology[22]. In our cases, a large confused and disorderly low level echo or a multiloculated partially cystic echo was detected by ultrasound. The cyst was characterized by hemorrhage and necrosis. In adults, the primary lesion mainly displayed cystic change, but the recurrent mass presented with solid-to-cystic change and solid type predominance. The identical result was found in one of our adult cases.

Tumor characteristics

The majority of UESL are located in the right lobe, but they can also arise in the left lobe or in the bilateral lobes

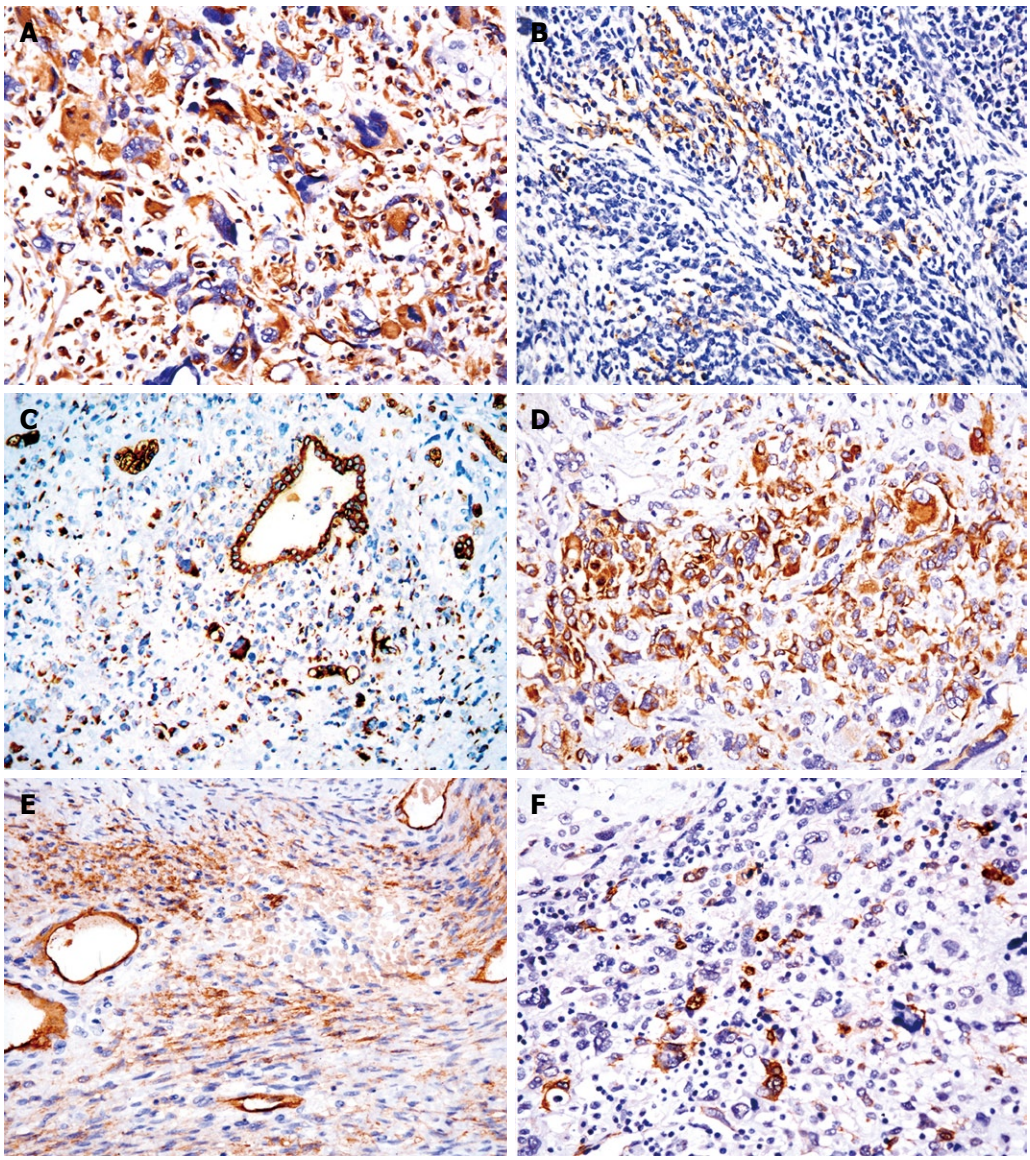

Figure 3 Immunohistochemistry showing tumor cells strongly reactive to vimentin (A), diffuse membranous immunostaining for CD56 in mesenchymal cells (B), diffuse multifocal cytoplasmic immunostaining with a distinct paranuclear dot-like staining using cytokeratin 19 (C), focal cytoplasmic positivity for desmin in some tumor cells (D), tumor cells focally positive for α-smooth muscle actin (E) and S100 (F) (EnVision+, × 400).

simultaneously (Table 1). Macroscopically, UESL is usually a large, solitary and well-circumscribed mass with variable areas of hemorrhage, necrosis and cystic degeneration. Microscopically, it is composed of loosely arranged, medium-large spindles, oval and stellate pleomorphic cells with poorly defined cell borders, and giant cells with severe atypia. Although its pathological origin remains unclear, ultrastructural and immunohistochemical studies have shown its fibroblastic, histiocytic, lipoblastic, myoblastic, myofibroblastic, rhabdomyoblastic and leiomyoblastic differentiation[23]. Most UESL are diffusely positive for vimentin, a1-AT, and focally positive for cytokeratin, desmin, α-SMA, muscle-specific actin, CD68, myoglobin, non-specific enolase, S100, and CD34, suggesting that embryonal sarcoma is an 'undifferentiated' sarcoma, since it may display partial differentiation[23,24]. In addition, genetic alterations are complex and heterogeneous[14]. Mutated p53 and non-expression of telomerase catalytic subunit, human telomerase reverse transcriptase, may also explain its poor biological behavior.

Differential diagnosis

UESL in adults should be differentially diagnosed from carcinosarcoma, sarcomatoid or spindle-cell carcinoma, mesenchymal hamartoma, mixed hepatoblastoma with spindle-cell features, angiomyolipoma, and various other sarcomas (such as malignant fibrous histiocytoma, leiomyosarcoma, osteosarcoma, angiosarcoma, liposarcoma, melanoma, rhabdomyosarcoma or malignant schwannoma[3,23]. Besides its large size, no other specific features can be used in differential diagnosis of UESL from other hepatic masses. However, the morphology and complete immunohistochemical profiles of other hepatic masses are different from those of UESL. Furthermore, the im-

munohistochemical profile of UESL is neither specific nor diagnostic, showing evidence of widely divergent differentiation.

Surgical management and adjuvant therapies

No consensus has been reached in standard treatment of UESL. Although the prognosis of UESL patients is very poor, it has been reported that the tumor is potentially treatable[25-28]. Complete resection with vigorous multiple approaches including chemotherapy remains the treatment of choice[29,30]. Some researchers suggested that recurrent UESL should be radically removed whenever feasible[3]. Our patients received a successful surgery, once or twice. TACE was performed in 2 cases. Recurrent UESL occurred at 30 and 14 mo, respectively, following resection, suggesting that UESL is highly sensitive to TACE. As the tumor is highly malignant and recurrent, it should be radically resected with TACE. However, careful evaluation of a larger number of patients is needed to confirm this treatment strategy.

In conclusion, UESL is still a therapeutic and diagnostic challenge. Radical resection is a treatment of choice. More attention should be paid to this peculiar disease, especially in adults.

COMMENTS

Background

Undifferentiated embryonal sarcoma of the liver (UESL) is a rare and highly malignant spindle cell tumor. Although it is generally a disease of children and young adults, middle-aged and elderly patients have rarely been reported. Previous reports describing its general features do not separate infants from adults, and few studies focusing on adult cases are available. Given that the majority of such patients are under the age of 30 years, adult cases over the age of 30 years are quite exceptional.

Research frontiers

In the past 50 years, less than 60 adult cases have been reported. Furthermore, its detailed clinical, radiological or pathological characteristics of adult cases based on particular immunohistochemistry remain unclear.

Innovations and breakthroughs

UESL in adults shows evidence of widely divergent differentiation into mesenchymal and epithelial phenotypes, and its diagnosis should be made based on morphological and immunohistochemical features. The survival time of patients who undergo complete tumor resection followed by adjuvant transcatheter arterial chemoembolization (TACE) is longer than those who undergo surgical treatment alone.

Applications

The findings in this study are helpful in defining the optimal treatment for UESL patients. Complete resection after adjuvant TACE may improve the survival time of such patients.

Peer review

This is a retrospective review of a relatively rare tumor, UESL. The report outlining its clinical and pathological features is well written but fails to focus on the management strategies specific to UESL. The illustrations are clear. The discussion appears logical.

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BRIEF ARTICLE

Ultrasonic elastography in clinical quantitative assessment of fatty liver

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Abstract

AIM: To investigate the clinical application of ultrasonic elastography in quantitative assessment of fatty liver grading.

METHODS: A total of 105 patients with fatty liver were divided into mild group (n = 46), moderate group (n = 39), and severe group (n = 20). Forty-five healthy individuals served as a normal control group. All patients who underwent routine ultrasound scan and further ultrasonic elastography were evaluated accordingly to the evaluation standards for ultrasonic elastography. The ratio of surface areas of blue region/total surface area in the desired region was measured.

RESULTS: Ultrasonic elastography technique, in comparison to traditional ultrasound, had a rather high consistence in grading of fatty liver [κ value = (95.3%-63.6%)/(1%-63.6%) = 0.87, P = 0.001]. The score of ultrasonic elastography increased with the severity of fatty liver with a sensitivity of 97.14% and a specificity of 91.11%. A significant difference was found in the ratio of surface areas of blue regions between different groups (P < 0.05).

CONCLUSION: Ultrasonic elastography can be used in quantitative assessment of the severity of fatty liver.

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Key words: Transient elastography; Ultrasonic elastography evaluation; Fatty liver; Quantitative diagnosis; Grading of fatty liver

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INTRODUCTION

Fatty liver is one of the important hepatic diseases in China and a threat to the public health. Moderate fatty liver can lead to significant necrosis and inflammation in hepatocytes. Severe fatty liver can lead to fibrosis and pseudolobe formation. If it is not immediately controlled or appropriately treated, the condition can progress to liver cirrhosis. No better and convenient auxiliary examination is currently available for the objective evaluation of fatty liver grading.

In recent years, ultrasonic imaging technique has transformed to functional imaging from anatomical imaging. Ultrasonic elastography technique is one of the new functional ultrasonic imaging techniques, which was developed in the past few years and can be used in quantitative and semi-quantitative assessment of diffused lesions in liver, such as cirrhosis after hepatitis, alcoholic cirrhosis, hepatic dysfunction after surgery, and other diseases[1-4]. Although

many researchers have employed ultrasonic elastography in study of chronic hepatic diseases, few studies are available on the diagnosis of fatty liver[5,6]. This study was to investigate its application in clinical quantitative assessment of fatty liver grading.

MATERIALS AND METHODS

Ultrasonic equipment

Color ultrasonic equipment model HV900 with a linear probe and a frequency of 4-9 MHz was purchased from HITACHI Company (Japan).

Patients

One hundred and five patients with diagnosed fatty liver in the First Affiliated Hospital of China Medical University between November 2008 and March 2009, were divided into mild group (n = 46), moderate group (n = 39), and severe group (n = 20). Forty-five healthy individuals served as a control group. Their fatty liver was graded as previously described[7-10]. Informed consent was obtained from each patient.

The inclusion criteria for normal liver were as follows: smooth hepatic capsule with a linear hyperechogenicity, left lobe with a sharp edge and left outer edge with an angle < 45°, evenly distributed iso-echo in hepatic parenchyma, intrahepatic pipeline system with a normal distribution and well sound-transparent power similar to the normal renal parenchyma echo, clear hepatic and portal vein with unobstructed blood flow, no expanded intrahepatic bile duct, normal liver function, and negative hepatitis test.

Ultrasonic elastography

Patients were placed in supine position and the 8th or 9th intercostal space was selected as the scanning site. Appropriate depth and enhancement were adjusted. The function of elastography was initiated, then the size of desired region was identified (2 cm above and below the boundary of desired region, and the width was not limited) with vascular branches avoided and pressure index strictly controlled at level 2 or 3.

Evaluation standards for ultrasonic elastography

Images were evaluated according to the following standards: one score: a few blue dots in green region of elasticity image with liver membrane blue colored, two scores: liver elasticity image primarily green colored with a few blue spots and liver membrane blue colored, three cores: obvious blue region (< 1/2 of desired region) in elasticity image and liver membrane green colored, four scores: more obvious blue region (> 1/2 of desired region) in elasticity image and liver membrane red colored (Figure 1). These images were evaluated by two physicians with 6-year experience.

Measurement of ratio of surface area in customized blue region

Ultrasonic elastography was performed and the image showed the quasi-circular blue area in the desired image region. The sum of surface area of each blue region was measured on 2D image, and defined as the total surface area of blue regions. The ratio of surface area of blue region was calculated according to the following equation: The ratio of surface area of blue region (BAR) = total surface area of blue regions/total surface area in desired region.

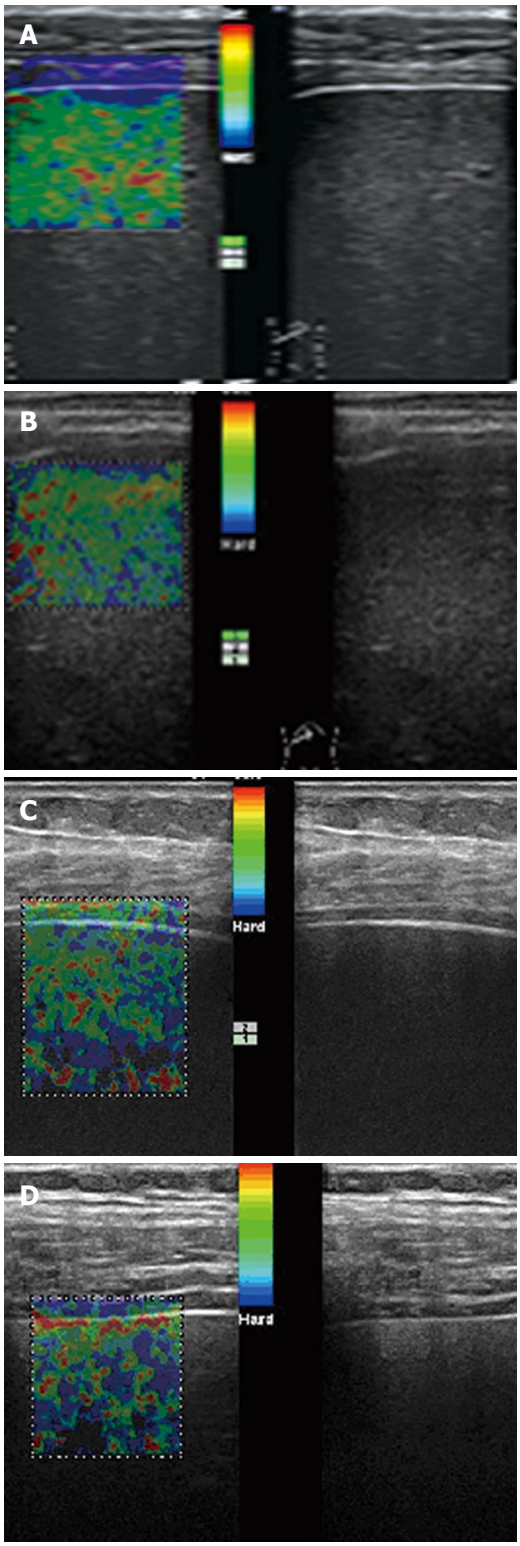

Figure 1 Scores 1 (A), 2 (B), 3 (C), and 4 (D) of ultrasound elastography.

Table 1 Grading of fatty liver shown by traditional ultrasound and elastography

Traditional ultrasound	Elastography				Total
	1 score	2 scores	3 scores	4 scores	
Normal	41	3	1	0	45
Mild	2	42	2	0	46
Moderate	1	3	34	1	39
Severe	0	1	2	17	20
Total	44	49	39	18	150

The consistency of two methods was tested by κ and the κ value was 0.87 (P = 0.001).

Table 2 Fatty liver detected by traditional ultrasound and ultrasonic elastography

Traditional ultrasound	Elastography		Total
	Positive	Negative	
Positive	102	3	105
Negative	4	41	45
Total	106	44	150

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Numeral data were analyzed *via* κ value to test the consistency of traditional ultrasound and ultrasonic elastography in grading of fatty liver. Quantitative data were expressed as mean ± SD. *t*-test was used for intergroup comparison of averages. $P < 0.01$ was considered statistically significant.

RESULTS

Consistency of ultrasonic elastography and traditional ultrasound in grading of fatty liver

The results of ultrasonic elastography were as follows. Of the 45 patients in control group, 41 had one score, 3 had two scores, and 1 had three scores. Of the 46 patients in mild group, 42 had two scores, 2 had one score, and 2 had three scores. Of the 39 patients in moderate group, 34 had three scores, 1 had one score, 3 had two scores, and 1 had four scores. Of the 20 patients in severe group, 17 had four scores, 2 had three scores, and 1 had two scores. The observed consistency rate was 95.3%, the chanced consistency rate was 63.8%, the κ value was 0.87 (P = 0.001). κ analysis showed that the consistency of ultrasonic elastography and traditional ultrasound was high in grading of fatty liver, where the κ value was 0.87 (Table 1).

Score of ultrasonic elastography increased with severity of fatty liver

The score of ultrasonic elastography increased with the severity of fatty liver (Figure 2). The primary score of ultrasonic elastography was 1, 2, 3 and 4, respectively in control, mild, moderate, and severe groups, accounting for 91.1%, 91.3%, 87.1%, and 85.0% of each group, respectively.

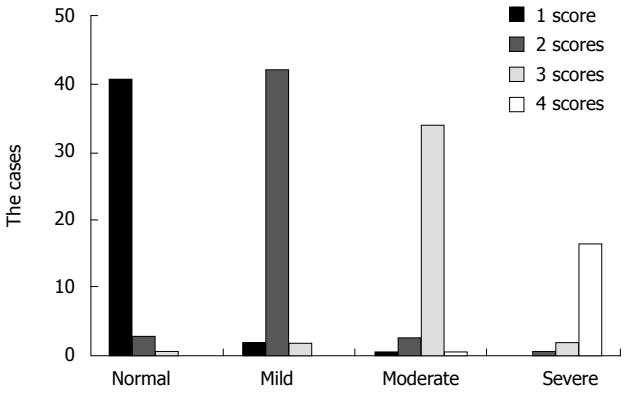

Figure 2 Distribution of scores in different gradings of fatty liver.

Sensitivity and specificity of ultrasonic elastography for fatty liver

All the 150 patients underwent examination. Of the 105 patients who were diagnosed as fatty liver by traditional ultrasound, 102 were diagnosed as fatty liver and 3 as normal by ultrasonic elastography, Of the 45 patients who were diagnosed as normal by traditional ultrasonic examination, 41 were diagnosed as normal and 4 as fatty liver by ultrasonic elastography with a sensitivity of 97.14% and a specificity of 91.11% (Table 2).

BAR value of ultrasonic elastography in different groups

The BAR value was 0.0943 ± 0.0851, 0.1947 ± 0.0582, 0.3242 ± 0.0662, and 0.5005 ± 0.0943, respectively, in control, mild, moderate, and severe groups ($P < 0.001$), which increased with the severity of fatty liver.

DISCUSSION

Types of ultrasound elastography and their clinical application

Ultrasonic elastography is a brand new ultrasonic technique. Its basic principle relies on the application of dynamic or static/semi-static stimulation from an intrinsic (including autonomous) or extrinsic source of tissues. Under physical regulation of elastic mechanics and biomechanics, tissues would generate a strain as a response to relocation, reactions, and possibly a certain change in the speed, which is shown as a disturbance in distribution. Therefore, ultrasonic elastography can obtain quantitative information on distributions of elasticity in tissues. Currently, these distributions are marked by various colors, including red, blue, yellow, and green, with blue representing sclerosis and red representing softness. Many types of ultrasonic elastography available at present, can be divided into strain elastography which produces an imaging of pressure by comparing differences in tissues before and after the operator applies a certain force, transient elastography which discovers relocation of tissues once transient vibration is applied at a low frequency, and vibration sonoelastography which produces

a resonance image of tissues once vibration is applied at a low frequency[11]. Different manufacturers of ultrasonic equipments would design software systems for ultrasonic elastography based on different imaging principles. The first technique, strain imaging, is more susceptible to human factors. Strain and relocation can vary greatly due to different pressures and frequencies of pressure. In order to compensate for such variations, the instrument is equipped with a display device to show the comprehensive indices such as pressures and frequencies of pressure.

Since the invention of ultrasonic elastography, it has been applied to the detection of masses and lesions in mammary and liver tissues, thus, more research results on mammary lesions are available[12,13].

Pathophysiology of fatty liver and characteristics of ultrasound elastography

Fatty liver, also known as intra-hepatic lipid degeneration, is caused by accumulation of lipid in liver due to various reasons. In fact, lipid is accumulated in normal liver, accounting for 5% of fresh liver. When the amount of lipid is over 5% in liver, it is defined as fatty liver, where lipids are mostly in a form of triacylglycerol. Based on the amount of lipids in liver, fatty liver is further divided into mild (accounting 5%-10% of fresh liver), moderate (accounting for 10%-25% of fresh liver), and severe (accounting for over 25% of fresh liver). With the aggravation of fatty liver, hepatic fibrosis also worsens. Currently, ultrasonic examination is the most preferable diagnostic method for fatty liver. However, it costs more and no objective index is available. Therefore, ultrasonic elastography was performed to detect hepatic fibrosis in patients with fatty liver in this study, which showed significant variations in different groups. The images of control group showed evenly distributed green color with few red dots. As fatty liver worsened, more blue regions gradually appeared and the color of liver enveloping membrane was also significantly changed, indicating that ultrasonic elastography can provide more direct real-time images. Therefore, ultrasonic elastography can be used in detection and diagnosis of fatty liver and scores of ultrasonic elastography can be used as an auxiliary diagnostic index for fatty liver.

Factors affecting ultrasound elastography

Liu *et al*[14] believed that subcutaneous fat is not related to the severity of fatty liver but is an interfering factor for elastography. When subcutaneous fat between skin and liver enveloping membrane is over 3 cm and the liver is situated in depth, it would be difficult to obtain good elastography images. In this study, the subcutaneous fat between skin and liver enveloping membrane was over 3.1 cm in 3 patients, and elastography images with a better resolution were not obtained. Del Poggio *et al*[15] also believed that instant elastography cannot reliably determine fatty liver if the patient is obese.

Consistency and sensitivity of ultrasound elastography

In this study, the consistency of traditional ultrasound and ultrasonic elastography was rather high in grading of fatty liver (κ value > 0.75) with a sensitivity of 97.14% and a specificity of 91.11%, indicating that ultrasonic elastography can be used in grading of fatty liver. Liver biopsy has been recognized as the gold standard for diagnosing hepatic fibrosis, but it leads to severe complications and false negative results[16-19]. The complications of liver biopsy include post biopsy pain, bleeding, organ perforation, and even death. Taking into account the complications of liver biopsy, we did not perform it.

Ultrasound elastography operation

Ultrasonic elastography relies on the strain imaging, which requires application of certain pressures before it is formed. This technique requires highly skilled operators due to its rigorous operating criteria, such as the size and location of sample window, range of scan, enhancement of 2D image, and control of pressure index, which would affect the research results.

Prospect of ultrasound elastography

Blue region in the desired region represents the hardness of tissues and the increased blue region indicates the severity of fatty liver. Our study showed that an increased BAR value when fatty liver worsened, suggesting that BAR value can be used as a quantitative index for the severity of fatty liver.

Friedrich-Rust *et al*[20] showed that ARFI imaging is a promising US-based method for the assessment of liver fibrosis in chronic viral hepatitis, with a similar diagnostic accuracy of TE. However, it also has some inadequacies.

In conclusion, ultrasonic elastography technique can be used as an auxiliary examination for the assessment of fatty liver. It provides new clinical diagnostic indicators and is able to reduce the false positive and negative rate, thus allowing the doctors to make the right diagnosis in a limited time.

COMMENTS

Background

Ultrasonic elastography technique is one of the new functional ultrasonic imaging techniques, which is developed over the past few years and can be used for quantitative and semi-quantitative assessment of liver sclerosis and diffused lesions.

Research frontiers

Ultrasonic elastography is a brand new ultrasonic technique. Since the invention of ultrasonic elastography, it has been applied to mammary tissues with more research results on mammary lesions available.

Innovations and breakthroughs

The clinical application of ultrasonic elastography in quantitative assessment of the severity of fatty liver was studied.

Applications

This research showed the area of blue region value can be used as a quantitative index for the severity of fatty liver.

Peer review

In this study, the authors showed that ultrasonic elastography can be used as an auxiliary examination for the quantificational assessment of fatty liver. It would be more interesting if biopsies were done in those with fatty liver.

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BRIEF ARTICLE

Mn-SOD and CuZn-SOD polymorphisms and interactions with risk factors in gastric cancer

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Abstract

AIM: To investigate the effects of superoxide dismutase (SOD) polymorphisms (*rs4998557*, *rs4880*), *Helicobacter pylori* (*H. pylori*) infection and environmental factors in gastric cancer (GC) and malignant potential of gastric precancerous lesions (GPL).

METHODS: Copper-zinc superoxide dismutase (SOD1, CuZn-SOD)-G7958A (*rs4998557*) and manganese superoxide dismutase (SOD2, Mn-SOD)-Val16Ala (*rs4880*) polymorphisms were genotyped by SNaPshot multiplex polymerase chain reaction (PCR) in 145 patients with GPL (87 cases of gastric ulcer, 33 cases of gastric polyps and 25 cases of atrophic gastritis), 140 patients with GC and 147 healthy controls. *H. pylori* infection was detected by immunoblotting analysis.

RESULTS: The SOD1-7958A allele was associated with a higher risk of gastric cancer [odds ratio (OR) = 3.01, 95% confidence intervals (95% CI): 1.83-4.95]. SOD2-16Ala/Val genotype was a risk factor for malignant potential of GPL (OR = 2.04, 95% CI: 1.19-3.49). SOD2-16Ala/- genotype increased the risk of gastric cancer (OR = 2.85, 95% CI: 1.66-4.89). SOD1-7958A/- genotype, SOD2-16Ala/- genotype, alcohol drinking, positive family history and type I *H. pylori* infection were associated with risk of gastric cancer, and there were additive interactions between the two genotypes and the other three risk factors. SOD2-16Ala/Val genotype and positive family history were associated with malignant potential of GPL and jointly contributed to a higher risk for malignant potential of GPL (OR = 7.71, 95% CI: 2.10-28.22). SOD1-7958A/- genotype and SOD2-16Ala/- genotype jointly contributed to a higher risk for gastric cancer (OR = 6.43, 95% CI: 3.20-12.91).

CONCLUSION: SOD1-7958A/- and SOD2-16Ala/-genotypes increase the risk of gastric cancer in Chinese Han population. SOD2-16Ala/-genotype is associated with malignant potential of GPL.

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Key words: Copper-zinc superoxide dismutase; Manganese superoxide dismutase; Gastric cancer; Gastric precancerous lesions; Gene polymorphisms; Interaction

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INTRODUCTION

Gastric cancer (GC) is the second most common malignancy worldwide and 42% of the cases occur in China[1]. Gastric carcinogenesis is a complex multi-factor and multi-step process involving various environmental carcinogens, *Helicobacter pylori* (*H. pylori*) infection and genetic variants. The involvement of reactive oxygen species (ROS) in the pathogenesis of gastric malignancies is well known[2,3]. ROS are molecules or ions formed by the incomplete one-electron reduction of oxygen, including singlet oxygen, superoxides, peroxides, hydroxyl radical, and hypochlorous acid, which contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, induce oxidative damage to nucleic acids, proteins and lipids, and affect membrane fluidity by altering the amounts of unsaturated fatty acids and proteins in the cell membrane[4]. ROS participates simultaneously in Ras-Raf-MEK1/2-ERK1/2 and the p38 mitogen-activated protein kinases (MAPK) signaling pathway that have inverse functions in tumorigenesis[4]. In addition, with aging, humans tend to have an increased affectability of lipid peroxides caused by ROS[5]. A recent research has indicated that ROS also plays a critical role in the energy dysfunction of mitochondria caused by ethanol induced gastric mucosal injury[6].

Superoxide dismutase (SOD) is a major antioxidant enzyme, which plays a vital role in clearance of ROS. Among the isoforms of SOD, copper-zinc superoxide dismutase (SOD1, CuZn-SOD) with copper (Cu) and zinc (Zn) in its catalytic center is localized in the intracellular cytoplasmic compartments, and manganese superoxide dismutase (SOD2, Mn-SOD) plays an important role as a primary mitochondria antioxidant enzyme[7]. In addition, many researches have shown that the activity and expression of SOD changed significantly in gastric cancer patients[2,8,9], which can either promote or suppress tumor formation in human gastric mucosa[10].

In recent years, host genetic factors are emerging as determinants of increasing risk for many cancers including GC[11,12]. Therefore, SOD genes are good candidates to evaluate the genetic susceptibility to gastric cancer. The gene polymorphism SOD2-Val16Ala (rs4880) has been evaluated widely for its association with various cancers including GC[13-18]. Moreover, Val16Ala significantly reduced SOD2 catalytic activity in hepatocytes[19]. However, there have been few studies on the correlations between SOD1 polymorphisms and cancer, and SOD1 polymorphisms have not been evaluated for its association with risk of gastric cancer.

Epidemiologic evidence has shown that gastric cancer is associated with *H. pylori* colonization[1,20]. *H. pylori*, which infects 50% of the world's population, is a major factor in both the induction of atrophic gastritis and histological progression to gastric cancer. Bacterial virulence factors such as cytotoxin-associated protein (CagA), vacuolating cytotoxin (VacA), and others have been associated with higher risks for gastric cancer development[21,22]. *H. pylori* vacuolating cytotoxin VacA can induce cellular vacuolation in epithelial cells and efficiently block proliferation of T cells by inducing a G_1/S cell cycle arrest[23], and CagA protein can promote signal transduction of oncogene and cell division[24]. *H. pylori* is also associated with ROS and SOD. It has been shown that *H. pylori* induced the production of intracellular ROS in gastric cells[25], resulting in changes in the activity and content of SOD[26,27]. *H. pylori* infection can also cause DNA oxidative damage by its main virulence CagA and VacA, which further implied the role of bacteria in tumorogenesis[28,29].

Gastric cancer is a multifactorial disease. Besides genetic susceptibility and *H. pylori* infection, gastric cancer is also associated with dietary and environmental factors[30]. It has been indicated that long-term massive alcoholic consumption could induce gastric mucosal injury such as hyperemia, edema and erosion, and promote bacterial multiplication and the synthesis of carcinogenic nitrosamines[31]. Smoking has also been found as a risk factor of gastric cancer[32]. In this study, we investigated the association between gastric cancer and the polymorphisms of SOD1-G7958A (rs4998557) and SOD2-Val16Ala (rs4880), and evaluated the relationship between gastric cancer and the epidemiological factors including age, sex, smoking, alcohol drinking, family history of gastric cancer and different types of *H. pylori* infection.

MATERIALS AND METHODS

Study population

From June 2007 to June 2009, 145 patients with gastric precancerous lesions (GPL) (87 cases of gastric ulcer, 33 cases of gastric polypus and 25 cases of atrophic gastritis), 140 patients with gastric cancer and 147 healthy controls were recruited from 3A grade hospitals in Hexi Region, Gansu Province of China. The recruiting criteria of cases include newly diagnosed, histopathologically confirmed and previously untreated patients in the oncology, gastroenterology and general surgery departments. Healthy controls were composed of 147 individuals who visited the outpatient department for physical examination, without tumors and gastrointestinal diseases. Subjects were informed of the detailed study protocol, and signed consent forms, and the study was approved by local ethics committees. A questionnaire given to each patient collected information on (1) demographic factors, such as age and sex; (2) smoking (at least one cigarette per day for 6 mo or longer) and alcohol drinking history (at least twice a week for 6 mo or longer and at least 100 g each time); and (3) family history of gastric cancer (first-

Table 1 Primers used for genotyping

SNP		Primers	Concentration (μmol/L)
rs4998557	Forward	5′-CGGTGTGGTGTGGATGTTGTG-3′	1.0
	Reverse	5′-GCCCCCAGGAGAGGACTGATT-3′	1.0
	SF	5′-TTTTTTTTTTTTTCCATTACCTGAATGGCTATACTGCTT-3′	0.8
rs4880	Forward	5′-CGGGCTGTGCTTTCTCGTCTT-3′	2.0
	Reverse	5′-GCCAACGCCTCCTGGTACTTC-3′	2.0
	SR	5′-TTTTGGAGCCCAGATACCCCAAA-3′	1.6

SNP: Single nucleotide polymorphism; SF: Single-base extension forward; SR: Single-base extension reverse.

degree relatives with gastric cancer including parents, brothers and sisters). Each subject was donated 3 mL peripheral vein blood in EDTA-K2 anticoagulative tube for DNA extraction and 2 mL serum for *H. pylori* infection test, and all specimens were kept frozen at -80℃.

Extraction of genomic DNA

Genomic DNA was extracted from the whole blood using Blood Genome DNA Extraction Kit (TaKaRa Bio, Dalian, China) according to the manufacturer's instructions. Concentration and purity of DNA were determined by SP-721 spectrophotometer (Eppendorf, Hamburg, Geman) at *A*260 nm and *A*280 nm. Integrity of DNA was confirmed by 1% agarose electrophoresis. The DNA samples were diluted to 5-10 ng/μL for genotyping. DNA was stored at -80℃ until use.

Detection of H. pylori infection

Presence and type of *H. pylori* infection were tested with *H. pylori* antibody Immunoblotting Kit (Blot Biotech, Shenzhen, China) following the manufacturer's instructions. Type I *H. pylori* infection was defined when either CagA or VacA was positive, or both were positive. Type Ⅱ *H. pylori* infection was defined if ureases (UreA/UreB) were positive. Patients were defined as *H. pylori* negative if CagA, VacA and ureases were negative.

Genotyping of SOD polymorphisms

SOD1 G7958A and SOD2 Val16Ala (T201C) polymorphisms were determined by multiplex SNaPshot technology. PCR and single-base extension primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1). Multiplex PCR was performed in a 20 μL reaction mixture containing 2 μL 10 × Buffer I, 1 U HotStarTaq polymerase (Qiagen, Dusseldorf, Geman), 0.3 mM dNTP, 3.0 mM $MgCl_2$ (Qiagen), 2 μL each PCR primer, and 5 ng template DNA in a thermal cycler (Applied Biosystems, Foster City, USA). PCR conditions were 95℃ for 15 min denaturation; 11 cycles at 94℃ for 20 s, 65-0.5℃ /cycle for 40 s, and 72℃ for 100 s; 24 cycles at 94℃ for 20 s, 59℃ for 30 s, 72℃ for 1.5 min; and 72℃ for 2 min. PCR product was stored at 4℃. One U SAP (Promega, Madison, USA) and 1 U Exonuclease I (Epicentre, San Diego, USA) were added into 10 μL PCR product for purification at 37℃ for 60 min, and 75℃ for 15 min. SNaPshot analysis was performed in a volume of 10 μL containing 5 μL SNaPshot Multiplex Kit (Applied Biosystems), 2 μL multiplex PCR product, 1 μL single-base extension primer mix and 2 μL ddH2O. Extension reactions were performed in a thermal cycler (Applied Biosystems) at 96℃ for 1min; 28 cycles at 96℃ for 10 s, 50℃ for 5 s, 60 ℃ for 30 s; and 60℃ for 1 min. Extension product was stored at 4℃. One U SAP (Promega) was added into 2 μL extension product for purification at 37℃ for 60 min, and 75℃ for 15 min. Single-base extension products after purification were mixed with deionized formamide containing GeneScan 120 LIZ Size Standard, denatured at 95℃ for 5 min and analyzed on an ABI Prism 3130XL genetic analyzer using GeneMapper 4.0 (Applied Biosystems).

For quality control, positive and negative controls and blinded duplicate samples were run. Alternative genotyping approaches were used as required to verify technical reliability and accuracy. Blinded repeat samples were run in 10% of the samples. A second scientist checked all laboratory interpretations independently.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Differences of measurement data and numeration data were assessed by single factor analysis of variance and Pearson χ^2 test, respectively. Goodness-of-fit χ^2 test was used to verify whether the distribution of SOD genotypes was in accordance with Hardy-Weinberg equilibrium. Non-conditional logistic regression analysis was performed to analyze the association of risk factors and genotypes of SOD with gastric cancer. According to the interaction model proposed by Khoury *et al*[33] and Ottman *et al*[34], we set up dummy variables in accordance with different genes-environmental exposure and analyzed genes-environment interaction in gastric cancer development by logistic regression. We determined the presence or absence of interactions by interaction coefficients (γ, $\gamma = \beta_{eg}/\beta_e$) (β, regressive coefficient; β_{eg}, regressive coefficient when genetic and environmental factors coexist; β_e, regressive coefficient when environmental factors exist alone) and judged the types of interaction by quantitative relationship of OR_{eg} (OR, odds ratio; OR_{eg}, OR when genetic and environmental factors coexist), OR_e (OR_e, OR when environmental factors exist alone) and OR_g (OR_g, OR when genes exist alone)[35-37]. Age and sex corrected odds ratios (ORs) with corresponding 95% confidence intervals

Table 2 Demographic characteristics and risk factors for gastric cancer and malignant potential of gastric precancerous lesion

Variable	HC (%) *n* = 147	GPL (%) *n* = 145	GC (%) *n* = 140	[1]*P*	GC *vs* GPL [2]OR (95% CI)/*P*	GC *vs* HC [2]OR (95% CI)/*P*
Age (yr), mean ± SD	54.8 ± 9.6	55.8 ± 11.4	56.5 ± 10.3	0.370		
Sex						
Female	50 (34.0)	35 (24.1)	37 (26.4)		1	1
Male	97 (66.0)	110 (75.9)	103 (73.6)	0.146	1.13 (0.66-1.93)/0.656	0.70 (0.42-1.16)/0.163
Smoking[3]						
No	114 (77.6)	104 (71.7)	102 (72.9)		1	1
Yes	33 (22.4)	41 (28.3)	38 (27.1)	0.484	0.95 (0.56-1.59)/0.831	1.29 (0.75-2.20)/0.358
Alcohol[4]						
No	100 (68.0)	69 (47.6)	55 (39.3)		1	1
Yes	47 (32.0)	76 (52.4)	85 (60.7)	< 0.001	1.40 (0.88-2.25)/0.158	3.29 (2.03-5.34)/< 0.001
FH[5]						
No	135 (91.8)	120 (82.8)	99 (73.8)		1	1
Yes	12 (8.2)	25 (17.2)	41 (26.2)	< 0.001	1.99 (1.13-3.49)/0.017	4.66 (2.33-9.32)/< 0.001
H. pylori						
Negative	76 (51.7)	39 (26.9)	26 (18.6)		1	1
Type Ⅰ	65 (44.2)	98 (67.6)	108 (77.1)	< 0.001	1.65 (0.94-2.91)/0.082	4.86 (2.83-8.35)/< 0.001
Type Ⅱ	6 (4.1)	8 (5.5)	6 (4.3)	0.132	1.13 (0.35-3.62)/0.843	2.92 (0.87-9.86)/0.084

[1]Gastric cancer (GC) *vs* gastric precancerous lesion (GPL) *vs* healthy control (HC), *P* values were calculated by One-Way ANOVA analysis, χ^2 test; [2]OR (odds ratio), 95% CI (95% confidence interval), *P* values were calculated by logistic regression; [3]Smoking history, at least one cigarette per day for 6 mo or longer; [4]Alcohol drinking history, at least twice a week for 6 mo or longer and at least 100 g each time; [5]Family history of gastric cancer, first-degree relatives with gastric cancer (parents, brothers or sisters). FH: Family history of gastric cancer.

(95% CI) and regressive coefficient (β) were calculated by logistic regression analysis. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Subject characteristics and analysis of risk factors for GC and malignant potential of GPL

The demographic characteristics and frequency distributions of smokers, alcohol drinkers and different types of *H. pylori* are shown in Table 2. There was no statistically significant difference among the three groups in terms of age, sex and smoking histories ($P > 0.05$). Alcohol drinking, positive family history and type I *H. pylori* infection were associated with an increased risk for GC development (OR = 3.29, 95% CI: 2.03-5.34; OR = 4.66, 95% CI: 2.33-9.32; OR = 4.86, 95% CI: 2.83-8.35, respectively). A positive family history was associated with an increased risk for malignant potential of GPL (OR = 1.99, 95% CI: 1.13-3.49).

Distribution of SOD1 and SOD2 polymorphisms

All genotypes in the healthy controls were in Hardy-Weinberg equilibrium ($P > 0.05$). The frequencies of AG and AA genotypes in SOD1 were significantly higher in patients with GC than in healthy controls ($P < 0.05$), and the risk of gastric cancer in carriers with SOD1 A/- genotype was 3.01 folds higher (95% CI: 1.83-4.95) than in carriers with GG genotype. The frequencies of Val/Ala and Ala/Ala genotypes in SOD2 were significantly higher in patients with GC than in healthy controls ($P < 0.05$), and the risk of gastric cancer in carriers with SOD2 Ala/- genotype was 2.85 folds higher (95% CI: 1.66-4.89) than in carriers with Val/Val genotype. The malignant potential of GPL in carriers with SOD2 16Ala/Val and 16Ala/- genotypes was 2.04 folds (95% CI: 1.19-3.49) and 2.19 folds (95% CI: 1.30-3.68) higher than in those with Val/Val genotype, respectively (Table 3).

Interaction of SOD2 Val16Ala and positive family history for malignant potential of GPL

A positive family history combined with SOD2 Ala/- genotype resulted in an increased risk for malignant potential of GPL (OR = 7.71, 95% CI: 2.10-28.22) (Table 4). The interaction coefficients (γ) was 2.96. SOD2 Ala/- genotype could generate the amplification effect on a positive family history, and the interaction accorded with the super-multiplication model.

Interaction of SOD1 G7958A and environmental factors for gastric cancer development

Interaction 1: Alcohol drinking coexisted with SOD1 A/- genotype resulted in an increased risk for gastric cancer development (OR = 16.50, 95% CI: 6.67-40.86). The γ value was 1.20. SOD1 A/- genotype could generate the amplification effect on alcohol drinking.

Interaction 2: A positive family history coexisted with SOD1 A/- genotype resulted in an increased risk for gastric cancer development (OR = 15.56, 95% CI: 5.57-43.50). The γ value was 1.35. SOD1 A/- genotype could generate the amplification effect on a positive family history.

Interaction 3: Type I *H. pylori* infection coexisted with SOD1 A/- genotype resulted in an increased risk for gastric cancer development (OR = 10.71, 95% CI: 4.92-23.33). The γ value was 1.27. SOD1 A/- genotype could generate the amplification effect on type I *H. pylori* infection. All the interactions accorded with the additive model (Table 5).

Table 3 Distribution of SOD1 G7958A and SOD2 Val16Ala polymorphisms

Variable	HC (%) *n* = 147	GPL (%) *n* = 145	GC (%) *n* = 140	[1]*P*	GC *vs* GPL [2]OR (95% CI)/*P*	GC *vs* HC [2]OR(95% CI)/*P*
SOD1						
GG	78 (53.1)	47 (32.4)	39 (27.9)		1.00	1.00
AG	57 (38.8)	72 (49.7)	72 (51.4)		1.16 (0.67-2.02)/0.593	2.60 (1.54-4.39)/< 0.001
AA	12 (8.2)	26 (17.9)	29 (20.7)	< 0.001[3]	1.32 (0.66-2.62)/0.429	4.94 (2.26-10.80)/< 0.001
A/-	69 (46.9)	98 (67.6)	101 (72.1)	< 0.001[4]	1.21 (0.72-2.03)/0.483	3.01 (1.83-4.95)/< 0.001
SOD2						
Val/Val	119 (81.0)	112 (77.2)	85 (60.7)		1.00	1.00
Ala/Val	27 (18.4)	31 (21.4)	48 (34.3)		2.04 (1.19-3.49)/0.009	2.57 (1.48-4.48)/0.001
Ala/Ala	1 (0.7)	2 (1.4)	7 (5.0)	0.001[5]	4.50 (0.91-22.29)/0.065	10.68 (1.26-90.79)/0.030
Ala/-	28 (19.0)	33 (22.8)	55 (39.3)	< 0.001[6]	2.19 (1.30-3.68)/0.003	2.85 (1.66-4.89)/< 0.001

[1]*P* values were calculated by χ^2 test; [2]Adjusted for age, sex, OR, 95% CI, *P* values were calculated by logistic regression; [3]GG *vs* AG *vs* AA; [4]GG *vs* A/-; [5]Val/Val *vs* Ala/Val *vs* Ala/Ala; [6]Val/Val *vs* Ala/-. A/-: AA + AG; Ala/-: Ala/Val + Ala/Ala. HC: Healthy control; GPL: Gastric precancerous lesion; GC: Gastric cancer.

Table 4 Interaction of family history and SOD2 Val16Ala for malignant potential of gastric precancerous lesion

Family history	SOD2	GPL (%) *n* = 145	GC (%) *n* = 140	[1]OR(95% CI)	β	γ = β_{eg}/β_e
Negative	Val/Val	89 (61.4)	58 (41.4)	1.00		
Negative	Ala/-	31 (21.4)	41 (29.3)	2.22 (1.24-3.97)	0.80	
Positive	Val/Val	24 (16.6)	27 (19.3)	1.99 (1.03-3.84)	0.69	
Positive	Ala/-	1 (0.7)	14 (10.0)	7.71 (2.10-28.22)	2.04	2.96

A/-: AA + AG; Ala/-: Ala/Val + Ala/Ala. β: Regressive coefficient; γ: Interaction coefficients; β_{eg}: Regressive coefficient when genetic and environmental factors coexist; β_e: Regressive coefficient when environmental factors exist alone. [1]Adjusted for age, sex, OR, 95% CI, β were calculated by logistic regression. GPL: Gastric precancerous lesion; GC: Gastric cancer.

Table 5 Interaction of SOD1 G7958A and environmental factors for gastric cancer development

Factors	SOD1	HC (%) *n* = 147	GC (%) *n* = 140	[1]OR (95% CI)	β	γ = β_{eg}/β_e
Alcohol						
No	GG	58 (39.5)	10 (7.1)	1.00		
No	A/-	42 (28.6)	45 (32.1)	6.00 (2.70-13.33)	1.79	
Yes	GG	21 (14.3)	29 (20.7)	10.30 (3.94-26.91)	2.33	
Yes	A/-	26 (17.7)	56 (40.0)	16.50 (6.67-40.86)	2.80	1.20
Family history						
Negative	GG	73 (49.7)	25 (17.9)	1.00		
Negative	A/-	62 (42.2)	74 (52.9)	3.58 (2.02-6.37)	1.28	
Positive	GG	6 (4.1)	14 (10.0)	7.70 (2.63-22.51)	2.04	
Positive	A/-	6 (4.1)	27 (19.3)	15.56 (5.57-43.50)	2.75	1.35
Type I *H. pylori*						
Negative	GG	55 (37.4)	10 (7.1)	1.00		
Negative	A/-	27 (18.4)	22 (15.7)	4.48 (1.84-10.91)	1.50	
Positive	GG	24 (16.3)	29 (20.7)	6.41 (2.69-15.27)	1.86	
Positive	A/-	41 (27.9)	79 (56.4)	10.71 (4.92-23.33)	2.37	1.27

A/-: AA + AG; Ala/-: Ala/Val + Ala/Ala. [1]Adjusted for age, sex, OR, 95% CI, β were calculated by logistic regression. HC: Healthy control; GC: Gastric cancer.

Interaction of SOD2 Val16Ala and environmental factors for gastric cancer development

Interaction 1: Alcohol drinking coexisted with SOD2 Ala/- genotype resulted in an increased risk for gastric cancer (OR = 9.46, 95% CI: 4.08-21.94). The γ value was 1.36. SOD2 Ala/- genotype could generate the amplification effect on alcohol drinking.

Interaction 2: A positive family history coexisted with SOD2 Ala/- genotype resulted in an increased risk for gastric cancer (OR = 12.86, 95% CI: 3.36-49.25). The γ value was 1.38. SOD2 Ala/- genotype could generate the amplification effect on a positive family history.

Interaction 3: Type I *H. pylori* infection coexisted with

Table 6 Interaction of SOD2 Val16Ala and environmental factors for gastric cancer development

Factors	SOD2	HC (%) *n* = 147	GC (%) *n* = 140	[1]OR (95% CI)	β	γ = β_{eg}/β_e
Alcohol						
No	Val/Val	89 (60.5)	33 (23.6)	1.00		
No	Ala/-	11 (7.5)	21 (15.0)	4.89 (2.11-11.36)	1.59	
Yes	Val/Val	34 (23.1)	52 (37.1)	5.26 (2.62-10.55)	1.66	
Yes	Ala/-	13 (8.8)	34 (24.3)	9.46 (4.08-21.94)	2.25	1.36
Family history						
Negative	Val/Val	114 (77.6)	58 (41.4)	1.00		
Negative	Ala/-	21 (14.3)	41 (29.3)	3.90 (2.09-7.27)	1.36	
Positive	Val/Val	9 (6.1)	27 (19.3)	6.34 (2.75-14.59)	1.85	
Positive	Ala/-	3 (2.0)	14 (10.0)	12.86 (3.36-49.25)	2.55	1.38
Type I *H. pylori*						
Negative	Val/Val	75 (51.0)	23 (16.4)	1.00		
Negative	Ala/-	7 (4.8)	9 (6.4)	4.90 (1.59-15.08)	1.59	
Positive	Val/Val	48 (32.7)	61 (43.6)	4.24 (2.30-7.09)	1.44	
Positive	Ala/-	17 (11.6)	47 (33.6)	9.07 (4.36-18.86)	2.21	1.53

A/-: AA + AG; Ala/-: Ala/Val + Ala/Ala. [1]Adjusted for age, sex, OR, 95% CI, β were calculated by logistic regression. HC: Healthy control; GC: Gastric cancer.

Table 7 Interaction of SOD1 G7958A and SOD2 Val16Ala for gastric cancer development

Genotype		HC (%)	GC (%)	[1]OR (95% CI)	*P*
SOD1	SOD2	*n* = 147	*n* = 140		
GG	Val/Val	72 (49.0)	31 (22.1)	1.00	
A/-	Val/Val	50 (34.0)	54 (38.6)	2.54 (1.43-4.52)	0.002
GG	Ala/-	7 (4.8)	8 (5.7)	2.69 (0.89-8.14)	0.079
A/-	Ala/-	18 (12.2)	47 (33.6)	6.43 (3.20-12.91)	< 0.001

A/-: AA + AG; Ala/-: Ala/Val + Ala/Ala. [1]Adjusted for age, sex, OR, 95% CI and *P* values were calculated by logistic regression. HC: Healthy control; GC: Gastric cancer.

SOD1 A/- genotype SOD2 Ala/- genotype resulted in an increased risk for gastric cancer (OR, 9.07, 95% CI: 4.36-18.86). The γ value was 1.53. SOD1 A/- genotype could generate the amplification effect on type I *H. pylori* infection. All these interactions accorded with the additive model (Table 6).

Gene-gene interaction for gastric cancer development

The combination of SOD1 A/- genotype and SOD2 Ala/- genotype resulted in an increased risk for gastric cancer (OR = 6.43, 95% CI: 3.20-12.91). The interaction accorded with the additive model (Table 7).

DISCUSSION

Reactive oxygen species (ROS) can damage DNA in the form of mutations, deletions, gene amplification and rearrangements, which may cause programmed cell death, or activation of several proto-oncogenes and/or inactivation of some tumor suppressor genes[16]. SOD, as a major antioxidant enzyme, plays a vital role in clearance of ROS. Previous studies have shown that the expression and activity of SOD played a role in the promotion or suppression of tumor formation in human gastric mucosa[10], and SOD2-Val16Ala polymorphism has been evaluated widely for its association with various cancers including gastric cancer[13-18].

In our study, we found that SOD2-Val16Ala polymorphism was associated with gastric cancer susceptibility and malignant potential of GPL. SOD2-Ala/- genotype carriers had a nearly 3-fold and 2-fold increased risk for developing gastric cancer and malignant potential of GPL compared with Val/Val genotype carriers. The results were in agreement with other studies on tumors such as adult brain tumors[38], prostate cancer[7], breast cancer[39], lung cancer[40] and pancreatic cancer[41]. Contrary to our results, the polymorphism of SOD2-Val16Ala was not found to be associated with gastric cancer in a Polish case-control study[18]. It implies that the distribution of polymorphism varies among different regions and races. However, a further study with a larger sample size and geographic range is needed.

SOD1 polymorphisms have been rarely evaluated for its association with risk of cancer occurrence. They were not found to be associated with the risk of breast cancer[16] and prostate cancer[7]. We found for the first time that the SOD1-7958A/- genotype was a risk factor for gastric cancer development. Subjects carrying 7958A/- genotype had a 3-fold increased risk for developing gastric cancer compared with carriers with G/G genotype,

but no association was found between the 7958A/- genotype and malignant potential of GPL. SOD1-G7958A polymorphism could be a susceptible biomarker for gastric cancer. Our results may provide a new target for gene-targeted therapy of gastric cancer.

Gastric carcinogenesis is a complex multi-factor and multi-step process involving interactions of various environmental carcinogens, bacterial and genetic variants. To our knowledge, there has been no study on the interactions between SOD1 and SOD2 polymorphisms and environmental factors, and gene-gene interactions of SOD1 and SOD2 in gastric cancer. In our study, we found that carriers with a positive family history of gastric cancer had a 2-fold increased risk for malignant potential of GPL. The combination of a positive family history and SOD2-16Ala/- genotype contributed to a higher risk for malignant potential of GPL. Meanwhile, we also found that alcohol drinking, a positive family history and type I *H. pylori* infection were significantly associated with gastric cancer, and there were additive interactions with SOD1-7958A/- genotype and SOD2-16Ala/- genotype for gastric carcinogenesis. *H. pylori* infection could result in changes in the activity and content of SOD[26,27] and DNA oxidative damage by its main virulence CagA and VacA[28,29]. Therefore, the interaction of *H. pylori* infection and the polymorphisms of SOD1 and SOD2 exists theoretically. Our results suggested that gastric carcinogenesis resulted from combined action of gene and environment but not the unitary effect of gene or environment. Similar to our previous researches, we found that CagA$^+$ *H. pylori* infection was a definite risk factor for gastric cancer and that CagA$^+$ *H. pylori* infection combined with 762Ala/Ala genotype in PARP-1 contributed to a higher risk for gastric cancer[42]. Therefore, the interaction of gene-environment has played a role in the gastric carcinogenesis. We also found that there was an interaction between SOD1-7958A/- genotype and SOD2-16Ala/- genotype in gastric carcinogenesis, which had a 6-fold increased risk for gastric cancer development. This discovery has practical implications for gene therapy, and confirms the importance of gene-gene interaction in targeted therapy for cancer.

In summary, gastric carcinogenesis involves a variety of factors including genetic factors, environmental factors and gene-environment and gene-gene interactions. SOD1-7958A/- genotype, SOD2-16Ala/- genotype, alcohol drinking, a positive family history of gastric cancer, and type I *H. pylori* infection could be risk factors for gastric cancer in Chinese Han population, and there were positive gene-gene and gene-environment interactions. The effects of the interactions contributed to a higher risk for gastric cancer. Therefore, control of alcohol drinking and eradication of *H. pylori* infection may help lower the incidence of gastric cancer to a certain extent.

Because the sample size and risk factors investigated were limited in our study, and the genetic and environmental factors involved in carcinogenesis are numerous and complex, our conclusion remains to be further confirmed by studies of larger sample size on more risk factors among different races and regions so as to determine whether the SOD1-G7958A and SOD2-Val16Ala polymorphisms would be a susceptible biomarker for gastric cancer, and to evaluate whether there are interactions between gene and environment. In addition, Ala variant of SOD2 significantly reduced SOD2 catalytic activity in hepatocytes[19] and allows more efficient SOD2 to import into the mitochondrial matrix and generates more active SOD2 compared with the Val variant[43]. Thus, the Ala variant of SOD2 may increase the risk of gastric cancer by altering the activity or content of SOD2 to lower the ability of scavenging ROS. However, the carcinogenic mechanism of SOD1-7958A/- variant in gastric cancer is still unclear. As SOD1-G7958A is located in intron 1 of SOD1 gene, we imagine that the 7958A/- variant may cause gastric carcinogenesis by regulating the expression and alternative splicing of SOD1. This hypothesis remains to be further elucidated by detecting the expression levels of SOD1 and function analysis.

COMMENTS

Background

Gastric cancer (GC) is the second most common malignancy worldwide. Epidemiological evidences show that gastric carcinogenesis results from gene-environmental interactions. Superoxide dismutases (SOD) are major antioxidant enzymes, which play vital roles in clearance of reactive oxygen species (ROS) *in vivo* and can either promote or suppress tumor formation in human gastric mucosa. There have been few studies on the relationship between gene polymorphisms of SOD and GC in Chinese Han population and interactions between SOD polymorphisms and environmental factors in GC.

Research frontiers

In this study, the authors investigated the associations between gene polymorphisms of SOD and GC, evaluated the relationship between GC and the epidemiological factors including age, sex, smoking, alcohol drinking, family history of gastric cancer and different types of *H. pylori* infection, and analyzed gene-environmental and gene-gene interactions according to the interaction model.

Innovations and breakthroughs

This study showed that copper-zinc superoxide dismutase (SOD1)-7958A/- genotype and manganese superoxide dismutase (SOD2, Mn-SOD)-16Ala/- genotype, alcohol drinking, a positive family history of gastric cancer, and type I *H. pylori* infection were correlated with increased risk of GC in Chinese Han population. The gene-gene and gene-environment interactions contributed to a higher risk for GC.

Applications

This study showed that gene-environmental interactions play a great role in the gastric carcinogenesis, and control of alcohol drinking and eradication of *H. pylori* infection may contribute to a decreased incidence of GC to a certain extent.

Terminology

SODs are major antioxidant enzymes, which play vital roles in clearance of ROS. SOD1 with copper (Cu) and zinc (Zn) in their catalytic center are localized in intracellular cytoplasmic compartments. *SOD2* plays an important role as a primary mitochondria antioxidant enzyme. The activity and expression of SOD change significantly in gastric cancer patients, which can either promote or suppress tumor formation in human gastric mucosa.

Peer review

The paper showed that SOD1 2809A/- genotype, SOD2 16Ala/- genotype, alcohol, positive family history and type I *H. pylori* infection were associated with risk of gastric cancer in Chinese Han population, and there were gene-gene and gene-environment interactions for gastric cancer development. SOD2 Ala/- genotype and a positive family history were risk factors for malignant potential of GPL, and there was a super-multiplication interaction. The described polymorphisms are novel with important scientific merit, but expanding the study with the mentioned aspects may improve the results.

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CASE REPORT

Gastrointestinal bleeding 30 years after a complicated cholecystectomy

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Abstract

Gastrointestinal bleeding from small-bowel varices is a rare and difficult to treat complication of portal hypertension. We describe the case of a 79-year-old female patient with recurrent severe hemorrhage from small-bowel varices 30 years after a complicated cholecystectomy. When double balloon enteroscopy was unsuccessful to reach the site of bleeding, a rendezvous approach was favored with intraoperative endoscopy. Active bleeding from varices within a biliodigestive anastomosis was found and controlled by endoscopic injection of cyanoacrylate. Intraoperative endoscopy should be considered in the case of life-threatening gastrointestinal hemorrhage that is not accessible by conventional endoscopy.

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Key words: Upper gastrointestinal bleeding; Intestinal varices; Intraoperative endoscopy; Cyanoacrylate

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INTRODUCTION

Hemorrhage from small-bowel varices is a rare but life-threatening condition. Despite sophisticated endoscopic techniques such as single- or double-balloon endoscopy, localization and treatment of the site of bleeding can be challenging. No controlled trials have evaluated the best strategies in these situations, however, successful endoscopic obliteration of jejunal varices by injection of cyanoacrylate has been described in individual case reports. We describe the rare case of a small-bowel variceal hemorrhage within a biliodigestive anastomosis in a 79-year-old female patient. Active bleeding was controlled by cyanoacrylate occlusion using an interdisciplinary approach.

CASE REPORT

A 79-year-old Caucasian woman was admitted to our hospital because of recurrent gastrointestinal bleeding. The patient had been well until 4 wk earlier when she presented to another hospital with melena and anemia (hemoglobin 7.2 g/dL). Upper and lower gastrointestinal endoscopy were negative without detection of the source of bleeding. Multiple units of blood were transfused. Repeated episodes of melena were observed dur-

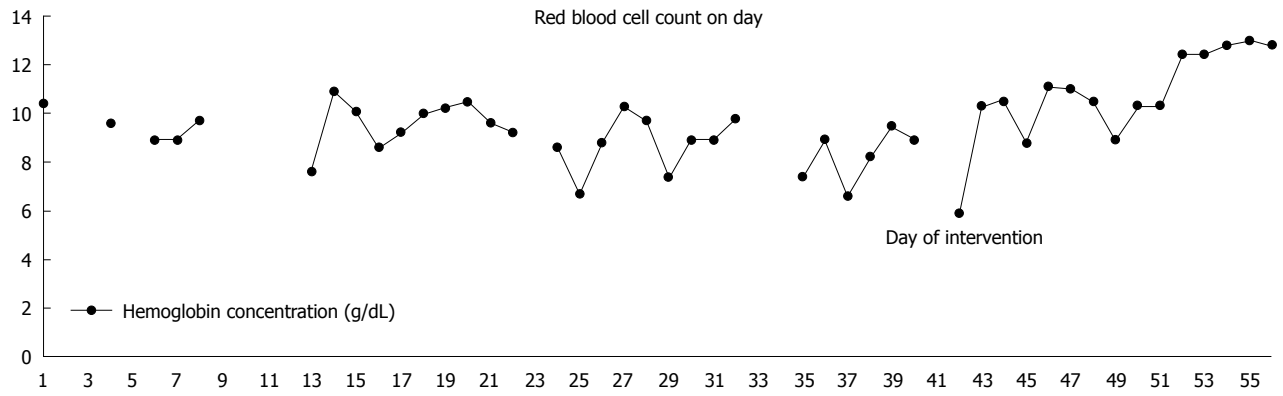

Figure 1 Course of red blood cell count.

Table 1 Laboratory tests on admission

Laboratory test	On admission
Creatinine (mg/dL)	1.0
Potassium (mmol/dL)	3.8
Total protein (g/dL)	3.4
AST (U/L)	25
ALT (U/L)	27
γ-GT (U/L)	22
INR	1
RBC (g/dL)	9.6
WBC (/nL)	5.5
Platelets (/nL)	290
IgG (mg/dL)	1140
IgA (mg/dL)	559
IgM (mg/dL)	98
Reticulocytes (0.1%)	41
ANA, AMA, AMA-M2, LKM-1, SLA, ANCA	Negative
Ferritin (μg/L)	9
Cobalamine (ng/L)	401
Folic acid (μg/L)	5.2

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GT: Gamma-glutamyl-transferase; INR: International ratio; RBC: Red blood cell count; WBC: White blood cell count; ANA: Antinuclear antibody; AMA: Anti-mitochondrial antibody; LKM: Liver-kidney-microsomal antibody; SLA: Soluble liver antigen antibody; ANCA: Anti neutrophil cytoplasmatic antibody.

ing the following weeks and the patient was transferred to our department.

Two years earlier, a diagnosis of autoimmune hepatitis was made with a positive antinuclear antibody titer of 1:2000, however, no biopsy was performed for confirmation. She had received prednisone, which was tapered and discontinued. At the time of presentation, she received no immunosuppressive therapy and liver function tests were normal. Her former history included hypertension, minor depression and a complicated cholecystectomy approximately 30 years earlier.

On admission to our hospital, the hemoglobin concentration was 10.4 g/dL. Rectal examination confirmed the presence of melena and a guaiac stool test was positive. Abdominal ultrasound showed a slightly enlarged liver with a prominent caudate lobe but normal echogenicity. Visualization of the hilar region and the pancreas was incomplete due to meteorism; the spleen was normal. Laboratory test results are shown in Table 1 and Figure 1.

Repeated conventional upper and lower endoscopy did not detect the site of bleeding. An axial computed tomography (CT) scan of the abdomen showed air within the intrahepatic bile ducts, and cavernous transformation of the portal vein. Therefore, bleeding within a biliodigestive anastomosis was suspected and double balloon enteroscopy (Fuji) was performed. Approximately 180 cm aborally fresh blood was seen in the jejunum, however, a biliodigestive anastomosis or any other potential source of bleeding could not be detected. A decision was made to take the patient to the operating room, and a laparotomy with intraoperative colonoscopy was performed. An end-to-side biliodigestive anastomosis was detected with active bleeding from varices within the anastomosed small-bowel loop (Figures 2 and 3). N-butyl-2-cyanoacrylate (histoacryl) 1.0 mL mixed with lipiodol 1.0 mL was injected at two sites (total volume 2.0 mL), after which, bleeding stopped (Figures 2 and 3). A CT scan of the abdomen obtained 5 d after the intervention showed contrast-enhancing histoacryl-lipiodol deposits within the varices (Figure 4). The patient recovered uneventfully and was in good health with no signs of recurrent gastrointestinal bleeding and a normal hematocrit at 6 mo of follow-up.

DISCUSSION

Small-bowel varices can be found in approximately 8% of patients with portal hypertension using video capsule endoscopy[1]. Varices within jejunal anastomoses have been reported in anecdotal cases only[2]. Hemorrhage from jejunal varices, although a rare event, is often severe and can be fatal due to limited endoscopic access[3,4]. To the best of our knowledge, this could be the first description of hemorrhage from varices within a biliodigestive anastomosis.

Successful endoscopic obliteration of jejunal varices by injection of cyanoacrylate has been described in individual case reports[5,6]. Transjugular intrahepatic portosystemic shunt (TIPS) is another treatment option with demonstrated efficacy[7-10]. Cavernous transformation of the portal vein precluded decompression by TIPS in our case. In addition, surgical intervention would have carried a high risk of bleeding. As far as we are aware, no prospective tri-

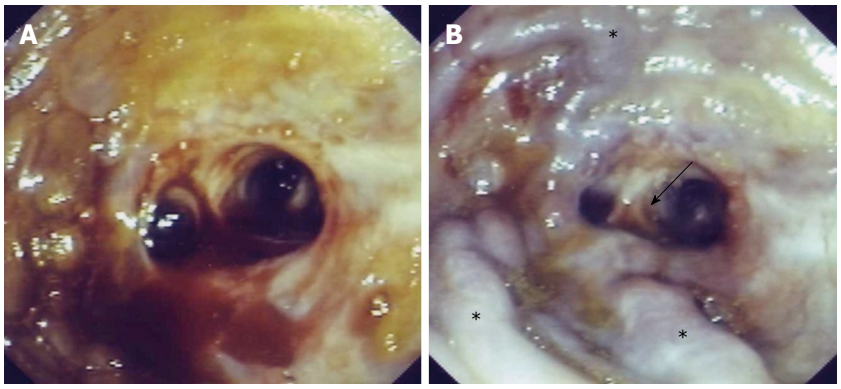

Figure 2 Endoscopic view of the biliodigestive anastomosis. Prominent varices with signs of active bleeding were visible (A); Arrow: Hepatic bifurcation; asterisk: Varices within jejunal loop (B).

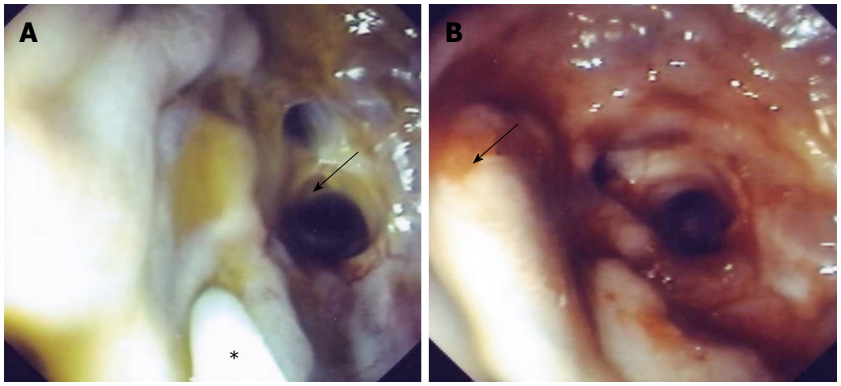

Figure 3 Obliteration of the varices by endoscopic injection of cyanoacrylate (histoacryl)-lipiodol. Arrow (A): Hepatic bifurcation; asterisk: Endoscopic needle; Arrow (B): Occluded varix with site of injection.

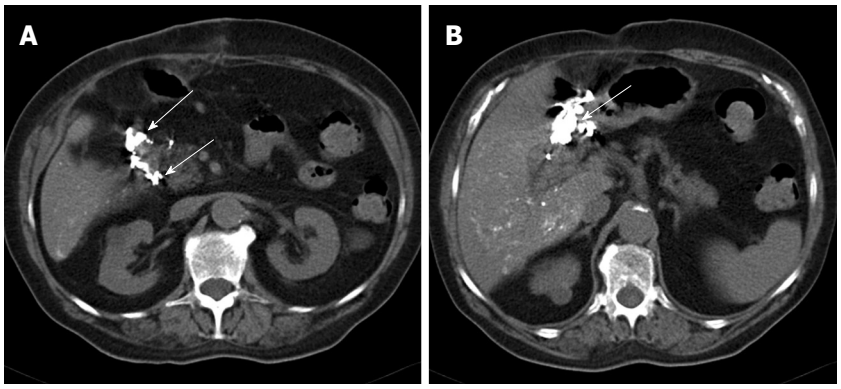

Figure 4 Computed tomography images of the abdomen on 5 d after variceal obliteration. The contrast-enhancing histoacryl-lipiodol deposits were detected within the varices (arrows, A). Small amounts of contrast were visualized intrahepatic (B).

als have explored the management of small-bowel varices. Therefore, treatment must be individualized and depends on the experience of the team and availability of resources. Endoscopy can be considered the first-line approach, however, endoscopic access of the site of bleeding might be difficult, even with the use of push enteroscopy. If conventional endoscopy or push enteroscopy is unsuccessful, as in the present case, a rendezvous approach with intraoperative endoscopy should be considered to limit the extent of surgery. Our case demonstrates the efficacy and tolerability of histoacryl blockage for small-intestinal varices, which is a standard procedure in the stomach and esophagus.

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CASE REPORT

Completely obstructed colorectal anastomosis: A new non-electrosurgical endoscopic approach before balloon dilatation

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Abstract

Benign stricture is a relatively common complication of colorectal anastomosis after low anterior resection. On occasion, the anastomosis may close completely. A variety of endoscopic techniques have been described, but there is a lack of data from controlled prospective trials as to the optimal approach. Through-the-scope balloon dilatation is well known and easy to perform. Some case reports describe different endoscopic approaches, including endoscopic electrocision with a papillotomy knife or hook knife. We report a case of a colorectal anastomosis web occlusion, treated without electrocision. Gastrografin enema and sigmoidoscopy showed complete obstruction at the anastomotic site due to the presence of an anastomotic occlusive web. In order to avoid thermal injuries, we decided to use a suprapapillary biliary puncture catheter. The Artifon catheter was inserted into the center of the circular staple line at the level of the anastomosis, and fluoroscopic identification of the proximal bowel was obtained with dye injection. A 0.025-inch guidewire was then passed through the catheter into the colon and progressive pneumatic dilatation was performed. The successful destruction of the occlusive web facilitated passage of the colonoscope, allowing evaluation of the entire colon and stoma closure after three months of follow-up. The patient tolerated the procedure well, with no complications. This report highlights an alternative non-electrosurgical approach that uses a new device that proved to be safe and useful.

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Key words: Anastomosis; Dilation; Balloon; Obstructed; Artifon

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INTRODUCTION

Benign stricture is a relatively common complication of colorectal anastomosis after low anterior resection, occurring in 5.8% to 20% of cases. On occasion, the anastomosis may close completely. Factors promoting development of benign anastomotic strictures are still poorly understood, but include ischemia, dehiscence, and radiation therapy. In the majority of patients, an anastomotic stricture is a serious condition that may require repeated endoscopic treatment or surgery. Direct digital dilatation or transrectal surgical treatment is possible if the

anastomosis is located in the lower rectum. Endoscopic balloon dilatation is the best method for all patients in whom these procedures for the lower rectum cannot be done[1-5].

Intervention with the colonoscope is the first treatment option. A variety of endoscopic techniques have been described, but there is a lack of data from controlled prospective trials as to the optimal approach. Through-the-scope (TTS) balloon dilatation is well known and easy to perform. Some cases reported in the literature describe different endoscopic approaches, including endoscopic electrocision with a papillotomy knife or hook knife[6,7].

We report a case of a colorectal anastomosis web occlusion, treated without electrocision by employing an Artifon catheter puncture before balloon dilatation.

CASE REPORT

In March 2009, a 70-year-old man, diagnosed with rectal cancer, underwent laparoscopy-assisted low anterior resection, with a protective loop ileostomy, at our institute.

Two months later, the patient developed *constipation and abdominal pain*. Prior to ileostomy takedown, gastrografin enema through both the ileostomy and anus was performed, showing complete obstruction at the anastomotic site (Figure 1). To better confirm the entity and site of the obstruction, sigmoidoscopy was performed, showing an occlusive web completely obliterating the anastomosis (Figure 1). In order to avoid thermal injuries[8], we decided to use a new puncture catheter (Figure 2). This device is a specially designed polyethylene catheter (Artifon Catheter; SCITECH, Goiania, Brazil) with an 18-gauge needle and a flexible metallic sheath at the distal end, which allows puncture of the bile duct and insertion of a 0.025/0.0018-inch diameter guidewire. Sigmoidoscopy was performed using a therapeutic gastroscope (GIF-1TQ160, Olympus America Corp., Melville, NY, USA). On radiology, the lumen of the distal colon and proximal rectum, made apparent by air contrast, seemed to be continuous but completely separated at the anastomotic level. The Artifon catheter was inserted into the center of the circular staple line at the level of the anastomosis, under endoscopic and fluoroscopic control, and fluoroscopic identification of the proximal bowel was obtained with dye injection (Figure 3). A 0.025-inch guidewire (*Tracer Metro*, Cook Endoscopy, Winston-Salem, NC) was then passed through the catheter into the colon (Figures 3 and 4). Progressive pneumatic dilatation was performed over the guidewire with a 10 mm × 4 cm biliary balloon dilatation catheter (Hurricane RX 10 × 4, 5.8/180; Microvasive Endoscopy, Boston Scientific Corp. Natick, Mass) and, successively, with a controlled radial expansion balloon dilator (CRE™ Balloon Dilator, Boston Scientific Cork Ltd, Ireland) until a 2 cm diameter dilation was achieved (Figures 3 and 4). The successful destruction of the occlusive web facilitated passage of the colonoscope, allowing evaluation of the entire colon and stoma closure after three months of follow-up. The patient tolerated the procedure well, with no complications.

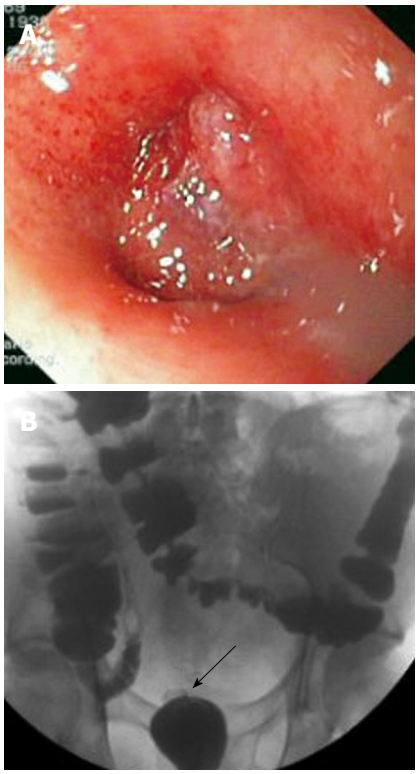

Figure 1 Endoscopic appearance of a totally occlusive web at the level of the anastomosis (A), and gastrografin enema showing complete obstruction at the rectal anastomosis (B, arrow).

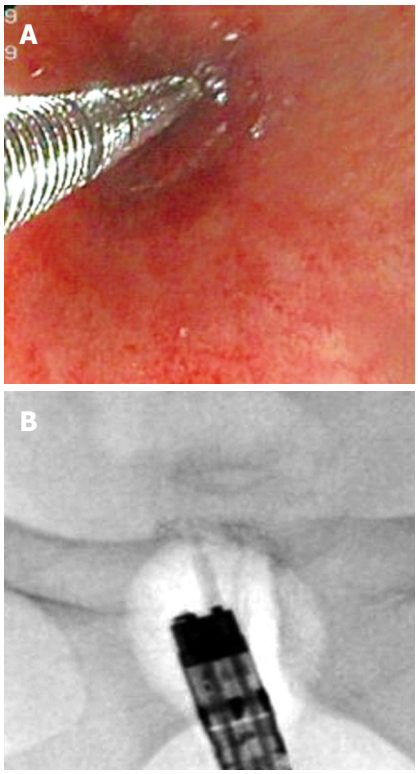

Figure 2 Appearance of Artifon catheter at colonoscopy (A) and fluoroscopy (B).

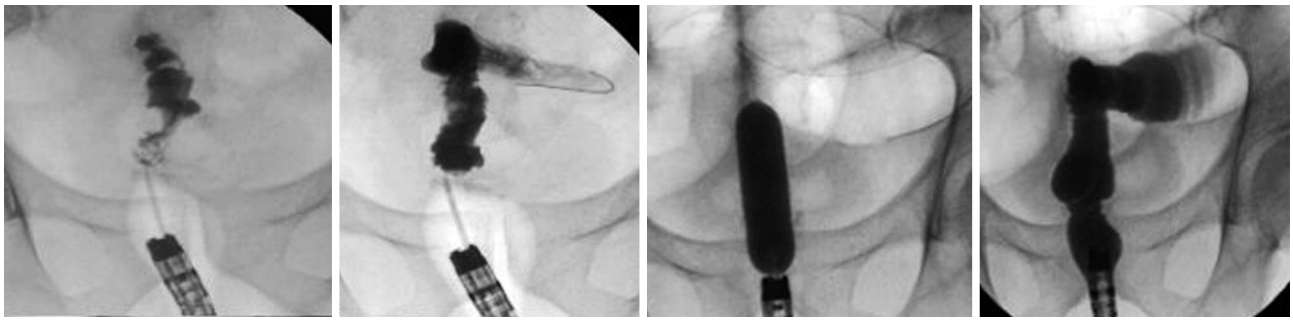

Figure 3 Fluoroscopy showing adjacency of the distal colon and proximal rectum, dye injection, guidewire insertion, progressive balloon dilatation, and restoration of colonic continuity.

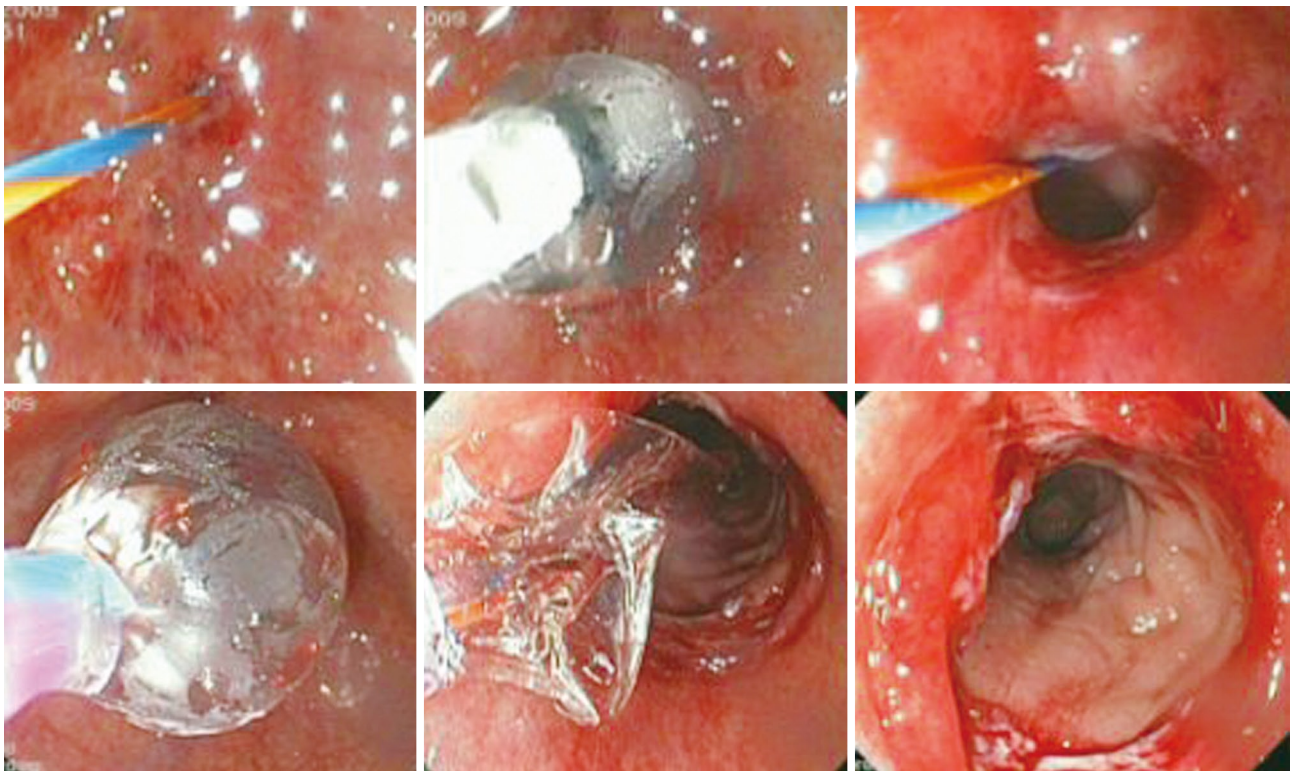

Figure 4 Endoscopic images of guidewire insertion, progressive balloon dilatation, and successful destruction of the occlusive web.

DISCUSSION

Benign colorectal anastomotic strictures are a challenging complication. Endoscopic dilatation is a valid and safe treatment, and is the preferred method for managing anastomotic colonic strictures, with surgery reserved for endoscopic failures. Endoscopic techniques center on the use of dilating balloons to disrupt scar tissue. Other techniques for endoscopic management include stricture incision with a needle knife, with or without balloon dilatation. In our report, we identified the proximal lumen by adapting a biliary approach to our aim. We used a suprapapillary biliary puncture catheter (Artifon Catheter®) to inject dye and insert a guidewire on which to perform balloon dilatation, without resorting to electrocision. This report highlights an alternative non-electrosurgical approach that uses a new device that proved to be safe and useful. However, it needs to be stressed that these procedures should be performed only by highly experienced endoscopists familiar with these specialized procedures.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

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ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 38 October 14, 2010

EDITORIAL

4755 Role of endoscopic retrograde cholangiopancreatography in pancreatic diseases

Christodoulou DK, Tsianos EV

TOPIC HIGHLIGHT

4762 Fat: A matter of disturbance for the immune system

Federico A, D'Aiuto E, Borriello F, Barra G, Gravina AG, Romano M, De Palma R

4773 Hepatic steatosis, low-grade chronic inflammation and hormone/growth factor/adipokine imbalance

Tarantino G, Savastano S, Colao A

4784 Noninvasive investigations for non alcoholic fatty liver disease and liver fibrosis

Fierbinteanu-Braticevici C, Dina I, Petrisor A, Tribus L, Negreanu L, Carstoiu C

REVIEW

4792 Current developments in natural orifices transluminal endoscopic surgery: An evidence-based review

Teoh AYB, Chiu PWY, Ng EKW

ORIGINAL ARTICLE

4800 Role of *CYP2E1* gene polymorphisms association with hepatitis risk in Northeast India

Deka M, Bose M, Baruah B, Bose PD, Medhi S, Bose S, Saikia A, Kar P

4809 A new index for non-invasive assessment of liver fibrosis

Ichino N, Osakabe K, Nishikawa T, Sugiyama H, Kato M, Kitahara S, Hashimoto S, Kawabe N, Harata M, Nitta Y, Murao M, Nakano T, Arima Y, Shimazaki H, Suzuki K, Yoshioka K

BRIEF ARTICLE

4817 *Helicobacter* species and common gut bacterial DNA in gallbladder with cholecystitis

Karagin PH, Stenram U, Wadström T, Ljungh Å

4823 Tumour budding predicts response to anti-EGFR therapies in metastatic colorectal cancer patients
Zlobec I, Molinari F, Martin V, Mazzucchelli L, Saletti P, Trezzi R, De Dosso S, Vlajnic T, Frattini M, Lugli A

4832 Liver stiffness measurements in patients with HBV *vs* HCV chronic hepatitis: A comparative study
Sporea I, Şirli R, Deleanu A, Tudora A, Popescu A, Curescu M, Bota S

4838 Erythropoietin ameliorates early ischemia-reperfusion injury following the Pringle maneuver
Kato M, Sawada T, Kita J, Shimoda M, Kubota K

4846 mPGES-1 expression in non-cancerous liver tissue impacts on postoperative recurrence of HCC
Nonaka K, Fujioka H, Takii Y, Abiru S, Migita K, Ito M, Kanematsu T, Ishibashi H

4854 Suspected uncomplicated cecal diverticulitis diagnosed by imaging: Initial antibiotics *vs* laparoscopic treatment
Park HC, Lee BH

4858 Comparative analysis of dideoxy sequencing, the KRAS StripAssay and pyrosequencing for detection of KRAS mutation
Gao J, Li YY, Sun PN, Shen L

4865 Possible key residues that determine left gastric artery blood flow response to PACAP in dogs
Wei MX, Hu P, Wang P, Naruse S, Nokihara K, Wray V, Ozaki T

4871 Clinical significance of C-reactive protein values in antibiotic treatment for pyogenic liver abscess
Gao HN, Yuan WX, Yang MF, Zhao H, Hu JH, Zhang X, Fan J, Ma WH

CASE REPORT

4876 Treatment of portal vein tumor thrombus using ^{125}Iodine seed implantation brachytherapy
Zhang L, Mu W, Hu CF, Huang XQ

LETTERS TO THE EDITOR

4880 Science of weight loss supplements: Compromised by conflicts of interest?
Lobb A

World Journal of Gastroenterology
Volume 16 Number 38 October 14, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		
FLYLEAF	I-VII	Editorial Board

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EDITORIAL

Role of endoscopic retrograde cholangiopancreatography in pancreatic diseases

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Abstract

Over the last 15 years, endoscopic retrograde cholangiopancreatography (ERCP) has evolved from a diagnostic tool to one that is primarily used to provide therapy. This development occurred first for biliary disorders and subsequently to a lesser extent for pancreatic diseases. Computed tomography, magnetic resonance imaging, magnetic resonance cholangiopancreatography and endoscopic ultrasonography suggest a diagnosis in the majority of patients with pancreatic diseases today and can help physicians and patients avoid unnecessary ERCP. However, a selected number of patients with pancreatic diseases may benefit from pancreatic endotherapy and avoid complex surgery and chronic use of medications. Pancreatic sphincterotomy, pancreatic stenting and pancreatic cyst drainage are some of the most effective and challenging endoscopic pancreatic interventions and should be performed with caution by expert therapeutic endoscopists. There has been a paucity of randomized studies investigating endoscopic techniques in comparison with surgery and medical therapy for the treatment of most benign and malignant pancreatic disorders due to the limited number of patients and the expertise required to attempt these procedures.

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Key words: Endoscopic retrograde cholangiopancreatography; Pancreas; Pancreatic disease; Pancreatic endotherapy; Therapeutic endoscopy

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INTRODUCTION

Pancreatic diseases are among the most challenging disorders of the digestive system. A wide range of benign conditions present both diagnostic and therapeutic challenges to the gastroenterologist and surgeon[1]. These include acute pancreatitis (including recurrent), chronic pancreatitis, pancreatic duct stones, pancreatic leaks, pseudocysts and strictures. Symptoms exhibited by patients with these disorders can be disabling. Endoscopic treatments for these benign disorders have evolved in the last decades and remain a viable, cost-effective alternative to more invasive surgical or radiological methods[2]. In addition, endoscopic therapy can provide palliation for inoperable malignant pancreatic diseases, such as pancreatic cancer with biliary and duodenal obstruction[3].

Endoscopic pancreatic therapy has been developed

much more slowly than the endoscopic treatment of biliary disorders. There are many reasons for this, but the main one appears to be a fear of inducing pancreatitis after even the contrast injection or just sphincteric manipulation. It has become clear however, that techniques initially restricted to biliary endotherapy can also be used in the pancreas in selected individuals[4]. Thus, sphincterotomy and attempts at stone retrieval and stricture treatment were first used in chronic pancreatitis patients in whom the procedure-related risk was much lower than in patients with normal anatomy or sphincter of Oddi dysfunction. More and more use of these techniques has resulted from studies showing that small-caliber stents placed into the pancreatic duct after a sphincterotomy or repeated manipulation of the papilla significantly reduce the incidence and severity of procedure-related pancreatitis. Despite this general observation, however, there have still been very few large series, controlled trials and critical reviews of these techniques.

The introduction of advanced radiological and imaging techniques has limited the diagnostic role of endoscopic retrograde cholangiopancreatography (ERCP), but sometimes the information provided during a therapeutic procedure is also useful for diagnostic purposes. In this editorial, we will focus on the current role of ERCP for the diagnosis and especially the treatment of pancreatic disorders.

ENDOSCOPIC DIAGNOSIS OF CHRONIC PANCREATITIS

ERCP and endoscopic ultrasonography (EUS) are the principal endoscopic methods to assess patients with chronic pancreatitis and complement radiologic methods [computed tomography (CT) scans, magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography]. Both ERCP and EUS can establish the diagnosis of chronic pancreatitis[1,5]. ERCP allows detection of pancreatic duct changes including ductal dilation, strictures, abnormal side branches, communicating pseudocysts, pancreatic duct stones and pancreatic duct leaks. ERCP is highly effective in visualizing these ductal findings (sensitivity for the diagnosis of chronic pancreatitis of 71%-93% and a specificity of 89%-100%). The Cambridge Classification, which assesses the main pancreatic duct and side branches is a widely accepted system for scoring ductal findings seen on ERCP[6]. Unfortunately, pancreatography is imperfect and care should be taken not to overinterpret minor findings seen on ERCP. Conversely, ERCP may not detect changes of less advanced chronic pancreatitis. When the diagnosis of chronic pancreatitis is sought, ERCP should be reserved for patients in whom the diagnosis is still unclear after non-invasive pancreatic function testing or other non-invasive (CT, MRI) or less invasive (EUS) imaging studies have been performed[7,8]. Although ERCP can be used to obtain information about ductal anatomy to define the level and degree of obstruction and the presence of strictures and stones, it does not provide information regarding the surrounding pancreatic parenchyma. EUS can provide high-resolution images of both the ductal structures and the parenchyma[9]. There is good interobserver agreement in the diagnosis of chronic pancreatitis by EUS, and EUS may detect early chronic pancreatitis in a reliable manner compared with ERCP[10].

PANCREATIC DUCT STRICTURES

The finding of a pancreatic duct stricture often poses a diagnostic dilemma regarding the specific cause. The cause of a pancreatic duct stricture is likely to include one or more of the following: chronic pancreatitis, pancreatic neoplasm (benign or malignant), pseudocyst or traumatic injury (blunt or penetrating)[2]. Filling defects such as protein plugs or stones may resemble a stricture. Cancer is the most feared cause of pancreatic duct stricture and should be considered in all patients in whom a pancreatic duct stricture is identified. Patients older than 50 years presenting with single or multiple episodes of acute pancreatitis, who have a pancreatic duct stricture, must have malignancy included in the differential diagnosis, particularly in the absence of alcohol abuse.

Changes in ductal anatomy other than the stricture should be looked for when examining the pancreatogram. This includes irregularity in contour or dilation of the pancreatic duct or of the secondary radicles. The presence of a single stricture with proximal dilation and normal distal ductal anatomy is suggestive of a neoplastic cause. Changes noted throughout the duct, particularly distally to the stricture, in addition to the anticipated proximal dilation, are usually suggestive of chronic pancreatitis. The presence of multiple strictures and dilations in a "chain-of-lakes" appearance is characteristic of chronic pancreatitis. Unfortunately, none of these features suggestive of a diagnosis of chronic pancreatitis is absolute in ruling out pancreatic cancer in individual patients because patients with chronic pancreatitis are at increased risk for pancreatic cancer. Therefore, the pancreatogram alone is not sufficient to rule out pancreatic cancer in patients with chronic pancreatitis, and if there is a clinical suspicion, aggressive attempts to obtain tissue should be made to establish a diagnosis[11]. Physicians should have a low threshold to perform EUS to more closely and thoroughly examine the pancreatic parenchyma, with fine-needle aspiration of any areas felt to be suspicious for possible malignancy. Obtaining serum CA 19-9 levels may be helpful in patients considered to harbor a malignancy, although levels can be elevated in patients with chronic pancreatitis in the absence of cancer.

Benign strictures of the main pancreatic duct are generally due to inflammation or fibrosis around the main pancreatic duct. Because ductal obstruction may lead to pain or acute pancreatitis superimposed on chronic pancreatitis, endoscopic therapy with balloon dilation or pancreatic duct stents for the treatment of dominant pancreatic duct strictures has been evaluated. Stricture dilation may be required to facilitate stent placement or stone removal.

Data regarding the role of endoscopic therapy in treating main pancreatic duct strictures are inconsistent. Some, but not all, authors have reported high success rates (75% to 94%) in treating pain by stenting of pancreatic duct strictures[2,4,12]. In addition, although some authors have correlated clinical improvement to a decrease in the diameter of the main pancreatic duct upstream, others have not. Pancreatic stents are prone to occlusion and patients undergoing endoscopic therapy for pancreatic duct strictures may require frequent stent exchanges. Symptomatic improvement may persist after pancreatic stent removal despite persistence of the stricture. Confounding factors in the literature on pancreatic stent therapy are other therapies performed at the time of stent placement (e.g. pancreatic sphincterotomy, pancreatic stone removal) and the tendency of the chronic pancreatitis pain to wax and wane and decrease with time as deterioration of pancreas function occurs[13]. The optimum duration of stent placement, stent number and diameter and degree of balloon dilation are not known. Complications related to endoscopic therapy of pancreatic duct strictures include pain, pancreatitis, stent occlusion, proximal or distal stent migration, duodenal erosions, pancreatic infection, ductal perforation, and bleeding from pancreatic sphincterotomy.

The role of placing multiple stents in the pancreatic duct has been assessed by Costamagna *et al*[12]. Nineteen patients with severe chronic pancreatitis and with a single pancreatic stent through a refractory dominant stricture in the pancreatic head underwent removal of this stent followed by balloon dilation of the stricture and insertion of the maximum number of stents allowed by the tightness of the stricture and the caliber of the pancreatic duct diameter. Stents were removed after 6-12 mo. The median number of stents placed through the major or minor papilla was three; their diameter ranged from 8.5 to 11.5 Fr and length from 4 to 7 cm. During a mean follow-up of 38 mo after stent removal, 84% of patients were asymptomatic, and 11% had symptomatic stricture recurrence. No major complications were recorded. This study showed that endoscopic multiple stenting of a dominant pancreatic duct stricture is feasible and safe.

PANCREATIC DUCT STONES

Obstructing pancreatic duct stones may contribute to abdominal pain or acute pancreatitis in patients with chronic pancreatitis. ERCP provides direct access to the pancreatic duct for evaluation and treatment of symptomatic pancreatic duct stones. In one randomized trial comparing endoscopic and surgical therapy, surgery was superior for long term pain reduction in patients with painful obstructive chronic pancreatitis[14]. However, because of its lower degree of invasiveness, endotherapy may be preferred, reserving surgery as second-line therapy for patients in whom endoscopic therapy fails or is ineffective. Pancreatic stone removal can be challenging. Frequently the stone configuration and size, coupled with pancreatic duct strictures, occlude the lumen. Adjuvant endoscopic approaches such as stricture dilation, intraductal lithotripsy and pancreatic sphincterotomy may be needed. Even when accessible, pancreatic duct stones (which are often dense and hardened) may be impacted, requiring extracorporeal shock wave lithotripsy (ESWL) to fragment the stones, before endoscopic removal can be achieved. Multiple ESWL sessions may be required and success rate in complete duct clearance and duct decompression exceeds 50%[3,15,16]. Intraductal lithotripsy guided by pancreatoscopy has also been used to fragment pancreatic stones.

Most series have shown improvement in pain with pancreatic endotherapy. Some encouraging short-term results and long-term 5 years follow-up results showing improvements in pain (77%-100% and 54%-86%, respectively) have been reported[17-19]. Although modest, these success rates are acceptable in the context of traditionally difficult-to-manage groups of patients.

ENDOSCOPIC PAIN MANAGEMENT IN CHRONIC PANCREATITIS

The ideal treatment for patients with pancreatic duct stones, dilated pancreatic ducts and pain is not known. The stones can be easily removed coincidently with the performance of a surgical drainage procedure, such as pancreaticojejunostomy. Alternatively, however, they can be fragmented by ESWL and removed endoscopically after sphincterotomy of the pancreatic duct. Stones can be cleared by this approach in roughly 80% of patients, and approximately 50% of these have long-term relief of their symptoms[20]. Dumonceau *et al*[21] conducted a randomized trial comparing pain relief after ESWL alone *vs* in combination with endoscopic drainage of the main pancreatic duct in patients with painful calcified chronic pancreatitis. Two years after trial intervention, 10 (38%) and 13 (45%) patients of the ESWL alone group and of the ESWL combined with endoscopy group, respectively, had presented pain relapse. In both groups, a similar and significant decrease was seen after treatment in the number of pain episodes/year (mean decrease, 3.7 episodes). Thus, there was no difference between the treatment groups, and the treatment costs per patient were three times higher in the ESWL combined with endoscopy group compared with the ESWL alone group.

An alternative involves the use of stents placed in the pancreatic duct endoscopically. Reports indicate that 30%-76% of patients receiving such stents have symptomatic improvement over a period of 14 to 36 mo of observation. Although these results seem encouraging, a criticism is that most of the data reported to date have been from relatively short-term, non-randomized studies. The issue is further complicated by the fact that pancreatic duct stents may not be entirely harmless; for example, they may cause further pancreatic duct changes and potentiation of chronic pancreatitis. Endoprosthesis occlusion and migration also seem to be relatively common.

There have been two randomized controlled trials comparing endoscopic therapy with surgery for the pallia-

tion of pain in chronic pancreatitis[14,22]. After 5 years of follow-up, pain was absent in 14%-16% of patients treated with endoscopy and in 36%-40% of patients treated with surgery. Based on these trials, it appears that surgery provides better pain relief compared to endoscopy, but even surgery fails to provide substantial pain relief in more than half of the patients. Due to its low degree of invasiveness, however, endotherapy can be offered as a first-line treatment, with surgery being performed in cases of failure and/or recurrence.

In cases of chronic pancreatitis with intractable pain where surgery is clearly indicated, ERCP can give valuable information regarding pancreatic duct configuration and exact ductal changes, according to the Cambridge classification[23,24]. In many cases, efforts such as decreasing smoking and alcohol use, taking oral pancreatic enzyme supplements, and receiving endoscopic therapies such as sphincterotomy and stent placement are usually effective in managing pain and inhibiting disease progression. Surgical options for chronic pancreatitis treatment include drainage procedures such as the Puestow procedure and resections such as pancreaticoduodenectomy, distal pancreatectomy, or total pancreatectomy. ERCP can serve as a preoperative bridge therapy to partial or total pancreatectomy with autologous islet cell transplantation. The latter procedure was developed for both pain management and maintenance of pancreatic endocrine function, especially glycemic control. A few institutes in the world have performed total pancreatectomy with autologous islet transplantation, since it requires special techniques for islet processing. The effectiveness of this procedure has been reported[25,26].

PANCREATIC DUCT LEAKS

Pancreatic duct disruptions or leaks can occur as a result of severe acute pancreatitis or chronic pancreatitis. The causes of the disruption are usually severe inflammation or obstruction of the duct, or severe pancreatic necrosis. Pancreatic leaks can result in pancreatic ascites, pleural effusions, pseudocyst formation and internal and external pancreatic fistulas. Pancreatic duct leaks can often be treated with endoscopic placement of transpapillary stents in a manner similar to the use of biliary stents for closing bile duct leaks[27]. Endoscopic therapy is successful in closing the leaks in approximately 60% of patients. Factors associated with a better outcome in duct disruption include a partial disruption, successfully bridging the disruption with a stent and longer duration of stent placement (approximately 6 wk). There are no comparative studies of surgical, medical and endoscopic therapy for treatment of pancreatic duct leaks.

A novel treatment approach using endoscopic injection of N-butyl-2-cyanoacrylate to achieve closure of the fistula has also been reported[28]. In total, 12 patients underwent ERCP with injection of tissue glue directly into the pancreatic fistulous tract, in addition to endoscopic drainage with stent placement when this was considered to be indicated by the endoscopist. A single session of glue injection was successful in seven patients, and a second session was required in one patient. Inadvertent injection of the cyanoacrylate into the pancreatic duct at the time of glue injection into a pancreatic fistula can be associated with chemical or obstructive pancreatitis. In contrast, the injection of glue to completely fill a disconnected ductal system usually results in glandular atrophy and has been used to avoid surgical resection in high-risk patients by some institutions[29].

PANCREATIC PSEUDOCYSTS

Pancreatic pseudocysts arise as a complication of chronic pancreatitis in 20%-40% of cases[5,18,30]. Endoscopic drainage and management of the pseudocyst is a less invasive alternative to surgical treatment and is safer when the site of the puncture is defined by EUS. Pseudocyst drainage should be considered (1) for symptomatic lesions due to pain, gastric outlet obstruction, early satiety, weight loss or obstructive jaundice; (2) when there are signs of infection of the pseudocyst; and (3) when progressive enlargement of the cyst takes place, even if it is asymptomatic. Special care must be taken to avoid drainage of cystic neoplasms, duplication cysts and other noninflammatory collections[5,31,32].

A retrospective study was conducted to determine the impact of procedure experience on patient outcomes after endoscopic drainage of endoscopic pancreatic fluid collections[33]. In that large review of 175 cases, endoscopic drainage was carried out to treat pancreatic necrosis (33%), acute pseudocysts (23%), or chronic pseudocysts (44%). There was a dramatic improvement in the resolution rates of chronic pseudocysts after the first 20 procedures in comparison with former procedures (45% *vs* 93%) and a reduction in days to resolution of the pseudocyst (50 d *vs* 33 d). In patients with pancreatic necrosis there was a statistically significant decrease in the median hospital stay with greater experience (23 d *vs* 15 d). While these findings require confirmation by other groups, this study for the first time documented the importance of operator experience for patient outcomes after these often technically challenging endoscopic procedures.

Several excellent literature synopses and technical reviews on pancreatic pseudocysts have been published in recent years. These include a technical review by Ballie regarding pseudocysts in general and a subsequent article by the same author on the endoscopic management of pseudocysts[34,35]; a technical review by Hawes[36] that distinguishes between pseudocysts and other types of pancreatic fluid collection; and an excellent article by Giovannini *et al*[37] describing the use of EUS for cystogastrostomy. Finally, Rosso *et al*[38] reviewed 466 cases of endoscopically treated pseudocysts which were reported in 17 publications, comparing the results with previously published surgical series. The authors correctly concluded that pseudocysts are best handled by an integrated multidisciplinary team including pancreatic surgical specialists, gastroenterologists and

interventional radiologists. The conclusions from all these review articles are that treatment of pseudocysts can be complicated but it requires patience, expertise, adequate clinical and endoscopic skills and appropriate endoscopic accessories.

BILIARY OBSTRUCTION IN CHRONIC PANCREATITIS AND PANCREATIC CANCER

Distal common bile duct strictures have been reported to occur in 2.7% to 45.6% of patients with chronic pancreatitis. These strictures can occur from inflammation, fibrosis, or compression from a pseudocyst or a pancreatic stone[17,39]. Because long-standing biliary obstruction can lead to secondary biliary cirrhosis or recurrent cholangitis, biliary decompression is recommended in patients with clinically significant obstruction (e.g. cholestasis or jaundice). Surgical biliary bypass is the standard approach for managing chronic common bile duct strictures. Endoscopic therapy has been used as an alternative to surgery[40]. Plastic biliary stents are a useful short-term treatment for chronic pancreatitis-induced common bile duct strictures in the setting of cholestasis, jaundice or cholangitis and may be used as a long-term treatment approach in poor surgical candidates. Unfortunately, long-term success rates are as low as 7.7%-10% in some studies when single large-bore stents are used[41,42]. The use of multiple stents with frequent stent exchanges and balloon dilations over a long period of time (up to 1-2 years) may be more efficacious than single stents for the treatment of these strictures. Patient selection is critical in this setting because patients need to return frequently for stent changes. Poor compliance to follow-up can lead to biliary sepsis from stent occlusion[43-45].

Self-expanding metal stents (SEMS) have been used for the treatment of benign biliary strictures. Uncovered metal stents have given good 3-year results for poor operative candidates, while reports for covered metal stents have given mixed results. The routine use of metal stents for benign biliary strictures is not recommended at this time[46-49].

Several randomized controlled trials have demonstrated the superiority of SEMS to polyethylene stents for the treatment of malignant distal biliary obstruction, because they have a longer duration of patency (plastic stents occlude at a median of 3 to 6 mo after placement) and consequently have been shown to be more cost-effective[50,51]. The choice of plastic (e.g. polyethylene) stents *vs* SEMS has been debated in the literature and data suggest that SEMS should be preferentially used when life expectancy exceeds 6 mo, whereas polyethylene stents are more cost-effective in patients who are expected to live less than 4 mo[52,53]. However, it is not always easy to predict patient survival at presentation.

There can be significant delays between diagnosis and surgery in patients with resectable pancreatic cancer and obstructive jaundice when neoadjuvant therapy is used or when there is limited access to surgery. In these instances, placement of SEMS at the time of initial ERCP has been advocated for relief of obstructive jaundice. Recently, it was reported that the costs of stenting alone were identical when using either plastic or metal stents for biliary obstruction drained for more than 30 wk before surgery in patients with resectable pancreatic cancer[54]. In the polyethylene group, 16 of 42 patients (38%) required 3 or more ERCPs before surgery and 7 more underwent palliative surgery in the setting of unresectable disease. If actual costs associated with stent-related complications had been included in the calculation, then the balance would have turned in favor of SEMS, because stent-related complications were 15% *vs* 93% after insertion of metal *vs* plastic stents, respectively.

With newly designed stents arriving on the market from different manufacturers, it remains to be established whether covered SEMS are more effective than uncovered in palliating obstructive jaundice and whether complications associated with SEMS (i.e. migration, cholecystitis and occlusion) can be reduced[55,56]. Only comparative multicenter studies can answer these questions.

CONCLUSION

ERCP is useful for the diagnosis of chronic pancreatitis but it should be reserved for patients in whom the diagnosis has not been established by non-invasive or less invasive procedures. ERCP and pancreatic endotherapy can be effective in patients with pancreatic strictures, pancreatic duct leaks, pancreatic duct stones and pancreatic pseudocysts. However, the most important advance with regard to ERCP is the palliative or preoperative treatment of biliary obstruction caused by chronic pancreatitis or malignant pancreatic disease. Metal stents offer better long-term relief compared to plastic stents and should be preferred in patients with a life expectancy of more than 4 to 6 mo. Expertise in ERCP is a prerequisite for effective pancreatic endotherapy.

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

Fat: A matter of disturbance for the immune system

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Abstract

Obesity is increasingly being recognized as a risk factor for a number of benign and malignant gastrointestinal conditions. However, literature on the underlying pathophysiological mechanisms is sparse and ambiguous. There is compelling evidence that both overnutrition and undernutrition negatively interfere with the immune system. Overnutrition has been found to increase susceptibility to the development of inflammatory diseases, autoimmune diseases and cancer. In the regulation of immune and inflammatory processes, white adipose tissue plays a critical role, not only as an energy store but also as an important endocrine organ. The obese state is characterised by a low-grade systemic inflammation, mainly as a result of increased adipocytes as well as fat resident- and recruited-macrophage activity. In the past few years, various products of adipose tissue including adipokines and cytokines have been characterised and a number of pathways linking adipose tissue metabolism with the immune system have been identified. Activation of the innate immune system plays a major role in hepatic steatosis. Non-alcoholic fatty liver disease includes a wide spectrum of diseases, from pure steatosis to non-alcoholic steatohepatitis in the absence of significant alcohol consumption. Although steatosis is considered a non-progressive disease, non-alcoholic steatohepatitis may deteriorate in advanced chronic liver diseases, cirrhosis, and hepatocellular carcinoma. An important parallel between obesity-related pathology of adipose tissue and liver pertains to the emerging role of macrophages, and growing evidence suggests that Kupffer cells critically contribute to progression of non-alcoholic fatty liver disease. Moreover, a close link between specific immune activation and atherosclerosis has been well established, suggesting that fat can directly trigger immune responses. This review discusses the role of fat as "a matter of disturbance for the immune system" with a focus on hepatic steatosis.

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Key words: Adipocytokine; Adipose tissue; Fat; Immune system; Kupffer cell; Natural killer; Steatosis

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INTRODUCTION

Obesity predisposes individuals to an increased risk of

developing many diseases, including atherosclerosis, diabetes, non-alcoholic fatty liver disease (NAFLD), cancer and immune-mediated disorders, such as asthma[1-3]. Obesity is typically assessed clinically with the surrogate measure of body mass index (BMI). Individuals with a BMI $\geqslant$ 30 kg/m^2 are considered obese. The incidence of obesity and its associated disorders is increasing markedly worldwide. Data from the most recent NHANES (National Health and Nutrition Examination Survey; 2005-2006) indicate that the prevalence of obesity was 33%-35% among US adults[4]. In another recent NHANES survey based on the combined years of 2003-2006, 16% of children or adolescents aged 2-19 years were obese[5]. In Europe, several surveys conducted since 2000 and using direct anthropometric measurements, showed that the prevalence of obesity ranges from 15% to 30% in men and from 11% to 34% in women, with considerable geographic variation (rates being higher in Central, Eastern, and Southern Europe)[6]. Urbanization and unbalanced diet, associated with genetic susceptibility have allowed the emergence of the obese phenotype.

In mammals, adipose tissue (AT) occurs in two forms: white adipose tissue (WAT) and brown adipose tissue (BAT). Most AT in mammals is WAT and this is thought to be the site of energy storage. In contrast, BAT is found mainly in human neonates and is important for the regulation of body temperature through non-shivering thermogenesis. In addition to adipocytes, which are the most abundant cell type in WAT, adipose tissue also contains pre-adipocytes or stromal vascular cells (which are non-fat cells): endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages. Body fat distribution, rather than adiposity *per se*, is an important risk factor for obesity-related disorders. An excess of intra-abdominal fat rather than subcutaneous fat (central *vs* peripheral obesity) is associated with metabolic syndrome (MS) and cardiovascular disease (CVD). The mechanisms responsible for this association are still unknown, but several hypotheses, which are not mutually exclusive, have been formulated[7]. The first hypothesis proposed a direct effect of visceral AT depots on insulin resistance, lipoprotein metabolism, and blood pressure. Metabolic products of omental and mesenteric AT depots are released into the portal vein, which provides direct delivery to the liver. Lipolysis of omental and mesenteric AT depots releases free fatty acids (FFAs) that can induce hepatic insulin resistance and provide substrate for lipoprotein synthesis and neutral lipid storage in hepatocytes. In addition, specific proteins and hormones produced by omental and mesenteric AT, such as inflammatory molecules, angiotensinogen, and cortisol can also contribute to MS and CVD. Another hypothesis suggests that the limited capacity of subcutaneous fat to store excess energy results in overflow of fatty acids to intra-abdominal fat and "ectopic" sites such as liver, muscle, and islets. In this paradigm, excess intra-abdominal fat is merely a marker of fatty acid overflow from subcutaneous depots.

FAT AND THE IMMUNE SYSTEM

Adipose tissue was once thought to be an inert mass whose sole function was the storage of fat. However, it is now recognized that AT is an active endocrine organ that secretes numerous adipokines, cytokines and chemokines including leptin, adiponectin, resistin, retinol binding protein 4 (RBP4), tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, and monocyte chemotactic protein 1 (MCP-1)[8,9]. All of these play a central role in the regulation of energy and vascular as well as immune system homeostasis by acting both locally and at distant sites influencing various metabolic and immune processes. Moreover, organs other than AT may contribute to systemic levels of some adipokines.

Obesity is associated with a low-grade inflammation of WAT resulting from chronic activation of the innate immune system, which can subsequently lead to insulin resistance, impaired glucose tolerance and even diabetes[10,11]. In addition to these associations between obesity and disease, research in the past few years has identified important pathways that link metabolism with the immune system and *vice versa*. Many of these interactions between metabolic and immune systems seem to be orchestrated by the complex network of soluble mediators derived from immune cells and adipocytes[1].

The effects of obesity on the immune system are not restricted to local effects within AT. Elevated levels of pro-inflammatory cytokines have been noted in the serum of asymptomatic obese individuals, the cytokine levels being related to the degree of obesity[12]. TNF-α is only present at very low levels in human blood suggesting that TNF-α released by adipose tissue has only autocrine/paracrine actions. IL-6, however, is present at much higher levels. Adipocyte-derived IL-6 has been estimated to comprise 30% of the circulating IL-6 suggesting an endocrine action[13]. Furthermore, these elevated levels of IL-6 are associated with increased circulating levels of C-reactive protein suggesting that although the elevation in levels is modest compared with those seen in sepsis, they could be having real effects on innate immune function.

Obesity is also associated with altered functioning of circulating immune cells[12,14]. Decreased T- and B-cell function, increased monocyte and granulocyte phagocytosis and oxidative burst, and an increase in leukocyte count have been described. More recently, circulating mononuclear cells from obese subjects have been shown to exhibit increased nuclear factor κB (NFκB) nuclear binding with decreased levels of NFκB inhibitor, together with increased mRNA expression of IL-6, TNF-α and migration inhibition factor. Furthermore, there is a good correlation between the markers of macrophage activation and plasma levels of FFAs[15]. It has previously been demonstrated that macronutrient challenges in normal subjects increase NFκB nuclear binding in circulating mononuclear cells, raising the possibility that the activated state of mononuclear cells is due to increased circulating levels of FFAs found in the obese. Indeed, hyperlipidaemia in mice mediates an inflammatory response by the same signalling cascade through which lipopolysaccharide activates the innate immune system (this engages a receptor complex comprising Toll 4 CD14, CD14 and MD-2)[16]. Table 1

Table 1 Adipocytokines, pro-inflammatory cytokines and chemokines, and other factors synthesised by adipocytes and macrophages in white adipose tissue

Adipocytes	Macrophages
Adiponectin	TNF-α
Leptin	IL-1β
Resistin	IL-6
RBP4	MCP-1
TNF-α	Resistin
IL-1β	
IL-6	
MCP-1	
Visfatin	
MIP	

RBP4: Retinol binding protein 4; TNF-α: Tumor necrosis factor α; IL: Interleukin; MCP: Monocyte chemotactic protein; MIP: Macrophage inflammatory protein.

summarises the secretion of adipokines, cytokines and other factors by adipocytes and macrophages in WAT. Finally, recent research has implicated the innate immune system in the pathophysiology of obesity-related liver damage[17,18].

Obesity is a high risk factor for NAFLD. Studies in an animal model of obesity-related liver disease revealed the involvement of dysfunctional hepatic immune cells[19]. In this review we analyse the relationship between hepatic steatosis and the immune system.

HEPATIC STEATOSIS AND THE IMMUNE SYSTEM

Hepatic steatosis is the histological hallmark of alcoholic liver disease (ALD) and NAFLD, which are among the commonest causes of cirrhosis and liver failure in the developed world[20-24]. Steatosis may also alter the natural history of other liver diseases such as chronic viral hepatitis[25]. Excessive consumption of alcohol in humans results in a spectrum of liver abnormalities, ranging from simple fatty liver to steatohepatitis and cirrhosis, which may be present independently or in combination. Infiltration of the liver by lymphocytes and neutrophils is an important feature of alcoholic hepatitis; it initiates a cascade of effector mechanisms that ultimately lead to hepatocyte death, fibrosis, and cirrhosis. Only a minority of consistently heavy drinkers with steatosis ever develop clinically important liver disease[24,26] implying that host or environmental factors determine the evolution of alcohol-related liver damage. Ingestion of alcohol leads to increased production of reactive oxygen species (ROS), which are generated during the metabolism of alcohol by cytochrome P450 2E1 enzyme, and excessive alcohol consumption is associated with increases in lipid, protein, and DNA peroxidation. Consistent with this disease model, risk factors for the development of progressive liver damage in alcohol drinkers include both polymorphisms in alcohol-metabolizing enzymes and polymorphisms in genes associated with a more vigorous inflammatory response in addition to exogenous factors including obesity, exposure to other hepatotoxins, and infection with hepatitis C and/or B virus[27-29].

NAFLD is increasingly recognized as a leading cause of liver dysfunction and cirrhosis in the developed world and is part of a spectrum of metabolic diseases associated with central (intra-abdominal) obesity, hypertension, dyslipidaemia, insulin resistance, and type 2 diabetes mellitus[22,30]. Similar to alcoholic liver disease, NAFLD is a spectrum of disorders, beginning as simple steatosis that is mostly considered an innocent condition. Being both the source and the result of insulin resistance, however, steatosis may be associated with an increased risk for cardiovascular morbidity[31]. Most importantly, in about 15% of all patients with NAFLD, steatosis may evolve into steatohepatitis (NASH), a medley of inflammation, hepatocellular injury, and fibrosis, often resulting in cirrhosis and even hepatocellular carcinoma[32]. Although this full sequence of progression is relatively rare, the overwhelming prevalence of NAFLD predicts a major healthcare burden. Epidemiology, pathogenesis, and approach to treatment of NAFLD follow the same trends as other metabolic disorders, and insulin resistance is the key event linking NAFLD to these diseases[33-35].

ROLE OF ADIPOCYTOKINES IN ALCOHOLIC AND NON-ALCOHOLIC STEATOHEPATITIS

Many of the initial proinflammatory changes seen in NAFLD may be the consequence of altered metabolism rather than the underlying immune pathogenic event, and adipokines provide a link between fat, inflammation, and immunity (for more details see review by Tilg *et al*[9]). More than 50 adipokines have been identified so far. Of these, leptin and adiponectin can influence the immune response, and their serum levels are increased and decreased, respectively, in NASH[9]. While many adipokines are associated with adverse biological functions, adiponectin, the most abundant adipose-derived hormone, seems to have a protective effect in NAFLD. Adiponectin inhibits TNF-α induced endothelial cell adhesion molecule expression, induces production of anti-inflammatory cytokines such as IL-10, and reduces T and B lymphocyte responses. In particular, full-length adiponectin (Acrp30) and its cleavage derivative, globular adiponectin (gAcrp), have been credited with anti-diabetic, anti-inflammatory and anti-atherogenic properties[36]. Adiponectin stimulates hepatic fatty acid oxidation and ketogenesis, while it inhibits cholesterol and triglyceride synthesis[36]. While these metabolic activities primarily occur in hepatocytes, adiponectin has potent anti-inflammatory effects in macrophages. Thus, adiponectin is able to suppress the effects of lipopolysaccharides (LPS) in macrophages, including activation of NFκB and ERK1/2[37-39]. Similarly, adiponectin prevents LPS-mediated inflammatory signalling in Kupffer cells[40]. These anti-inflammatory effects of adiponectin may involve IL-10 signalling pathways[41]. Interestingly, NADPH oxidase is a

major IL-10 target in various cell systems including macrophages[42].

Decreased levels of adiponectin are definitely related to a variety of unfavourable effects, but the precise origin of adiponectin reduction has not been clarified. TNF-α has been demonstrated to suppress the transcription of adiponectin in an adipocyte cell line, which might explain the lower levels of serum adiponectin in obese individuals[9]. Expression of adiponectin is also regulated by other pro-inflammatory mediators such as IL-6, which suppresses adiponectin transcription and translation in an adipocyte cell line[9].

In a recent study, Kolak *et al*[43] evaluated subcutaneous AT biopsies obtained from healthy women both with and without increased liver fat (LFAT) (2.3% ± 0.3% *vs* 14.4% ± 2.9%, respectively), with similar BMIs and percentage body fat. Expression of cytokines and chemokines including CD68 (which correlates with the number of macrophages), MCP-1, macrophage-inflammatory protein (MIP-1α), and PAI-1 were significantly increased, whereas peroxisome proliferator-activated receptors (PPAR)-γ and adiponectin were significantly decreased in women with high levels of LFAT compared with women with normal levels of LFAT, even though subcutaneous fat cell size, BMI, and percentage body fat were similar.

Leptin activates neutrophils, stimulates proliferation in human circulating monocytes, and appears to induce Th1-type cytokine production while inhibiting Th2-type cytokines. In addition, leptin has marked effects on the innate immune response by promoting activation and phagocytosis of macrophages, presumably through JAK/STAT signalling[44]. Expansion of adipocytes in obesity leads to the recruitment of macrophages and the release of TNF-α, IL-6, and MCP-1 from macrophages and lymphocytes. TNF-α and IL-6 suppress the transcription of adiponectin, and TNF-α and IL-1 stimulate the production of leptin[9,44]. Accordingly, hyperleptinaemia associated with obesity may contribute to progression of NAFLD, although this issue remains controversial[45].

Resistin is another pro-inflammatory adipokine secreted by monocytes/macrophages and adipocytes in response to pro-inflammatory signals. Resistin induces NFκB-dependent secretion of TNF-α and IL-6 by monocytes and increases ICAM-1 and VCAM-1 expression in endothelial cells, suggesting that it contributes to endothelial activation and leukocyte recruitment[46]. In particular, in pure steatosis there is no significant increase in adhesion molecule expression but distinctive patterns are associated with both alcoholic hepatitis and cirrhosis, and in murine models of NASH elevated ICAM-1 expression is seen[47]. Alcoholic hepatitis is characterized by increased expression of E-selectin and ICAM-1 on portal and hepatic venous endothelium and of ICAM-1, VCAM-1, and VAP-1 on sinusoidal endothelium as a consequence of local pro-inflammatory cytokines, particularly TNF-α[48-51]. In alcoholic cirrhosis, increased expression of endothelial adhesion molecules including ICAM-1, VCAM-1, and P-selectin is largely restricted to portal and septal vessels. Endothelial ICAM-1 expression is increased in periseptal areas where LFA-1 is also increased in leukocytes, however, in contrast to alcoholic hepatitis, there is little increased ICAM-1 expression on hepatocytes[48].

Visfatin, the characteristic adipokine of mesenteric AT, was previously identified as a protein involved in immune B-cell maturation (pre-B colony enhancing factor)[52]. More recently, visfatin was described to be a highly expressed protein with insulin-like functions that was predominantly found in visceral AT, from which the name visfatin was derived[53]. Thus, visfatin was identified as nicotinamide phosphoribosyltransferase, the rate-limiting enzyme that converts nicotinamide (a form of vitamin B3) to nicotinamide mononucleotide, a NAD precursor[54]. Visfatin also has pro-inflammatory properties by inducing TNF-α and IL-6 in monocytes[55]. Further studies are needed to fully understand the effect of this adipokine in Kupffer cells.

Figure 1 summarises the effects of adipocytokines on the regulation of the immune response.

HEPATIC STEATOSIS AND NATURAL KILLER CELLS

One experimental model which has generated a significant body of evidence regarding potential mechanisms of NAFLD pathogenesis and its relationship with the immune system is the *ob/ob* mouse. *Ob/ob* mice, which are leptin deficient as a result of a spontaneous mutation in the leptin gene, exhibit a number of metabolic and inflammatory features which mimic human NAFLD[56] including insulin resistance, hyperlipidaemia, hepatic steatosis, and TNF-α elevation. One of the principal applications of the *ob/ob* mouse has been the identification of susceptibility of the steatotic liver to inflammatory insult (exemplified by the response to LPS) as a key factor in the development of NASH[57]. A number of immuno-regulatory abnormalities have been identified in *ob/ob* mice which may contribute to their increased susceptibility to inflammatory damage. These include selective depletion in the liver (but not other organs) of Natural Killer (NK) T cells, a key population of immuno-regulatory/effecter lymphocytes which express phenotypic features of both "classical" T cells (CD3) and NK cells [NK1.1 (CD161 in humans)][58,59]. In their most characteristic form, NKT cells show specificity, through a semi-invariant surface T-cell receptor, for highly conserved glycolipid antigens presented by the MHC class I homolog CD1d. NKT cells, which are specifically enriched within the liver, have characteristic cytokine release patterns {Th-1 dominant [interferon (IFN)-γ], mixed, and Th-2 dominant (IL-4) depending on the mechanism of stimulation} which endow, in addition to their effector function, significant immuno-regulatory properties[60]. The observation that liver NKT cells are depleted in steatosis in *ob/ob* mice has led to the suggestion that these cells play a key role in mediating and/or regulating inflammatory effects critical to the development of NAFLD. Although of potential value in the understanding of the pathogenesis of NAFLD, conceptual problems arise with regard to the *ob/ob* mouse

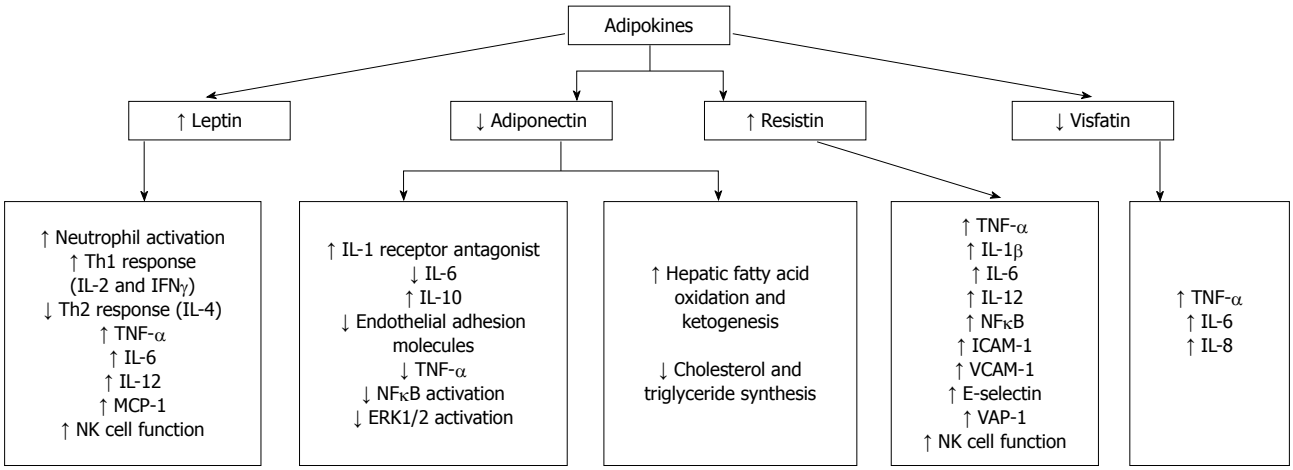

Figure 1 Effects of adipocytokines on regulation of the immune response. IL: Interleukin; IFN: Interferon; MCP: Monocyte chemotactic protein; NK: Natural Killer; TNF-α: Tumor necrosis factor α; NFκB: Nuclear factor κB.

as a model for human disease due to its markedly different leptin phenotype (absent *vs* elevated), and to the fact that leptin is itself a key immunomodulatory cytokine[61]. There are, therefore, potential mechanisms whereby leptin deficiency could modulate the immune response independent of its effects on hepatic fat accumulation. Li *et al*[62] used a natural obese/steatosis model to study the effects of hepatic steatosis on hepatic innate immune system function in leptin complete animals. C57Bl/6 mice fed a high-fat diet showed excess weight gain and the development of hepatic steatosis[63]. Although total hepatic mononuclear cell levels were similar in the high- and low-fat diet groups, the percentage of hepatic (but not splenic) NKT cells was significantly reduced. Within both the hepatic T-cell and NKT fractions, the numbers of cells showing cytoplasmic staining for TNF-α and IFN-γ were, conversely, increased in the high-fat diet group (and serum IFN-γ levels were elevated), suggesting Th1 skewing of the response phenotype resulting from induced NKT cell effects. Finally, the livers of obese mice appeared to be sensitized to LPS injury, presumably reflecting the augmented Th1-type inflammatory cytokine response. These observations suggest that the development of hepatic steatosis *per se* can be associated with significant changes in liver NKT cell function. This finding would be compatible with the NKT cell changes seen in the *ob/ob* mice occurring as a result of hepatic steatosis that occurs in these animals, rather than the specific absence of leptin. The findings do, however, raise a number of issues which will determine whether this model is suitable for the study of human NAFLD. The first issue is the mechanism responsible for liver NKT cell "loss", and Th-1 skewing of the residual cells, in obese C57Bl/6 mice. Theoretically, a reduction in liver NKT cells in obese C57Bl/6 mice could result from a decreased rate of NKT cell recruitment to, or development in, the liver, an increased rate of NKT cell death or migration from the liver, a loss of surface markers identifying the cells as NKT cells or any combination of these effects. The liver recruitment aspect of NKT cell homeostasis was not addressed in the Li's study[60,64,65]. Instead, the authors argue that increased cell loss is the dominant effect, with evidence presented to suggest increased NKT cell apoptosis and increased hepatic expression of IL-12 (postulated to be a promoter of NKT cell apoptosis). There is an emerging consensus, however, that NKT cells are in fact relatively resistant to activation-induced cell death[66]. An alternative (albeit non-mutually exclusive) explanation for the Li's data would be that endogenous IL-12 released by Kupffer cells (KC) at elevated levels in the context of obesity[62,67] acts as a cofactor for the stimulation of IFN-γ release (as opposed to IL-4 release which occurs in the absence of IL-12) by physiologically activated NKT cells, with the resulting "loss" of cells occurring as a consequence of post-activation surface phenotypic shift[68]. If elevation of KC-released IL-12 in response to steatosis were to prove to be a factor in human fatty liver development[69], its well-established ability to promote breakdown of self-tolerance may explain the increasingly recognised tendency towards autoantibody formation reported in NASH patients[70,71]. The possibility that NKT cell activation is responsible, through activation-induced cell death and/or post-activation phenotypic change, for "reduction" in hepatic NKT cells in obese C57Bl/6 mice, and through cytokine release, for liver damage, raises the important question of the mechanism of this activation. Most previous work on NKT cell activation has used non-physiological ligands (anti-CD3 and anti-TCR). Although the recent identification of α-galactosylceramide has highlighted the potential importance of glycolipids as natural ligands for NKT, it is unlikely, given its marine sponge origin, that this agent is a physiological ligand in mice or humans. At present, the identity of the *in vivo* physiological ligand for NKT cells, the extent to which TCR-mediated as opposed to cytokine-driven mechanisms (such as *via* IL-12) are required for activation, and the extent to which different activation pathways result in different cytokine response phenotypes, remain areas of speculation. One potentially highly intriguing link between hepatic steatosis and NKT cell activation has emerged

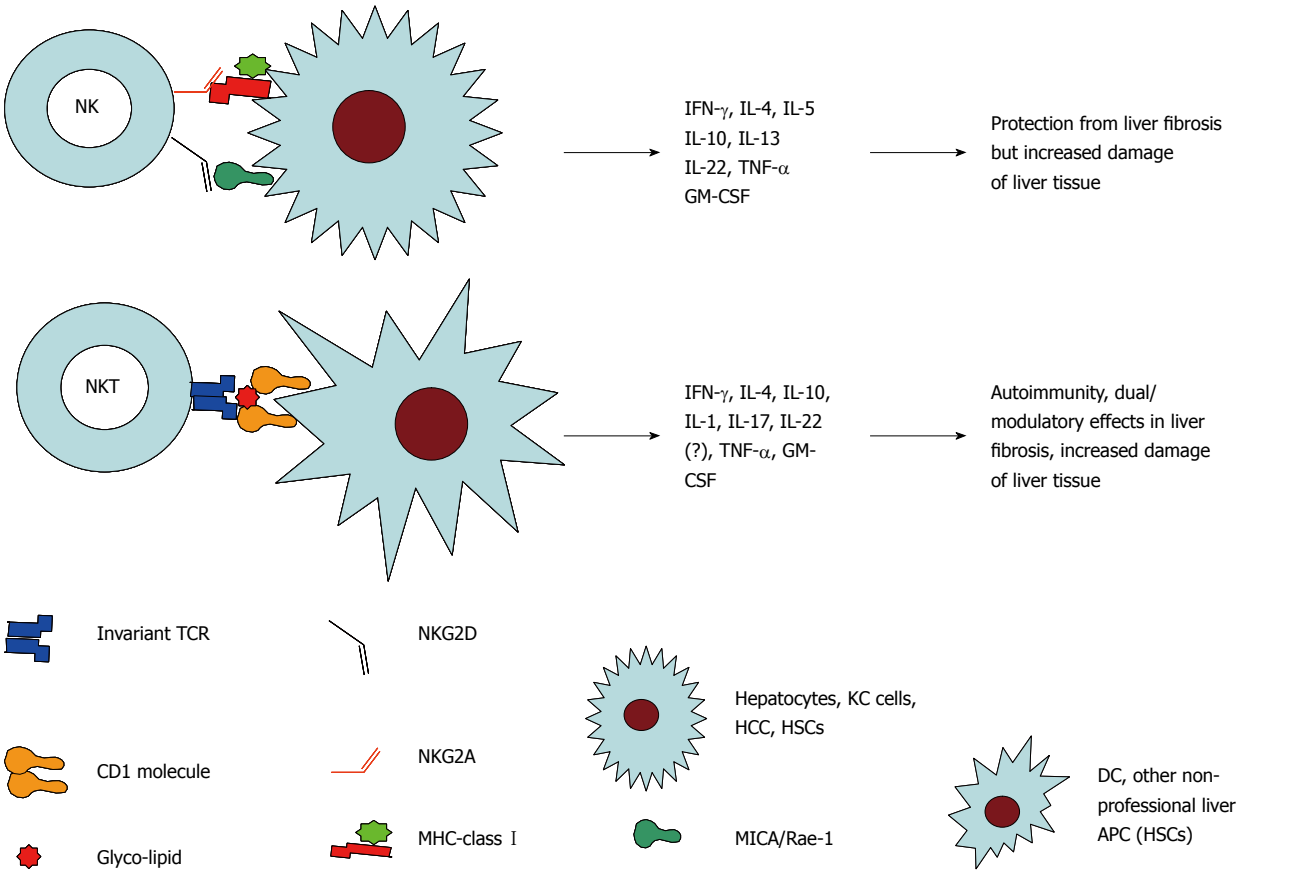

Figure 2 Simplified scheme of Natural Killer/Natural Killer T cell role in liver diseases. Natural Killer (NK) interacts with major and minor histocompatibility antigens expressed on several liver cells and kill and/or produce cytokines having several effects on the tissue. More complex is the role of NKT cells. These cells specifically recognize an antigen expressed in the context of a CD1 molecule and, upon recognition through an invariant TCR, secrete a large amount of cytokines having pleiotropic, sometimes controversial effects, whose overall results are due to the cytokine milieu and to the conditioning of the functions of other immune cells. This scenario is further complicated by the fact that many soluble factors (for instance cytokines) and hedgehog ligands may activate NK or NKT. IL: Interleukin; TNF-α: Tumor necrosis factor α; HCC: Hepatocellular carcinoma; HSCs: Hepatic stellate cells; GM-CSF: Granulocyte-macrophage colony stimulating factor; APC: Antigen presenting cell.

with the observation that microsomal triglyceride transfer protein, plays a key role in the acquisition of glycolipid antigens by CD1d[72]. In partial support of this concept, deficiency of microsomal triglyceride transfer protein in mice is associated with hepatic steatosis, and functional polymorphisms of its encoding gene have shown significant associations with NASH in humans[73]. One approach to dissecting out the mechanisms of NKT cell activation and loss in obese C57Bl/6 mice would be to utilize NKT cell adoptive transfer and tracking methodologies in recombinant NKT cell-deficient mice in combination with NKT cell activation and appropriate cytokine blocking.

In a recent paper, Hua *et al*[74] examined the mechanism of dietary fatty acid induced hepatic NKT cell deficiency and its causal relationship to insulin resistance and NAFLD, and found that dietary saturated fatty acids (SFA) or monounsaturated fatty acids (MUFA), but not polyunsaturated fatty acids (PUFA), caused hepatic NKT cell depletion with increased apoptosis. Dietary SFA or MUFA also impair hepatocyte presentation of endogenous, but not exogenous, antigen to NKT cells, indicating alterations of the endogenous antigen processing or presenting pathway. *In vitro* treatment of normal hepatocytes with fatty acids also demonstrates impaired ability of CD1d to present endogenous antigen by dietary fatty acids. Furthermore, dietary SFA and MUFA activate the NFκB signaling pathway and lead to insulin resistance and hepatic steatosis.

Recently, a new subset of T helper cells, named Th17 due to the ability to produce IL-17 and other cytokines, has been correlated to processes underlying hepatic steatosis. In particular, Th17 largely express a NKT marker, CD161, and they have been described to be closely involved in the immune responses in several anatomical sites including skin, liver and gut[75,76]. Th17 produce cytokines besides IL-17 such as IL-22 which is indicated to play a pivotal role in hepatic steatosis as recently shown[77].

Figure 2 shows a simplified Scheme of NK/NKT cell role in liver diseases.

KUPFFER CELLS AND STEATOSIS

Hepatocellular accumulation of lipids is a key morphologic feature of NAFLD. Lipidomic analysis of human liver tissue is a promising novel approach to associate abnormal fat composition with various stages of NAFLD. Thus, total and damaged phospholipids are more abundant in simple steatosis at the expense of triglycerides[78], while the increased ratio of stearic to arachidonic acid in NASH may

correlate with fibrosis[79]. Altered abundance and composition of liver tissue lipids may modulate the biological activity of KC in NAFLD through a number of mechanisms. First, the space-occupying effect of fat-laden hepatocytes may lead to impaired sinusoidal perfusion[80]. Leukocytes trapped in narrowed sinusoids may increasingly engage KC in the microvascular inflammatory response[80]. Second, excessive exposure of KC to fatty acids may modulate pathways of inflammation and insulin resistance through interaction with cell surface receptors and intracellular mediators[81]. Third, anomalous deposition of lipids in the plasma membrane may alter the structure of lipid raft domains and interfere with clustering and function of cell surface receptors[82]. Altered lipid composition may also affect proper functioning of intracellular membranes as seen with free cholesterol loading of mitochondria[83]. Finally, abundant or abnormal lipids may confound recognition of fatty hepatocytes as dangerous and promote adverse interactions with KC[17]. Nevertheless, the existence of a lipid-derived quintessential alarm expressed or released by steatotic hepatocytes remains speculative.

Recent findings indicate that TLR-mediated recognition of fatty acid moieties is an important mechanism by which lipids regulate pathways of inflammation and innate immunity[82]. Depending on fatty acid composition, the outcome of this effect may be highly variable. Saturated fatty acids, implicated in the development of chronic conditions such as atherosclerosis, have been shown to activate TLR4 signalling in adipocytes and macrophages through both Myd88-dependent and TRIF-dependent pathways[84,85]. In contrast, polyunsaturated fatty acids inhibit these events in several cell types including macrophages[85]. Consequently, TLR4 is a sensor of endogenous fatty acid levels and composition, and KC most likely benefit from this ability.

Emerging evidence indicates that altered cholesterol metabolism may directly affect the function of KC. Thus, high-fat diet fed to LDL receptor deficient mice rapidly results in significant hepatic inflammation, but only if the diet contains cholesterol[86]. The presence of "foamy" KC suggests that scavenging of modified lipoproteins may induce this early inflammatory response[86]. While these findings need to be extrapolated to human NAFLD with caution, they point to the importance of altered cholesterol metabolism. In addition, some of these observations challenge the "second-hit" concept since steatosis is not necessarily a forerunner of hepatic inflammation as these events may develop simultaneously[86,87].

There is evidence that steatosis promotes Th1 polarization of the cytokine balance favouring innate or classic activation of macrophages in NAFLD[88]. PPAR-α, PPAR-γ, and PPAR-σ and liver X receptors LXR-α and LXR-β are members of the nuclear hormone receptor superfamily of transcription factors that coordinate complex genetic programs of metabolism[89,90]. Therapeutic use of synthetic ligands to target these receptors and exploit their biological functions is increasing. The beneficial effects of PPAR-γ in hepatocellular lipid homeostasis have prompted large clinical trials to assess impact on NAFLD and these efforts have been recently reviewed elsewhere[91]. However, the recognition that nuclear hormone receptors link lipid metabolism to alternative activation of macrophages adds a new dimension to their potential use in the treatment of NAFLD[88,92]. While PPAR-γ promotes alternative activation of macrophages that contribute to valuable metabolic changes such as improved insulin sensitivity[93,94], recent research indicates that PPAR-σ is specifically required for a similar program in KC[95,96]. Thus, signature gene expression of PPARσ-deficient KC is greatly reduced in the livers of obese mice and in response to IL-4 stimulation[95,96]. Moreover, PPARσ ablation results in severe steatosis and insulin resistance[95,96]. Notably, the effect of PPARσ in KC is modulated by fatty acids[95] and may fail due to altered lipid homeostasis and hepatic microenvironment in NAFLD. Thus, hepatocytes as a previously unsuspected source of Th2 cytokines stimulate M2 gene expression in KC and this important regulatory circuit may be altered in steatosis[96]. These findings raise the intriguing possibility that specific targeting of PPAR-σ in KC to induce alternative activation may improve both inflammation and steatosis in NAFLD. One important caveat is that the M2 phenotype includes stimulation of the extracellular matrix that may contribute to hepatic fibrosis[97]. Figure 3 shows the effects of activation of Kupffer cells in NAFLD.

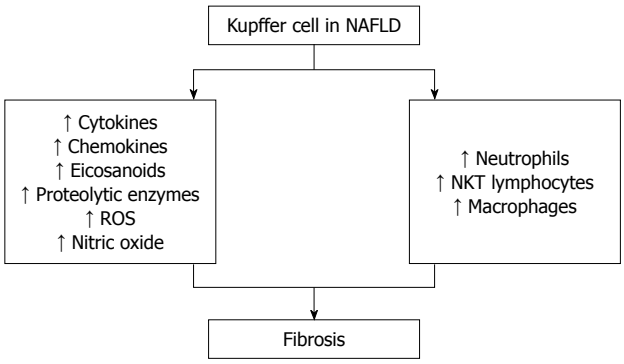

Figure 3 Effects of the activation of Kupffer cells in non-alcoholic fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; NKT: Natural Killer T cells; ROS: Reactive oxygen species.

In the last few years, there is increasing evidence that ligands of Hedgehog (Hh) may have a critical role in processes leading to liver fibrosis. The Hh mediated activity is quite low in healthy liver but increases during the course of several liver diseases, as recently reviewed[98]. In particular, it has recently been shown that damaged/dying hepatocytes may produce Hh ligands that mediate proliferation of myofibroblasts in the liver, thus promoting fibrosis[99]. Moreover, Hh seems to be critical due to its properties in regulating NKT growth and functions in liver fibrosis[100,101].

IMMATURE MYELOID CELLS AND STEATOSIS

Immature myeloid cells ($CD11b^{+}Gr\text{-}1^{+}$) play a role in the

induction of inflammatory cytokines[102] through activation of innate immune pathways. The role that immature myeloid cell populations play in obesity-related liver disease is unknown. In a recent study, Deng *et al*[103] hypothesize that accumulation of immature myeloid cells in the liver may be an important component in the development of inflammatory responses in liver tissue that are triggered by obesity, which in turn contributes to metabolic consequences, such as steatohepatitis. In this study, the liver of obese mice was demonstrated as the major organ where $CD11b^{+}Ly6C^{+}$-$Ly6G^{-}$ immature myeloid cells accumulate. It is not clear why these cells are preferentially recruited into the liver. Chemotactic cytokines and chemokines could direct the migration of immune cells including myeloid cells and may be responsible for the cell accumulation. Several hepatic cell populations, including hepatocytes, KC, sinusoidal endothelial cells, and hepatic stellate cells, can secrete chemokines upon activation. High-fat diet-derived products could activate one of these cells in the liver, resulting in the recruitment of these circulating activated immature myeloid cells into the liver. IL-6 is overexpressed in the NAFLD patient[104], and IL-6 has been shown to block immature myeloid cell differentiation[105]. As a result, these activated cells are accumulated in the liver. The specific role of chemokines or other factors in the recruitment of these cells to the liver warrants further investigation.

CONCLUSION

It is now recognized that adipose tissue is an active endocrine organ that secretes numerous molecules that play a central role in the regulation of energy and vascular as well as immune system homeostasis by acting both locally and at distant sites influencing various metabolic and immune processes. Many of these interactions between metabolic and immune systems seem to be orchestrated by this complex network of soluble mediators derived from immune cells and adipocytes that are briefly summarized in Figure 4.

NAFLD is becoming an increasingly relevant clinical issue, especially in the developed world. One of the unmet challenges of NAFLD is to satisfactorily predict its progression from simple steatosis into steatohepatitis. This transition represents a milestone in the natural history with a considerable probability for developing end-stage liver disease. Elucidation of molecular and cellular events that may lead to this outcome is therefore critically important. Fortunately, the past few years have brought remarkable advances in our understanding of NAFLD pathogenesis, often by extension of research in adipose tissue biology, obesity, and insulin resistance. These efforts point to the intricate relationship of the innate immune system and lipid homeostasis in NAFLD with a prominent role for Kupffer, myeloid and NKT cells and a number of biochemical and cellular mechanisms involved.

However, a number of questions regarding the role of macrophage infiltration in human obesity remain to be answered. For example, what is the cause/s of macrophage infiltration? Does moderate fat gain alter macrophage number and/or macrophage phenotype in humans? Are some individuals predisposed to this? Is macrophage infiltration causal in the development of insulin resistance? The activation of NKT cells exacerbates macrophage infiltration in adipose tissue and glucose intolerance with obesity. Therefore, NKT cells enhance chronic inflammation in visceral adipose tissue and contribute to the development of metabolic disorders in obesity. The NKT cells may be the novel therapeutic targets in atherosclerosis, metabolic syndrome, and type 2 diabetes.

Further studies are needed to fully understand the interaction between fat, the immune system and steatosis.

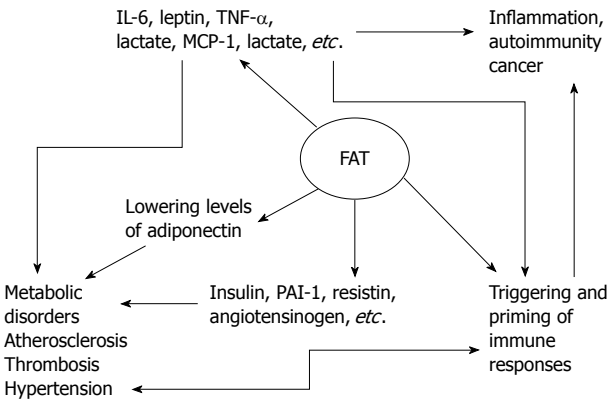

Figure 4 Complex network of soluble mediators derived from immune cells and adipocytes. MCP: Monocyte chemotactic protein; TNF-α: Tumor necrosis factor α; IL: Interleukin.

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

Hepatic steatosis, low-grade chronic inflammation and hormone/growth factor/adipokine imbalance

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Abstract

Non-alcoholic fatty liver disease (NAFLD), a further expression of metabolic syndrome, strictly linked to obesity and diabetes mellitus, is characterized by insulin resistance (IR), elevated serum levels of free fatty acids and fatty infiltration of the liver, which is known as hepatic steatosis. Hepatocyte apoptosis is a key feature of this disease and correlates with its severity. Free-fatty-acid-induced toxicity represents one of mechanisms for the pathogenesis of NAFLD and hormones, growth factors and adipokines influence also play a key role. This review highlights the various pathways that contribute to the development of hepatic steatosis. Circulating concentrations of inflammatory cytokines are reckoned to be the most important factor in causing and maintaining IR. Low-grade chronic inflammation is fundamental in the progression of NAFLD toward higher risk cirrhotic states.

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Key words: Hepatic steatosis; Low-grade chronic inflammation; Adipokines; Hormones; Growth factors

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INTRODUCTION

There are many genetic, evolutionary, environmental, behavioral and physiological factors that induce and exacerbate obesity. Teleologically, our early ancestors had a survival advantage if they were able to store energy to be used in famine or during very stressful situations. Those individuals with 'thrifty genes' survived and reproduced more metabolically efficient offspring. In modern society, thrifty genes are coupled with increased food availability and sedentary lifestyle. This often results in a net positive energy balance. The current view is that obesity leads to hyperinsulinemia/insulin resistance (IR) and IR exacerbates obesity, which frustrates most attempts at weight loss. Adipocytes, while utilizing glucose and fat excess during nutritional affluence and storing them as triglycerides, release energy as free fatty acids (FFAs) and glycerol by lipolysis. These FFAs do not produce significant metabolic disturbances as long as they are oxidized in target tissues by the leptins of adipocytes. Long-chain fatty acids (LCFAs) in non-adipocytes, which cannot undergo mitochondrial β-oxidation, cause signal transduction defects[1] or result in apoptosis *via* accumulation as cytosolic triglycerides. The consequences of increased lipid delivery to peripheral tissues are multiple and the integrated response

to central adiposity is complex and involves several organs and tissues.

Concerning the cardiovascular risk, some observations suggest that different lipid accumulation processes begin in the subendothelial or deep intimal regions, which contributes to complicated atheroma core formation in peripheral arteries. Furthermore, it has been demonstrated that the presence of deep intimal lipid accumulation is associated with reduced endothelium-dependent relaxation in large arteries. The functional and morphological abnormalities might contribute to human coronary atherogenesis that progresses slowly with age[2]. Besides, adipocytes can also prevent atherosclerotic vascular damage by its product adiponectin[3].

LIPOTOXICITY: THE CLASSICAL VIEW

Acute elevations in FFAs can provoke peripheral IR in humans[4]. In addition, acute lowering of FFAs with the antilipolytic drugs can enhance peripheral insulin-mediated glucose uptake[5]. A defect at the level of reduced glucose transport *per se* results from an effect of FFAs to inhibit proximal insulin signaling steps, including tyrosyl phosphorylation of insulin receptor substrates[6]. Obesity results in the accumulation of muscle (intramyocellular) triglycerides and activated lipids in the form of long-chain fatty acyl-CoA esters[7]. This accumulation is also implicated in the impairment of insulin signaling, possibly *via* activation of selected protein kinase C isoforms[8]. Similar effects occur in the liver in association with hepatic lipid accumulation, a ubiquitous finding in obese patients. Lipids can also accumulate in pancreatic islets, which impairs insulin secretion, such as in the development of diabetes in ZDF rats[7]. In addition, decreased catabolism contributes to tissue lipid accumulation, because hepatic and intramyocellular lipid content is associated with reduced mitochondrial oxidative and phosphorylation activity in muscle of elderly humans[9]. Obesity is clearly associated with increased levels of circulating FFAs. Patients with various grades of obesity and IR are generally resistant to the antilipolytic effects of insulin[10]. Furthermore, adipocyte constituents of visceral fat are more metabolically active and have a higher rate of lipolysis[11]. Increases in FFAs can provoke peripheral IR in animals and humans[4]. In addition to the role of FFAs in producing IR in muscle, the impaired ability of insulin to suppress FFA release boosts hepatic glucose production, because insulin-mediated anti-lipolysis contributes to insulin regulation of hepatic glucose output[12]. The glucose-fatty acid cycle that has the potential to increase fatty acid utilization to inhibit glucose oxidation in muscle was first proposed by Randle *et al*[13] in 1963.

LOW-GRADE CHRONIC INFLAMMATION: THE MAIN ROLE OF INTERLEUKIN 6

Growing evidence links a low-grade, chronic inflammatory state to obesity and its coexisting conditions such as IR, type 2 diabetes, metabolic syndrome and non-alcoholic fatty liver disease (NAFLD)[14,15] that includes a large spectrum that ranges from fatty liver (FL), non-alcoholic steatohepatitis (NASH) and cryptogenetic cirrhosis. The spleen also plays a key role in this chronic process[16]. Anti-inflammatory drugs can reverse IR[17], which suggests that inflammation is directly involved in its pathogenesis. Inflammatory mediators that are biosynthesized in the liver and increased in NAFLD patients include C-reactive protein (CRP)[15], interleukin (IL)-6[16], fibrinogen and plasminogen activator inhibitor-1 (PAI-1)[18]. Fat in the liver represents a site beyond adipose tissue that independently contributes to synthesis of inflammatory mediators. In support of a sequence of cellular and molecular events that mediate hepatic IR in NAFLD, recent data lend credence to the fact that hepatic steatosis activates IκB kinase (IKK)-β and nuclear factor (NF)-κB[19]. Among the inducible transcription factors that control inflammatory gene expression, NF-κB plays a central and evolutionarily conserved role in coordinating the expression of various soluble pro-inflammatory mediators (cytokines and chemokines) and leukocyte adhesion molecules. In non-stimulated cells, NF-κB is sequestered in the cytosol by the inhibitor of NF-κB (IκB) that masks the nuclear localization signal present along the NF-κB protein sequence. Treatment of cells with pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1, or with bacterial products such as lipopolysaccharide, leads to the activation of a specific-IKK complex that phosphorylates IκB and thereby tags it for ubiquitination and degradation by the proteasome[20]. The degradation of IκB thus allows NF-κB to translocate into the nucleus where it can act as a transcription factor that upregulates IL-6 production and secretion. IL-6 works locally through paracrine and/or endocrine mechanisms to activate IL-6 signaling in the liver. IL-6 is known to induce IR in hepatocytes[21]. Hepatic production of IL-6 also provides a further pathogenic link to extrahepatic organs such as muscle. NF-κB target genes are not upregulated in transgenic mouse muscle, but IL-6 target genes are, including suppressor of cytokine signaling (SOCS) and signal transducer and activator of transcription (STAT) proteins. These genes are reversed during IL-6 neutralization, which is consistent with the pathogenic involvement of IL-6. Activation of NF-κB leads to a severe syndrome of muscle wasting, without IR[22].

VISCERAL ADIPOSITY AND ADIPOCYTE DIFFERENTIATION

Although many studies have reported strict correlations between insulin sensitivity and visceral fat deposition[23], associations between the amount of subcutaneous fat on the trunk and IR have also been reported in obese non-diabetic men[24] and those with type 2 diabetes[25]. Thus, subcutaneous fat, not draining into the portal vein, determines IR by a mechanism that is not linked to the liver. At

the same time, IR in obese women is strictly related to increased overall fat mass, or to an elevation in truncal subcutaneous fat mass as measured by skinfold thickness[26] or magnetic resonance imaging[27]. Moreover, IR is predicted independently by an enlargement of truncal subcutaneous fat mass and an increased amount of visceral fat[26]. Total and subcutaneous fat mass is important to the IR syndrome; in fact, adipocytes, while responding sensitively to systemic influences, affect important target tissues with their secretions. They specifically detect the changes in energy equilibrium of the organism and adequately respond by drawing excess glucose and lipids from the bloodstream[28], storing them as triglycerides[29], releasing depot fat to non-adipocytes as FFAs and glycerol[30], and increasing fatty acid oxidation in adipose and non-adipose tissues to maintain fat homeostasis and cellular integrity[31]. The main transcription factor designating the characteristics of adipocytes is most likely the adipocyte determination and differentiation factor 1 (ADD1)/sterol regulatory element binding protein-1c (SREBP-1c)[32]. Its regulatory functions consist in sensing the glucose and fat excess and drawing them into the adipocytes to preserve energy and maintain constant blood levels. Otherwise, fat accumulation in non-adipocytes could be deleterious to their functions[33]. An inverse correlation has been found between cytosolic cholesterol concentration and ADD1/SREBP-1c, meanwhile, plasma insulin and glucose levels have a positive impact on ADD1/SREBP-1c[34].

Insulin crucially controls almost all aspects of adipocyte pathobiology. Almost all anabolic effects of insulin in the adipocytes are regulated by the transcription factor ADD1/SREBP-1c that also controls other mature adipocyte markers *via* transactivation of peroxisome proliferator activated receptor (PPAR)-γ and leptin[35]. PPAR-γ has major influences on various aspects of adipogenesis, such as adipocyte differentiation from preadipocytes and differentiation of fibroblasts into mature adipocytes[36]. ADD1/SREBP-1c and PPAR-γ also regulate the genetic expression of the enzymes for *de novo* lipogenesis and glucose transporter GLUT4[37].

Adipocytes do not have unlimited capacity for expansion by storing fat as triglycerides. When adipocytes reach a critical fat cell size, adipogenesis is triggered that increases the number of fat cells[38]. ADD1/SREBP-1c, and in particular PPAR-γ, efficiently cause the differentiation of pre-adipocytes and even fibroblasts or myoblasts into mature adipocytes[32]. Old adipocytes are protected by diverting the fuel excess to more competent (in terms of lipogenesis) younger adipocytes. When adipogenesis cannot occur, fat cells produce factors that strongly inhibit the anabolic actions of insulin. Two of these are TNF-α[39] and resistin[40]. These adipocyte products might result in the development of metabolic syndrome by creating insensitivity to insulin action, mainly in the fat tissue, and partly in the liver and muscle[33]. Resistin, a novel signaling molecule isolated in mice has been suggested as the putative hormone that links obesity with type 2 diabetes. Research confirms the expression of resistin in human adipose tissue and increased expression in abdominal fat[41]. Glucose firstly determines the fate of nutritional energy, whether it is oxidized or stored as triglycerides[34]. It then inhibits oxidation of LCFAs, which causes accumulation of LCFAs and their metabolites in the cytosol, which results in impaired signal transduction[42], and finally stimulates apoptosis, when unoxidized LCFA metabolites continue to accumulate in the long term (so-called glucolipotoxicity)[43].

ADD1/SREBP-1c, which is mostly dependent on insulin as outlined above, has central importance in fat tissue for the regulation of energy metabolism[30]. The amount and type of fat and associated cholesterol content of food generally inhibit ADD1/SREBP-1c expression *via* an effect on cytosolic cholesterol level[44]. Although activation of this transcription factor changes mainly according to the same intracellular cholesterol concentrations[45], the glucose component of the nutritional excess and insulin are the predominant stimulators for the genetic expression of ADD1/SREBP-1c in fat tissue[34]. It has been proposed that the amount and quality of nutritional carbohydrate, which determines the glycemic index and the responding insulin level, control the activation of ADD1/SREBP-1c. Extracellular glucose level is not only the operating lipogenic machinery of fat cells, but also controls the adipocyte secretions that are effective in non-adipocytes and their energy regulation, which determines fuel partitioning, and oxidation or storage in cells, including fat tissue. Ultimately, chronic over-nutrition, particularly rich in carbohydrates, causes impairment in transcriptional regulatory effects of ADD1/SREBP-1c, which leads to the development of a variety of metabolic disorders. In this way, IR has the potential to provide information on healthy and non-healthy obesity[46].

GROWTH FACTORS AND ADIPOKINES

Differentiation of precursor cells into mature fat cells is stimulated by multiple hormones, including glucocorticoids, growth hormone (GH), insulin-like growth factor (IGF)- I , and insulin.

As reported in a recent published review[47], a relevant role for GH in metabolism has been known since the late 1940s, when its effects on lipid and protein metabolism were demonstrated in experimental animal models[48]. After the binding of GH to specific monomeric or dimeric receptors, members of the cytokine receptor superfamily, the GH intracellular signal that is involved in lipid metabolism results in activation by trans-phosphorylation of adjacent Janus-kinase 2, with subsequent recruitment of the STAT pathway[49]. The GH signal cascade induces ultimately the transcription of specific genes, such as those for IGF- I , IGF-binding proteins (IGFBPs), acid-labile subunit (ALS), or SOCS proteins[47].

IGF- I , the main anabolic mediator of GH effects, is primarily GH-dependent and influences GH secretion through a negative feedback system[50]. IGF- I exhibits a 45% amino acid homology with insulin, acts as an insulin

sensitizer, and is a member of the IGF family, along with IGF-Ⅱ. Consequently, IGF-Ⅰ and insulin, apart from high affinity for their specific receptors, share lower affinity for their cognate insulin and IGF-Ⅰ receptors, respectively. The IGFBPs, present in serum, other biological fluids, and tissue extracts, bind IGF-Ⅰ and IGF-Ⅱ with affinities comparable to those of IGF-Ⅰ receptors. IGF-Ⅰ, in particular, circulates in plasma as a ternary complex along with IGFBP-3 or -5 and ALS, which prolongs the half-life of IGF-Ⅰ and modulates its bioavailability to peripheral tissues[51]. IGF-Ⅰ intracellular signal triggers metabolic mechanisms different from GH, by inducing tyrosine phosphorylation of the insulin receptors substrate proteins, with subsequent activation of phosphatidylinositol-triphosphate kinase and mitogen-activated protein kinase pathways[50,52]. However, a close interplay between the signaling pathways activated by GH, IGF-Ⅰ, and insulin has been demonstrated *in vitro*[47].

Human adipocytes express GH receptors. Adult patients with GH deficiency (GHD) characteristically develop an increase in abdominal obesity, total cholesterol, triglycerides and fibrinogen levels, and a decrease in high-density lipoprotein (HDL)-cholesterol levels, which indicates the metabolic syndrome[53]. GHD is correlated with the severity of alterations in lipid metabolism[54] and GH treatment in GHD patients is associated with improved lipid profiles and cardiovascular risk[55]. GH exerts insulin-like and insulin-antagonistic metabolic effects[56], which include increased gluconeogenesis, enhanced lipolysis, and inhibition of insulin action. In particular, GH displays its lipolytic effect mainly in the visceral adipose tissue, by increasing adipose tissue hormone-sensitive lipase activity *via* enhanced stimulation of the β-adrenergic receptors[49]. This effect results in increased FFA flux from the adipose to peripheral tissues[48]. No definite effects have been reported on lipoprotein lipase (LPL), thus suggesting that GH might not affect triglyceride uptake in adipose tissue. GH might also directly induce adipogenesis *via* activation of STAT-5/PPAR-γ pathway[47], although its role has been demonstrated only during the early phase of the process[47]. Finally, GH inhibits serum leptin and increases circulating resistin levels, while the effect on adiponectin remains controversial[47]. In this context, it is still not yet clear whether GH metabolic actions are exerted directly, or indirectly *via* IGF-1, or are part of GH antagonism of insulin signaling.

The effects of IGF-Ⅰ on lipolysis, gluconeogenesis, and SOCS protein are the opposite of those of GH. In particular, metabolic IGF-Ⅰ effects are similarly to those of insulin, and mainly consist of increased tissue glucose uptake, inhibition of gluconeogenesis, and enhanced adipogenesis[56]. IGF-Ⅰ has been suggested to be a major regulator of cell proliferation, differentiation and metabolism, thus regulating, among other biological processes, adipose tissue growth and differentiation of pre-adipocytes into adipocytes. The role of IGF-Ⅰ in the accumulation of adipose tissue has been investigated using transgenic mice that overexpress the *IGFBP-1* gene. In response to a sucrose-enriched diet, the transgenic mice gain significantly less body weight, and adipocyte size and epididymal fat mass are significantly reduced compared with wild-type mice[57]. Moreover, fewer colonies are generated from adipose tissue of transgenic mice, and the mitogenic response of these cells to IGF-Ⅰ is significantly lessened compared with those from wild-type mice[57]. Finally, the induction of glycerol-3-phosphate dehydrogenase, a measure of adipocyte differentiation, is reduced in pre-adipocytes from transgenic mice by IGF-Ⅰ, but not insulin. In line with the lipogenic properties of IGF-Ⅰ, long-term IGF-Ⅰ treatment of patients with GH insensitivity syndrome results in increased adipose tissue[58]. Although it has been shown that *in vitro* GH treatment of 3T3-L1 pre-adipocyte cultures is associated with a concomitant increase in IGF-Ⅰ expression[57], the effects of GH on lipolysis are not mediated by IGF-Ⅰ, because there are no functional IGF-Ⅰ receptors in adipocytes[59]. Nevertheless, a direct and independent effect of GH-induced IGFBP-3 on adipocytes has also been reported[60]. These data indicate that IGF-Ⅰ has a crucial role in the proliferation of adipocyte precursors, the differentiation of pre-adipocytes, and the development of obesity in response to caloric excess[61]. However, in healthy individuals, IGF-Ⅰ levels are inversely related to the percentage of body fat[62], and epidemiological studies have demonstrated the relationship, in subjects without pituitary or cardiovascular diseases, between low IGF-Ⅰ and hypertension and type 2 diabetes[63], and cardiovascular risk[64].

Hepatic GH signaling is also essential to regulate intrahepatic lipid metabolism. In contrast with its effects on adipose tissue, GH induces triglyceride uptake in the liver by increasing LPL and hepatic lipase expression in a STAT-5 independent manner; however, the net effects of GH in intrahepatic lipid metabolism might be affected by GH antagonism of insulin signaling in the liver, or by GH-mediated secretion of IGF-Ⅰ[47]. Intrahepatic lipid accumulation and other histological liver markers characterize patients with NAFLD. NAFLD represents a spectrum of disease that ranges from simple steatosis to NASH, NAFLD-associated cirrhosis and end-stage liver disease. Ninety percent of circulating IGF-Ⅰ originates in the liver, and hepatocytes are the largest source of IGFBP-1 and IGFBP-3. Thus, both NASH and liver cirrhosis result in a progressive decline of hepatic IGF-Ⅰ output[65]. In this context, a possible link between hepatic steatosis, GH/IGF-Ⅰ axis, and inflammatory cytokines, probably *via* SOCS signaling, might be suggested as one of the mechanisms involved in the development and/or progression of metabolic syndrome and its cardiovascular and hepatic consequences[66-68].

Transforming growth factor (TGF)-β1, in addition to playing a certain role as a pro-fibrogenetic cytokine mainly in NAFLD[69], is an anti-proliferative and pro-apoptotic factor for mammary epithelial cells, in which it acts in an auto/paracrine manner and is thus considered an important local regulator of mammary tissue involution. A recent study has supported additional evidence that stimu-

lation of IGF- I is associated with complete abrogation of TGF-β1-induced activation of pro-apoptotic Bad and Bax and in the consequent protection against apoptosis. In conclusion, apoptotic effects of TGF-β1 are mediated by IGFBPs and occurs through IGF- I sequestration, which results in inhibition of the protein kinase B/Akt-dependent survival pathway[70].

Leptin, which has autocrine, paracrine and endocrine effects, is one of the most important substances secreted by fat cells[71]. Leptin controls peripheral fatty acid oxidation *via* PPAR-α stimulation, and plays a key metabolic regulatory role in fat tissue more than in muscle, liver and pancreatic β cells. Leptin levels correlate directly with the severity of hepatic steatosis but not with inflammation or fibrosis in NAFLD patients[72].

Adipocytes that increase lipogenic activity by sensing the fuel excess also secrete leptin to prevent cytosolic fat accumulation that would compromise functions of non-adipocytes[31]. Leptin limits the lipid accumulation by its autocrine effect in adipocytes, thus maintaining cellular fat balance[30]. Its fatty acid oxidative effects are also enhanced by upregulation of mRNA of uncoupling protein-2 (UCP-2) in adipocytes and in some non-adipocytes[73], such as muscle and β cells[74]. UCP leads to energy loss by converting the energy from the Kreb's cycle to thermogenic heat dissipation in the mitochondrial electron transport chain[75]. Recent studies have indicated that leptin is an independent risk factor for coronary artery disease[73]. Although leptin is highly correlated to the overall adipose tissue, leptin is associated with IR independently of fat mass, which suggests that hyperleptinemia is an independent component of the metabolic syndrome[76,77].

Although the exact interactions between insulin and leptin are still confusing, a putative leptin resistance, like IR, has been postulated in obesity[78], and a few data also suggest peripheral leptin resistance[79]. A soluble form of the soluble leptin receptor (sOb-R) has been demonstrated. sOb-R represents the main leptin-binding compound in plasma, which results in a fraction of bound and free leptin in plasma[80]. The exact function of the sOb-R is not clear. In obesity, levels of the sOb-R are decreased compared with lean controls, which resulted in an increased fraction of free leptin[81]. A reduction in body weight through diet or bariatric surgery significantly increases the concentration of circulating sOb-R, and therefore, increases the fraction of bound leptin[82].

Thus, sOb-R might act as a modulating factor of leptin action and plays an important role in leptin resistance. The high concentrations of free leptin are indicative of leptin resistance[83]. Recent findings suggest that the insulin-degrading capacity of morbidly obese patients is linked to venous leptin levels. Insulin controls leptin synthesis but leptin can, in turn, influence insulin cleavage and thus insulinemia. If insulin cleavage is to be interpreted as a way to decrease hyperinsulinemia, leptin could be a signal that limits the extent of insulin physiological actions. The fact that leptin is produced in the same insulin-degrading tissue (i.e. visceral adipose fat pad) supports this association[84]. IR and clustering of components of the metabolic syndrome decrease the concentration of the sOb-R and increase leptin levels in obese or overweight middle-aged men, which results in a decreased fraction of bound leptin, which further emphasizes the close relationship between the insulin and leptin axes. It is likely that low levels of sOb-R and a high concentration of free leptin are independent components of the metabolic syndrome.

Adiponectin is a novel adipose-specific molecule that possesses possible anti-atherogenic and anti-inflammatory properties. The plasma levels of adiponectin are lower in obese subjects and in patients with type 2 diabetes, which contributes to the development of atherosclerotic complications[85]. It has been demonstrated that secretion of adiponectin from adipocytes is stimulated by insulin[86]. Furthermore, ADD1/SREBP-1c has been recognized as being responsible for the control of adiponectin at the transcriptional level[87].

In NAFLD patients, adiponectin and adiponectin receptor Ⅱ (AdipoRⅡ) staining is less evident in biopsies from those suffering from NASH than FL. Hepatic expression of adiponectin and AdipoRⅡ is reduced in NAFLD[88]. Patients with NASH have significantly lower levels of serum adiponectin than do controls. Although no significant correlation exists between serum adiponectin and anthropometric data, it is independently associated with age, HDL, and triglycerides. Type of meal has no effect on serum adiponectin either in patients with NASH or in controls. There is no expression of adiponectin mRNA in liver samples. However, AdipoRⅡ mRNA expression is higher in NASH than in FL and normal liver tissue[89]. Obesity, particularly visceral adiposity, might also contribute to IR by altering the levels of key adipocyte-derived circulating proteins, referred to as adipokines, including adiponectin and resistin. Adiponectin is produced by adipocytes, and its levels are generally lower in patients with IR and the metabolic syndrome[90]. A role in the regulation of insulin sensitivity and glucose homeostasis was demonstrated in studies that have shown that recombinant adiponectin lowers glucose in diabetic rodents and enhances insulin action in hepatocytes[91]. Exogenous adiponectin, or transgenic adiponectin overexpression, also can reduce lipid accumulation in muscle and liver and enhance insulin sensitivity in mice. Importantly, adiponectin-deficient mice display delayed FFA clearance and are sensitive to diet-induced IR[72]. The mechanisms that mediate the beneficial effects of this protein might involve the activation of AMP-activated protein kinase in muscle or liver; adiponectin signaling *via* this pathway might involve two recently identified, distinct receptors[91].

Administration of exogenous resistin to rodents increases plasma glucose and hepatic glucose production, whereas resistin-null mice have reduced fasting glucose levels[92]. Resistin is reckoned as an adipocyte-specific secretory factor that can cause IR and decrease adipocyte differentiation. Conversely, based on various studies, IGFs can improve IR and stimulate adipocyte adipogenesis. Whether IGFs exert their effects by controlling resistin

production or modulating resistin action is not known. These data demonstrate that IGF- I downregulates resistin gene expression *via* IGF-1R-dependent and MEK1-, p38 MAPK-, and phosphoinositide 3-kinase-independent pathways, and probably modifies the distribution of resistin protein between the intracellular and extracellular compartments *via* a p38 MAPK-dependent pathway. Decreases in resistin production and secretion induced by IGF- I might be related to the mechanism by which IGF- I modulates body weight and diabetes in animals[93].

Adipose tissue was once thought of as a reservoir for surplus energy, but more recently, it has been recognized as an active endocrine organ that contributes to metabolic homeostasis by secreting several adipokines such as leptin, adiponectin, TNF-α, IL-6, PAI-1 and resistin. Initially, resistin was reported as an adipose-tissue-specific protein[40] but ensuing studies *in vitro* and *in vivo* have shown conflicting data regarding the expression of resistin in relation to IR or obesity[94]. Moreover, a longitudinal analysis has shown that serum resistin is higher in obese than in lean subjects, and that changes in serum resistin are positively correlated with changes in body mass index (BMI), fat mass, plasma glucose and insulin levels after a weight reduction program entailing dieting and exercise[95]. In normal control rats, *in vivo* insulin infusion and *ex vivo* administration of TNF-α to cultured fat pads increases resistin gene expression significantly. These results imply that hyperinsulinemia and increased TNF-α levels might upregulate the adipose resistin gene in bile-duct-ligation-induced liver cirrhosis[96].

CARDIOVASCULAR RISK

Obesity is considered to be a major contributor to overall and cardiovascular morbidity and mortality[97]. Epidemiological studies have demonstrated that the incidence and prevalence of obesity are increasing. Metabolic syndrome, which comprises IR, visceral obesity, hypertension, dyslipidemia, and microalbuminuria, is considered a major risk factor for atherosclerosis in obesity[98,99]. Fat accumulation in the visceral depot and liver are strongly correlated, and both are highly correlated with the development and severity of IR[100].

Given that CRP level is a strong predictor of cardiovascular events in men[101], the mechanisms that underlie elevated CRP levels among unhealthy subjects are important. CRP is the main acute phase protein and is a marker of systemic inflammation. Adipose tissue secretes pro-inflammatory cytokines, such as IL-6 and TNF-α. The synthesis of CRP, mostly under the control of IL-6[102] and TNF-α, can stimulate the production of CRP[103]. About 30% of total circulating levels of IL-6 originate from adipose tissue in healthy Caucasian subjects[104]. Adipose tissue is an important factor in the increased CRP levels, *via* IL-6.

VISCERAL ADIPOSITY

Given that omental adipose tissue is a pure depot of visceral adipose tissue, it is of interest to investigate the regulation of lipid metabolism in human omental tissue *in vivo*. It has been proposed that IR of the liver derives from a relative increase in the delivery of FFA from the omental fat depot to the liver (*via* the portal vein). Increased delivery results from: (1) more stored lipid in the omental depot; (2) severe IR of the central fat depot; and (3) possible regulation of visceral lipolysis by the central nervous system. The significance of portal FFA delivery results from the importance of FFAs in the control of liver glucose production. Insulin regulates liver glucose output primarily *via* control of adipocyte lipolysis. Thus, because FFAs regulate the liver, it is expected that visceral adiposity will enhance delivery of FFAs to the liver and make the liver relatively insulin resistant. It is of interest how the intact organism compensates for IR secondary to visceral fat deposition. Although part of the compensation is enhanced B-cell sensitivity to glucose, an equally important component is reduced liver insulin clearance, which allows for a greater fraction of β-cell insulin secretion to bypass liver degradation, to enter the systemic circulation, and to result in hyperinsulinemic compensation. The signals that result in β-cell upregulation and reduced liver insulin clearance with visceral adiposity are unknown, but it appears that the glucagon-like peptide hormone plays an important role[105].

In patients who have undergone abdominal surgery, two specific adipokine concentrations have been measured in venous blood from the omentum to obtain information on some processes of synthesis in the presence of abdominal obesity. Although vascular endothelial growth factor (VEGF) and IL-6 concentrations are increased in the systemic circulation, the contribution of visceral adipose tissue to circulating levels of VEGF and IL-6 is modest[106]. In contrast, in a recent study on rat tissues, the omentum has been found to have the greatest VEGF concentrations of those examined and the highest VEGF secretion rate. Fractionation studies of the omentum furthermore have demonstrated that omental adipocytes, rather than the stromal-vascular cells, are the primary source of VEGF. An endothelial cell mitogenic assay has showed that a major portion of the mitogenic activity of heparin-binding proteins and conditioned media derived from omentum is abolished by VEGF antibody. Additional studies with the transcription inhibitor actinomycin D have demonstrated that the VEGF gene is continuously transcribed in the rat omental adipocytes. Incubation of the omental adipocytes under hypoxic conditions has induced approximately a 1.7-fold increase in VEGF protein expression, which is abolished by actinomycin D[107]. However, what is the importance of VEGF? Liver regeneration is dependent upon coordinated proliferation of hepatocytes and endothelial cells. VEGF promotes angiogenesis. Hepatic steatosis increases liver resection morbidity and delays regeneration. As a counter-reacting mechanism, serum VEGF concentration increases in more severe forms of NAFLD. Some researchers have hypothesized that VEGF overexpression stimulates hepatic regeneration[108].

Another debate has arisen over the so-called "portal hypothesis" that implicates increased lipolytic activity in visceral fat, and therefore, increased delivery of FFA to the liver, which ultimately leads to hepatic IR. The mechanism by which increased central adiposity causes hepatic IR has been clarified by research at the transcriptional level, by studying the expression of several genes that are involved in glucose and lipid metabolism in the fat-fed canine model. Northern blot analysis has revealed an increase in the ratio of visceral to subcutaneous mRNA expression of LPL and PPAR-γ. In addition, the ratio for SREBP-1 tends to be higher in fat-fed dogs, which suggests enhanced lipid accumulation in the visceral fat depot. The visceral to subcutaneous ratio of HSL increases significantly, which implies a higher rate of lipolysis in visceral adipose tissue despite hyperinsulinemia in obese dogs. Liver SREBP-1 expression is increased significantly, with a tendency for increased fatty-acid-binding protein expression. In addition, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase increases significantly, consistent with enhanced gluconeogenesis[109].

MITHOCONDRIAL INVOLVEMENT

ATP is crucial for maintaining cellular integrity, therefore, abnormal production might predispose to hepatocellular injury, and mitochondrial dysfunction could be the key mechanism. Estimates of energy metabolism are mainly based on basal metabolic rate (BMR). BMR involves measurement of subjects at rest, under thermo-neutral temperatures (i.e. no thermogenic stress), in a post-absorptive (not digesting food) and inactive state. The underlying machinery that fuels BMR is identical to that which fuels all the other sources of energy utilization, namely, oxidative phosphorylation. ATP is generated in mitochondria, and is subsequently hydrolyzed to ADP and phosphate to release energy for useful work. This process of electron transport during oxidative phosphorylation is the primary source of oxygen radical species. Total energy expenditure (TEE) is commonly predicted on the basis of patient weight, activity level, and degree of metabolic stress (metabolic demands). BMR accounts for about 70% of TEE; the remainder is provided by energy dissipated by metabolism of food (10% of TEE), and energy expended during physical activity (20% of TEE). Conditions that increase metabolic stress, such as infection, critical illness, or trauma, having inflammation in common, can increase BMR. BMR in obese patients is generally augmented, in contrast to common belief, and it is a strong body response to overfeeding, probably cytokine-mediated. BMR is generally measured by indirect calorimetry using a canopy system and single-frequency bio-impedance analysis. Increased energy expenditure, observed in morbidly obese patients with NAFLD as a consequence of a systemic, low-grade, inflammatory process, might explain progression from obesity to metabolic syndrome, independent of the presence of NAFLD. In this context, increased BMR might be indicative of metabolic syndrome, strictly linked to IL-6 levels[110]. Indeed, energy expenditure in obese patients is increased not only because the increased fat-free mass results in a rise in BMR, but also because of the higher energy cost of weight-bearing activities. NAFLD, which is characterized by mitochondrial dysfunction, can predispose to drug-induced hepatotoxicity that probably shares the same pathophysiological mechanism[111].

An up-to-date study has documented that hepatic mitochondrial dysfunction precedes the development of NAFLD and IR in Otsuka Long-Evans Tokushima fatty rats. This evidence suggests that progressive mitochondrial dysfunction contributes to the natural history of obesity-associated NAFLD[112].

INTRINSIC FACTORS LEADING TO HEPATIC STEATOSIS

Although we have previously focused on adipocyte biology and development of obesity, with an emphasis on IR, steatosis of the liver could be independently influenced by some aforementioned transcription factors. It is now clear that several members of the nuclear receptor superfamily are co-expressed by macrophages, lymphocytes and other cell types that are involved in the regulation of inflammatory and immune responses. Beyond PPAR-γ and SREBP-1c, nuclear liver X receptors[113] are members of this family that are known to be activated by lipid-derived endogenous (such as fatty acids, eicosanoids and cholesterol) and pharmacological ligands. Such transcription factors, as well as PPAR-γ co-activator 1α (PGC-1α)[114], farnesoid X receptor[115] and AMP-activated protein kinase[116], a key regulator of fatty acid oxidation in the liver, represent fundamental issues in the development of NAFLD and hepatic IR. In addition to peripheral IR and pancreatic β-cell dysfunction, it should be emphasized that type 2 diabetes mellitus is also characterized by aberrant hepatic gluconeogenesis. cAMP response element-binding protein (CREB), a key regulator of hepatic gluconeogenesis, mediates its actions through transcriptional induction of the nuclear hormone receptor PGC-1α. Recently, CREB-induced activation of the NR4A orphan nuclear receptor family, including the three highly homologous isotypes, NR4A1, NR4A2, and NR4A3, has been identified as a novel PGC-1α-independent mechanism for regulating hepatic gluconeogenesis.

CONCLUSION

It remains to be established whether IR is also a phenotypic expression and to what extend it has a genetic determinant. Although it is generally thought that organ fat deposition begins when visceral and subcutaneous abdominal adipose tissue stores are full, a recent study has not been able to confirm this. Given that IR is not related to fat deposition, it has been hypothesized that the chain of events does not presuppose that obesity is the cause of IR. This is supported by the clear association between inflammatory status (CRP level and spleen volume) and the hepatic score

at ultrasound[15]. Could the high fat liver content be the breaking point between benign and progressive obesity? This is the first intriguing question that could be answered by successive follow-up of the obese population. A possible confirmation of these findings is found in a study that suggests that the contribution of visceral fat to inflammation might not be completely accounted for by clinical measures of obesity (BMI and waist circumference)[117]. A second point to stress is whether weight control can slow down the progression of IR and the worsening of fat deposition in organs in obese patients and overweight subjects as soon as possible, such as in adolescence. Could a possible anti-inflammatory approach be used to cure metabolic syndrome and NAFLD? In fact, inflammatory mechanisms are fundamental to the progression of NAFLD towards higher-risk cirrhotic states. Finally, does hepatic IR follow peripheral IR[118]? In other words, are hepatocytes the last adipocytes? It is likely that they are[119].

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

Noninvasive investigations for non alcoholic fatty liver disease and liver fibrosis

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Abstract

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of diseases that have insulin resistance in common and are associated with metabolic conditions such as obesity, type 2 diabetes mellitus, and dyslipidemia. NAFLD ranges from simple liver steatosis, which follows a benign course, to nonalcoholic steatohepatitis (NASH), a more severe entity, with necroinflammation and fibrosis, which can progress to cryptogenic cirrhosis and end-stage liver disease. Liver biopsy remains the gold standard for evaluating the degree of hepatic necroinflammation and fibrosis; however, several noninvasive investigations, such as serum biomarkers, have been developed to establish the diagnosis and also to evaluate treatment response. These markers are currently neither available in all centers nor validated in extensive studies. Examples include high-sensitivity C reactive protein and plasma pentraxin 3, which are associated with extensive liver fibrosis in NASH. Interleukin-6 correlates with inflammation, and cytokeratin-18 represents a marker of hepatocyte apoptosis (prominent in NASH and absent in simple steatosis). Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH.

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Key words: Non-alcoholic fatty liver disease; Biomarkers; Necroinflammation; Liver fibrosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a group of conditions ranging from simple liver steatosis, usually asymptomatic, to nonalcoholic steatohepatitis (NASH), which is characterized by the presence of apoptosis/inflammation and fibrosis, and also by a progressive course, evolving to cryptogenic cirrhosis.

Non-alcoholic steatohepatitis (NASH) was first described in 1980 by Ludwig *et al*[1] in patients with abnormal liver tests and fatty infiltration accompanied by inflamma-

tory changes at histological examination. Prior to 1980, hepatic steatosis was documented in patients with associated metabolic conditions, especially in obese patients who underwent liver biopsy before and after bariatric surgery[2].

The prevalence of NAFLD has increased over the last two decades and it affects approximately 30% of adults in the United States[3] and almost a third of the general population[4]. The most common form of NAFLD encountered in clinical practice is liver steatosis, also known as non-alcoholic fatty liver (NAFL), if it occurs in the absence of significant alcohol consumption (more than 10 g/d in females and 20 g/d in men). Data analysis from two recent prospective cohort studies concluded that raised body mass index (BMI) and alcohol consumption are both related to liver disease, with evidence of a supra-additive interaction between the two[5]. The study by Liu *et al*[6] confirmed that excess body weight increases the incidence of liver cirrhosis. In middle aged women in the UK, an estimated 17% of incident or fatal liver cirrhosis is attributable to excess body weight, as compared with an estimated 42% attributable to alcohol[6].

Fatty liver disease has a benign clinical course, as long as inflammatory injury of the liver does not develop. It is essential to differentiate between this form of simple steatosis, which is associated with a favorable long-term prognosis, and NASH, with a different natural history; approximately 20% of patients with NASH will develop cryptogenic cirrhosis and even end-stage liver disease[7]. Cirrhosis associated with NASH might even progress to hepatocellular carcinoma[8] and death related to NASH was reported to be approximately 12%-25% over a 7-10 years period[9]. NAFLD is an important cause of cryptogenic cirrhosis, as Powell *et al*[7] suggested, although other disorders could progress to this type of cirrhosis[10]. Another important issue concerning NAFLD is related to the inner mechanisms of the disease. Even though steatosis is a benign condition and NASH a progressive one, the basic mechanisms of both entities seem to be the same. This is supported by the study of Tarantino *et al*[11] who reported similar levels of transforming growth factor-β1 in serum of patients with simple steatosis and those with NASH.

PATHOGENESIS OF NAFLD

The pathogenesis of NAFLD, and especially of NASH, is not completely understood; however, a few mechanisms were proposed to explain the liver injury associated with metabolic syndrome[12]. Identification of these mechanisms has therapeutic importance, because targeted therapies might prevent the progression of NAFLD to fibrosis and cirrhosis[13].

Insulin resistance plays a central role in NASH pathogenesis. Insulin resistance is the main feature of metabolic syndrome, which embodies obesity, hypertension, diabetes, and dyslipidemia[14]. NAFLD is considered to be the hepatic component of metabolic syndrome[15-17]. Although overweight and obesity are present in the majority of patients with NASH, steatohepatitis can also occur in subjects with normal body weight[18]. A direct correlation between the degree of obesity and NASH development has been observed[19]; however, not all obese patients will have NAFLD. Hepatic steatosis evidenced by ultrasound is clearly more pronounced in cases of insulin resistance compared with healthy subjects[20]. Insulin resistance promotes disturbances in lipid metabolism, with increased delivery of free fatty acids to the liver, impaired mitochondrial β oxidation, "*de novo*" lipogenesis, and decreased β export from the liver[19], all of which result in fatty liver development. Some authors suggest that hyperinsulinemia of NAFLD is the result of the decreased insulin extraction by the liver[21,22]. NASH is also associated with mitochondrial abnormalities, such as swollen or elongated mitochondria with crystalline inclusions[18,23]. Liver overloading with lipids initiates multiple pathways including lipid peroxidation, generation of reactive oxygen species, oxidative stress, and production of inflammatory cytokines. In fact, oxidative stress is a trigger for lipid peroxidation in hepatocytes, with subsequently secretion of proinflammatory cytokines and activation of fibrosis-developing stellate cells, which are the main mediators of liver fibrosis. tumor necrosis factor (TNF)-α is increased in NAFLD patients and it has a central role in liver injury and disease progression from fatty liver to steatohepatitis and hepatic fibrosis, by activating both Kupffer and stellate hepatic cells[12,24]. Taking into account this hypothesis, targeted therapies against TNF-α might be beneficial in NASH treatment[25,26].

Another concept involves adipocytokines, which are secreted by the adipose tissue (WAT) known as white adipose tissue and are related to visceral obesity. WAT is responsible for secretion of a several adipokines and cytokines, such as adiponectin, leptin, TNF-α, and interleukin (IL)-6, which are involved in hepatic inflammatory process[27,28].

A theory concerning iatrogenic NAFLD induced by several medications has emerged. Drugs like diltiazem, amiodarone, tamoxifen, steroids, and antiretrovirals are involved in fatty liver or insulin resistance development[18].

Besides the well-recognized risk factors for NAFLD, such as type 2 diabetes, insulin resistance, hyperlipemia, and obesity, other metabolic conditions have been associated with fatty liver disease, namely polycystic ovary syndrome, and lipodystrophy[29,30]. Other rare conditions associated with NAFLD are hypobetalipoproteinemia, Weber-Christian syndrome, total parenteral nutrition, toxic exposure at organic solvents, dimethylformamide, gastric by-pass, and jejunoileal bypass[18].

DIAGNOSTIC PROCEDURES

The goal of diagnostic procedures is to identify the patients with NASH before the onset of advanced fibrosis. Liver biopsy is now considered the "gold standard" for the assessment of liver fibrosis. It is important to take into account that a needle biopsy is merely a sample of the entire liver and that fibrosis presents a diffuse pattern in chronic liver disease. The liver biopsy removes only about 1/50 000th of the liver and carries substantial interpretation errors. Liver biopsy is an invasive procedure with certain unavoidable risks and complications[3].

Therefore, the development of noninvasive tests for assessing hepatic inflammation and fibrosis has become an active area of research.

NAFLD is first of all a diagnosis of exclusion, so other

specific causes of liver diseases should be ruled out: viral hepatitis, alcoholic liver disease, Wilson disease, hemochromatosis, and autoimmune hepatitis[15]. The most challenging of them is exclusion of alcoholic liver disease, because the histological picture of both conditions is similar. It is necessary to obtain an accurate history concerning the patient's daily alcohol intake; knowing that consumption of more than 10 g/d in females and 20 g/d in males are responsible for liver injury in the absence of other risk factors, such as obesity, diabetes, and viral hepatitis[30].

The clinical presentation of patients with either NAFLD or NASH is proteiform. The majority of subjects are asymptomatic, but some of them can present with fatigue or right upper quadrant discomfort. Hepatomegaly is discovered in 50% of patients at physical examination[31]. The presence of fatigue does not correlate with the severity of liver injury[31]. These patients share a common clinical feature, obesity, and potentially other features of metabolic syndrome: hyperglycemia, dyslipidemia, and hypertension. Liver dysfunction might be discovered incidentally during a routine check-up, or a work-up for other conditions (Table 1).

Table 1 Clinical features of nonalcoholic fatty liver disease

Symptoms	Signs
None	Hepatomegaly (50% of patients)
Fatigue	Obesity
Right upper quadrant discomfort	Hypertension

Laboratory tests

Approximately 80% of patients with NAFLD have liver function tests in normal ranges; only a small proportion exhibits mild elevation of aminotransferases[32]. The ratio between aspartate aminotransferase (AST) and alanine aminotransferase (ALT) is predictive for the severity of the liver disease, with an AST/ALT ratio > 1 suggesting cirrhosis or advanced fibrosis[33,34]. The degree of aminotransferases elevation is not higher than four times of the upper limit of normal and does not correlate with the severity of steatosis or fibrosis[18]. In the majority of cases, ALT/AST ratio is > 1.

Can serum aminotransferases levels distinguish between NASH and NAFLD? Higher AST and ALT levels, and AST/ALT ratio are all significantly associated with NASH. Serum AST presents a stronger association and has a higher likelihood of discriminating NASH from other forms of NAFLD. However, a multivariable model using both AST and ALT, showed that the discriminating score for distinguishing the patients with NASH from those without NASH was only 26%. This result indicates that additional noninvasive methods are needed for an accurate diagnosis. Fracanzani *et al*[35] studied 455 patients with NAFLD, divided in two groups according to their serum ALT levels. They compared clinical and histological features of patients with and without increased serum ALT. NASH was diagnosed in 62% and 74% of patients with normal or increased ALT levels, respectively. There were no significant differences in advanced fibrosis between the two groups, underlying the need for liver biopsy for diagnosis and staging of fibrosis in NASH[35].

Laboratory signs of advanced liver disease, such as hyperbilirubinemia, hypoalbuminemia, and abnormal prothrombin time are seen only in cases associated with cirrhosis. Other biological abnormalities, such as hyperglycemia and hypertriglyceridemia are related to the coexistent metabolic conditions.

Laboratory assessment of dyslipidemia and insulin resistance should also be performed. A simple laboratory test was designed to evaluate the insulin profile. It is known as homeostasis model assessment (HOMA), and is defined as the fasting insulin level (μU/mL) multiplied by the fasting glucose level (mmol/L) and divided by 22.5[20]. Although HOMA is not a perfect measure of insulin resistance, it is an easy way to estimate insulin resistance. A possible link between HOMA and hepatic steatosis has been demonstrated[20].

The major problem remains to determine the consequences of small amounts of alcohol intake in a patient with liver disease. Taking into account the difficulty of distinguishing between alcoholic and nonalcoholic liver disease based on patient's history, many attempts have been made to assess alcohol consumption using serum markers. Over time, several surrogate markers for alcoholism have been determined: high serum concentration of γ-glutamyltransferase, increased mean corpuscular volume, increased AST levels, AST/ALT ratio > 2, and a desialylated transferrin/total transferrin ratio > 1[18,36].

NAFLD is also accompanied by changes in serum iron markers, such levels of ferritin in 20%-50% of patients and increased transferrin saturation in 5%-10% of cases[33]. Hemochromatosis gene testing is recommended if the ferritin level is significantly elevated.

Overall, none of these tests have specificity for the diagnosis of NAFLD, pointing out only a liver dysfunction. The pattern of aminotransferase elevation does not provide an etiological clue for the hepatic disease, nor does it make a distinction between simple fatty liver and NASH[18].

In fact, the differentiation between steatosis and steatohepatitis can be made only by a histological approach[33]. Besides, the amount of lipid accumulated in the liver cannot be assessed using functional liver tests; however, the degree of liver infiltration with fat can be diagnosed using a variety of imaging methods.

Imaging studies

The most common and less invasive imaging technique used for NAFLD diagnosis is ultrasonography. Ultrasonography, the first-line imaging technique, assesses the presence of steatosis, showing a hyperechogenic liver parenchyma, known as "bright liver" and "blurring of the vascular margins". The increased hepatic echogenicity is diffuse and easy to appreciate by comparison with the lower echogenicity of the kidney or spleen.

The hepato-renal contrast is an ultrasound index for quantification the liver steatosis[37,38]. Normal liver exhibits an echostructure similar to that of renal parenchyma. In fatty liver, the increased hepatic echogenicity creates hepato-renal contrast. Webb *et al*[38] assessed the severity of liver steatosis in a study of 93 patients with positive histol-

ogy for chronic liver disease, according to the discrepancy in ultrasonographic liver-kidney densities. They reported that the hepato-renal index could quantify the severity of liver steatosis to a lower limit of 5%.

A simple parameter, noninvasive and easy to perform, is spleen longitudinal diameter. As the study of Tarantino *et al*[17] recently showed, spleen diameter could differentiate between NAFLD and NASH better than both IL-6 and vascular endothelial growth factor, with values greater than 116 mm predicting NASH[17].

Another technique that might be helpful in the diagnosis of steatosis is Doppler ultrasound. NAFLD is associated with hepatic parenchyma perfusion abnormalities. Several parameters have been described that reflect altered hepatic hemodynamics, among them, the most important is the hepatic vein Doppler pattern[3]. Recently, a new parameter was used in NAFLD evaluation: Doppler perfusion index (DPI), a ratio between hepatic arterial blood flow and total liver blood flow. DPI has been used in the detection of overt liver metastatic disease[39]. In a small trial, Dugoni *et al*[40] reported that DPI was highly predictive of fatty liver in patients with NAFLD. Larger studies are required to evaluate the role of DPI in the diagnosis of NAFLD.

The sensitivity of ultrasonography in detecting steatosis varies between 60% and 94%[18], and also varies depending on steatosis degree. Sensitivity is very low when the degree of steatosis is less than 30%[41]. Another difficulty consists of the impossibility of identifying the inflammatory changes of the hepatic parenchyma and to differentiate simple steatosis from steatohepatitis. It is also difficult to differentiate steatosis from liver fibrosis, because both of them have similar appearance on ultrasound[18]. This limitation was overcome by a superior technology, contrast-enhanced ultrasonography. Lim *et al*[42] studied the role of hepatic vein transit times (HVTT) using a microbubble contrast agent as a tracer and reported that HVTT can predict disease severity in patients with hepatitis C. Moreover, Iijima *et al*[43] evaluated the utility of contrast ultrasound with levovist for the diagnosis of NASH. The signal intensity from regions of interest on the contrast images was measured and estimated using time intensity curves. They found a statistically significant decrease of signal intensity in NASH, when compared with NAFLD, due to reduced uptake of levovist mediated by cell injury. Because this method has only been applied in small trials, larger studies are needed to establishing the role of contrast ultrasonography in the diagnosis of NASH in clinical practice.

The sensitivity of ultrasonography decreases in morbid obesity, because the ultrasonographic examination is difficult to perform in such circumstances[3]. Ultrasonography is inexpensive, simple, easily reproducible, and can be used repetitively to assess steatosis changes over the time, in conjunction with ALT fluctuations and BMI variation. The specificity of the method in detecting fatty infiltration of the liver is high, around 90%[18] (Table 2).

Computed tomography and magnetic resonance imaging are other alternatives, but their use is limited because they are expensive and the information they provide is limited. Compared with ultrasound, CT scans and MRI are superior when the fat deposition is focal[18], otherwise, abdominal ultrasound is more sensitive in diagnosing fatty liver disease[18,44,45] (Table 2).

Table 2 Noninvasive diagnosis of non-alcoholic fatty liver disease (adapted from Lewis *et al*[45]) (%)

Imaging	Sensitivity	Specificity	PPV	NPV
Ultrasound	91-100	93-100	62-89	94
Ultrasound (levovist)	100	95-100	N/A	N/A
Ultrasound (elasticity)	91	84	47	97
CT	93	N/A	76	N/A
MRI	N/A	N/A	N/A	N/A
MR (spectroscopy)	N/A	N/A	N/A	N/A
MR (elastography)	85	86	73	94

N/A: Not available; PPV: Positive predictive value; NPV: Negative predictive value; MRI: Magnetic resonance imaging; MR: Magnetic resonance; CT: Computed tomography.

CT scan technology can be also used to evaluate thickened abdominal subcutaneous adipose tissue and to measure the liver fat[46]. Non enhanced CT can identify steatosis using changes in signal intensity. The density of the liver, as visualized by CT, decreases as the severity of steatosis increases. CT can also visualize splenomegaly in the presence of portal hypertension, which is suggestive for advanced fibrosis in patients with NAFLD. CT allows grading of steatosis, by calculating the liver-to-spleen attenuation ratio[47]. Noncontrast CT is preferred for detecting steatosis because the images appear enhanced[48]. Focal fatty lesions can be identified by dual-energy CT scans. The limitations of CT consist of the difficulty to identify intermediate stages of fibrosis and its use in follow-up purposes, owing to the radiation exposure.

Magnetic resonance imaging (MRI) provides an accurate and rapid assessment of hepatic steatosis to a lower limit of 3%[49]. Phase-contrast imaging correlates with the quantitative assessment of fatty infiltration across the entire range of liver diseases. Loss of intensity on T1-weighted images can be useful in identifying focal fat deposition[49].

A new MRI technique, proton magnetic resonance spectroscopy (MRS), measures the fat proton fraction and hepatic triglyceride levels (HTGC). HTGC > 5% is the diagnostic level of hepatic steatosis[50,51]. MRS characterizes metabolic processes involved in cellular regeneration, and thus it can evaluate the disease severity in NASH. An increased ATP/phosphate ratio might be a signal for progression to an advanced stage of fibrosis in NASH. MRS is probably more accurate than previous imaging procedures for the diagnosis of NAFLD but it needs *in vivo* human validation.

Multi-echo magnetic resonance (MR) imaging, acquired at in-phase and out-of-phase echo times, allows simultaneous fat content and T2 quantification. This technique could be used to determine the fat-to-water ratio and the T2 values[52].

None of these imaging techniques is able to distinguish between liver steatosis and steatohepatitis; thus liver biopsy is required for definitive assessment of the hepatic disorder[3].

Unfortunately, the new imaging procedures, magnetic resonance spectroscopy, and contrast enhanced ultrasound cannot, as yet, be used routinely.

Table 3 Serological markers for nonalcoholic steatohepatitis and fibrosis

Serological markers	Advantage	Disadvantage
C reactive protein[53]	Independent risk factor for progression of NAFLD	Lack of specificity for NASH
Plasma pentraxin 3[54]	Can differentiate between NASH and non progressive NAFLD Fibrosis marker in NAFLD	Lack of specificity for NASH
Hyaluronic acid[34]	Identify fibrosis at a cut-off value of 45 ng/mL	Cannot differentiate NASH from simple steatosis
Tissue inhibitor of metalloproteinases[34]	Fibrosis marker	Cannot differentiate NASH from simple steatosis
Cytokeratin 18[3]	Marker of hepatocyte apoptosis Independent predictor of NASH and severity of disease	Limited utility in clinical practice
Polypeptide specific antigen[57]	Marker in differentiating NASH from pure fatty liver	Marker for various cancers
Endothelin 1[59]	Can differentiate NASH from simple steatosis	Lack of specificity for NASH

NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

BIOMARKERS FOR ASSESSMENT OF STEATOHEPATITIS AND FIBROSIS

Over time, several biological markers have been studied for evaluating the extent of steatosis, the presence of necroinflammation, and the development of fibrosis to avoid performing liver biopsy, an invasive procedure that still represents the gold standard of diagnosis. The most important parameter to be identified through non-invasive methods is inflammation, as it plays a central role in NAFLD progression.

Several biomarkers of inflammation were extensively studied in relation to fatty liver disease. The C reactive protein (CRP) is an acute-phase reactant produced by the liver and has an increased serum concentration in a variety of inflammatory conditions. The assessment of plasma levels of CRP proved to be useful in differentiating between simple steatosis and NASH. Moreover, it seems that high concentrations of high-sensitivity CRP are associated with extensive liver fibrosis in NASH[53].

Plasma pentraxin 3 (PTX3) is a novel marker that seems to be promising in distinguishing between NASH and non-NASH patients, and also in assessing the severity of fibrosis[54]. Plasma pentraxin 3 is an acute-phase reactant and together with CRP is a member of the pentraxin family of proteins[54]. The PTX 3 level is increased in NASH, but also in other diseases, such as vasculites, cardiovascular, and inflammatory conditions[54].

Another biomarker with a significant role in the fatty liver is IL-6. IL-6 is a chemokine that rises in NAFLD, and it is synthesized by hepatocytes and by immune cells, endothelial cells, and adipocytes[12,55]. Plasma levels of IL-6 vary in proportional with the hepatic concentration and indicate inflammatory activity and the degree of fibrosis[55].

TNF-α has long been recognized for its proinflammatory properties, and its role in NASH progression is clearly established, as well as in other inflammatory diseases. TNF-α is highly expressed in NASH and it has been shown that anti-TNF therapy with pentoxifylline is associated with improvement of liver histology and normalization of aminotransferases[56].

Cytokeratin 18 is a relatively new marker that derives from the caspase-3 pathway; however, to date, it has limited utility in clinical practice and is used only for research purposes. Cytokeratin-18 represents a marker of hepatocyte apoptosis, and its value as a potential biomarker for NASH is based on the observation that apoptosis is prominent in NASH and absent in simple steatosis[3].

Tarantino *et al*[57] found that the polypeptide specific antigen, a protein released during apoptosis, is an important marker of fibrosis, and is more accurate than ALT levels. Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH, because a significant decrease in serum concentration of this marker was associated with weight loss[58].

Oxidative stress has been documented to play a part in NASH pathogenesis, and several parameters have been assessed in different studies: glutathione peroxidase activity, superoxide dismutase activity, and vitamin E levels[3]. None of these markers seemed to have a significant value in evaluating the histological picture of NASH[3]. The clinical usefulness of these biomarkers is yet not established, and their accuracy in noninvasive assessment of steatohepatitis is under debate.

Fibrosis assessment is crucial in NASH because it represents an advanced stage of liver injury. Several studies evaluated certain matrix components, such as transforming growth factor β, hyaluronic acid, tissue inhibitors of metalloproteinases, and others[33]; however, none of them have entered routine use. Endothelin-1 is another mediator of fibrosis in NASH, with an established correlation between serum levels and the degree of fibrosis[59].

Serological markers for NASH and fibrosis are shown in Table 3.

DIAGNOSTIC PANELS FOR ASSESSMENT OF STEATOSIS, STEATOHEPATITIS AND FIBROSIS

Noninvasive panels of serological markers have been developed to evaluate the presence of steatosis and hepatic necroinflammation to avoid liver biopsy. Avoiding liver biopsy is desirable because it has certain disadvantages: it is an invasive procedure, is prone to sampling errors, and suffers from inter-observer variability[60]. The NASH-test imagined by BioPredictive was validated for the assessment of

steatohepatitis in patients without significant alcohol consumption and takes into account the following parameters: total bilirubin, GGT, α2-macroglobulin, apolipoprotein A1, haptoglobin and ALT, and is adjusted for age and gender plus[61] weight, height, AST, serum glucose, triglycerides, cholesterol and SteatoTest. The NASH test should be performed only if the SteatoTest is positive. The SteatoTest is a quantitative test that estimates liver steatosis, particularly in cases of associated metabolic syndrome[62]. The NASH test is a variation of the SteatoTest-ActiTest for the differentiation of steatosis from NASH. The Acti Test was designed for staging necroinflammation in viral hepatitis C and B[61]. Performing these biomarker tests should reduce the need for liver biopsy[63].

Serological markers for fibrosis assessment are frequently used in Europe, in contrast with the United States where liver biopsy is preferred. Different tests have been used for evaluating fibrosis, such as the AST/ALT ratio and the APRI test, which assesses platelets and AST levels[64]. At the moment, the most commonly used are the FibroTest (BioPredictive) in Europe, and FibroSpect and FibroSure in the United States[64]. FibroTest was first developed for patients with viral hepatitis C, and was then extended for NAFLD[33]. The advantages over liver biopsy are: entire examination of the liver and lack of risks due to the noninvasive procedure. FibroSpect evaluates liver fibrosis by analyzing the following markers: hyaluronic acid, tissue-inhibited matrix metalloproteinase inhibitor-1, and α-2 macroglobulin[64]. FibroTest takes into account GGT, haptoglobin, bilirubin, apolipoprotein A, and α-2-macroglobulin. The most important deficiency of these types of tests is their inability to distinguish between mild and moderate fibrosis, knowing that early detection of fibrosis is valuable for preventing disease progression[64]. The utility of these tests is limited in cases with advanced fibrosis.

Fibroscan

Fibroscan, or transient elastography, is a noninvasive method that evaluates liver stiffness using pulse-echo ultrasound[33,64]. Transient elastography measures liver stiffness in a painless and reproducible manner. It has several advantages over liver biopsy: it is noninvasive, evaluates a larger part of the liver, and seems to be more sensitive than serological markers[64]. The main weakness of Fibroscan is interference by steatosis with the wave velocity, as liver stiffness due to fibrosis might be counterbalanced by the presence of fatty infiltration[33]. Some authors state a positive correlation between liver stiffness assessed by Fibroscan and the degree of fibrosis in NAFLD[65,66]. When liver elasticity is used for fibrosis measurement in NAFLD, it is important to take into account that fatty liver can make the liver less stiff and therefore the reference ranges might be different. Fibroscan might also be unreliable in obese people because of technical reasons[67].

Acoustic radiation force impulse (ARFI) sonoelastography has recently been proposed as an alternative method to Fibroscan to assess liver elasticity. This alternative technique utilizes acoustic waves to interrogate the mechanical stiffness properties of the liver. One advantage of ARFI imaging is that it is integrated into a conventional ultrasonography (US) system and can thus be performed during standard US examinations of the liver, which are routinely performed in patients with chronic liver disease. Preliminary results indicate that ARFI imaging technology can be applied for the diagnosis of significant liver fibrosis[68,69]. The role of ARFI elastography for the diagnosis of NAFLD has not yet been established.

Another technique used for detecting moderate to severe hepatic fibrosis in obese individuals with NAFLD is magnetic resonance elastography. It has a higher diagnostic accuracy in fibrosis staging that is not related to BMI[70]. Further studies are needed to clearly define the role of liver elastography in patients with fatty liver disease.

Total overnight salivary caffeine assessment test

An interesting idea concerning the assessment of liver function in chronic liver diseases was elaborated by a working group conducted by Tarantino *et al*[71]. Systemic caffeine clearance, evidenced by measuring salivary caffeine concentration can be used as a hepatic function test in compensated cirrhosis. The total overnight salivary caffeine assessment is a reliable test for evaluating liver function and it can also differentiate between cirrhosis type, such as viral and cryptogenic (likely metabolic) cirrhosis.

Dynamic breath tests

Dynamic breath tests can detect specific alterations in different metabolic pathways. Braun *et al*[72] combined two tests to assess the extent of hepatic injury in patients with NAFLD: the ^{13}C-methacetin breath test (MBT) and the ^{13}C-octanoate breath test (OBT), which evaluate cytochrome P450 activity and mitochondrial dysfunction. Both mechanisms increase oxidative stress, which is clearly implicated in NASH pathogenesis. The noninvasive OBT reliably distinguish between fatty liver and NASH, and the MBT can predict the extent of liver fibrosis.

Additional studies are required to establish the role of these tests as an alternative to liver biopsy in the diagnosis and follow-up of hepatic injury in patients with NAFLD.

CONCLUSION

Currently, the standard procedure for evaluating the degree of necroinflammation and fibrosis, and for quantifying hepatic steatosis remains liver biopsy. However, this is an invasive procedure with unavoidable risks and limitations. Moreover, in most cases of NAFLD, the results of liver biopsy are not relevant to the choice of treatment, which remains that of metabolic syndrome. Hence the need for noninvasive strategies to cover the whole spectrum of NAFLD. Noninvasive investigations, such as various biomarkers, fibrosis scoring panels, and imaging techniques, offer considerable promise in their ability to detect steatosis and to stage liver fibrosis. Further testing and validation are needed for these noninvasive procedures to refine their role of clinical practice and supplant the need for liver biopsy in NAFLD.

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REVIEW

Current developments in natural orifices transluminal endoscopic surgery: An evidence-based review

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Abstract

Tremendous advances have been made in recent years addressing the key obstacles to safe performance and introduction of human natural orifice transluminal endoscopic surgery (NOTES). Animal studies have focused on identifying optimal solutions to these obstacles, in particular methods of creating transluminal access, safe closure of the point of access, and development of a multitasking platform with dedicated instruments. Whether the performance data generated from these animal studies can be reproduced in humans has yet to be determined. Reports of human NOTES procedures are emerging, and the possibility of accomplishing human NOTES based on existing technology has been demonstrated. However, dedicated platforms and devices are still lacking to allow for pure NOTES procedures, and whether NOTES can deliver the postulated benefits of earlier recovery and improved cosmesis remains uncertain.

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Key words: Natural orifice transluminal endoscopic surgery; Endoscopic surgery; Minimally invasive surgery; Vaginal surgery

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INTRODUCTION

The natural orifice transluminal endoscopic surgery (NOTES) white paper released in 2005 stated that a number of key issues had to be overcome before NOTES could be fully implemented in human subjects[1] (Table 1). Since then, there has been an exponential growth in the number of NOTES-related publications in the literature, from less than 10 articles in 2006 to over 180 in 2009 (through a PubMed search). NOTES has been demonstrated to be a feasible approach for the performance a wide variety of procedures in animal studies, and reports of human studies are emerging[2-16]. This paper aims to provide an evidence-based review of the current developments in NOTES, with particular emphasis on recent advances in tackling the key obstacles, and to provide an update on the latest development in human NOTES procedures.

METHODS

Search strategy

Studies were identified by performing electronic searches of MEDLINE, EMBASE, Current Contents, the Cochrane Library, and Entrez PubMed from January 2000 to January 2010. The search terms "natural orifice" or "transluminal or translumenal" and "endoscopy or endoscopic surgery or surgery" were used. Additional articles were identified by a manual search of the references in key articles. Amongst the identified studies, articles in English describing randomized controlled trials (RCTs) were considered first. In areas with limited or no RCTs, nonrandomized comparative studies and case series were also included. Only fully peer-reviewed articles were selected.

Inclusion and exclusion criteria

Only studies reporting the outcomes of NOTES-related procedures or devices were selected. Case reports were excluded except in human studies, where only a limited selection of studies was available.

ANIMAL STUDIES

Methods of access to the thoracic or peritoneal cavity

The thoracic and peritoneal cavity can be accessed by the transluminal approach and the method of transluminal access represents the first barrier to NOTES. In mediastinal/thoracic NOTES, the only site of access is through the thoracic esophagus. While for the abdominal cavity, NOTES accesses can be made *via* the transgastric, transcolonic, transvaginal, or transvesical approach.

Note that the thoracic esophagus is surrounded by a number of critical structures, including the descending thoracic aorta, the azygous vein, the pulmonary veins, and the heart. Locating a point of safe access is of paramount importance to avoid catastrophic vascular complications. Endoscopic ultrasound (EUS) has been shown to be a valuable tool for locating sites of safe accesses[17-19]. In the mediastinum, EUS can help identify landmarks such as the aortic arch, which facilitates optimal entrance sites for forward-viewing exploration and intervention[17].

With the site of entry located, one then needs to consider the method of creating a transluminal incision. In early transgastric NOTES procedures, published by Kalloo *et al*[20] and other authors between 2004 and 2005, the majority of transgastric gastrotomies were created by a needle-knife, followed by progressive enlargement of the incision using a pull-type sphincterotome or dilating balloon[20-24]. Both methods are effective ways of creating a transgastric gastrotomy. Nevertheless, using the sphincterotome is quicker than balloon dilation, and it also prevents spontaneous closure of the gastrotomy[22]. Thus, the sphincterotome method is more advantageous if repeated gastric crossing is required. In an attempt to further improve the ease of creating direct transgastric accesses, a prototype one-step needle sphincterotome has been developed by the authors' unit (Figure 1)[25]. The instrument consists of a retractable needle knife and a pull-type sphincterotome on the same instrumental shaft, which allows extension of the gastrotomy incision created by the needle knife without the need of changing instruments. It has been shown to allow significantly quicker creation of a gastrotomy than the balloon dilation method without increasing the risk of complications.

Though direct incision on the gut wall is a simple method of creating transluminal accesses, concerns regarding peritoneal contamination and the difficulty in closure of the opening have prompted the development of a submucosal tunneling technique[26-30]. This technique is the preferred method of transluminal access in mediastinal NOTES and it is also feasible in transgastric procedures[30]. In brief, it involves an initial mucosal incision, followed by creation of a submucosal tunnel using either high-pressure carbon dioxide inflation and balloon dilation, or by submucosal dissection, in a manner similar to endoscopic submucosal dissection. The length of the tunnel is reported to be between 5 and 10 cm. At the end of the tunnel, a myotomy is made to gain access into the peritoneal cavity or the mediastinum. This method was shown to be least susceptible to immediate leakage after

Table 1 Summary of human natural orifice transluminal endoscopic surgery procedures

Authors	Type of procedure	No. of patients	Organ of access
Marescaux *et al*[2] (2007)	Cholecystectomy	1	Transvaginal
Zorrón *et al*[3] (2007)	Cholecystectomy	1	Transvaginal
Gettman *et al*[4] (2007)	Peritonoscopy	1	Transvesical
Hazey *et al*[5] (2008)	Peritonoscopy	10	Transgastric
Lacy *et al*[6] (2008)	Sigmoidectomy	1	Transvaginal
Ramos *et al*[7] (2008)	Sleeve gastrectomy	1	Transvaginal
Zorrón *et al*[8] (2008)	Peritonoscopy	1	Transvaginal
Zornig *et al*[9] (2009)	Cholecystectomy	68	Transvaginal
Decarli *et al*[10] (2009)	Cholecystectomy	12	Transvaginal
Gumbs *et al*[11] (2009)	Cholecystectomy	4	Transvaginal
Auyang *et al*[12] (2009)	Cholecystectomy	1	Transgastric
Horgan *et al*[13] (2009)	Cholecystectomy	1	Transvaginal
Kaouk *et al*[14] (2009)	Nephrectomy	1	Transvaginal
Fischer *et al*[15] (2009)	Sleeve gastrectomy	1	Transvaginal
Lacy *et al*[16] (2009)	Sleeve gastrectomy	1	Transvaginal

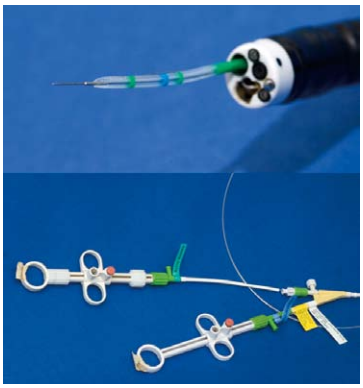

Figure 1 The one-step needle sphincterotome.

closure, with a leak pressure rivaling hand-sewn sutures in study involving 34 *ex vivo* porcine stomachs[31]. However, in one study, partial necrosis of the overlying mucosa was observed in up to four out of eight surviving swine (50%), and one swine suffered from severe peritonitis[27]. The cause of necrosis might be due to the high-pressure CO_2 bursts used for dissection, leading to impairment of blood supply. Other groups using the same technique without the device did not report such a complication[28,29]. Pauli *et al*[29] also reported that submucosal tunneling might increase the ease of in-line endoscope positioning to predetermined abdominal positions.

On the other hand, a number of hollow viscera are available for making accesses to the abdominal cavity. These include the stomach, colon, vagina, and the urinary bladder[2,20,32,33]. To avoid the limited maneuverability of a retroflexed endoscope, one will need to consider the access organ and the effect on the in-line position of the endoscope. Theoretically, the transgastric approach should facilitate in-line positioning of the endoscope to pelvic organs, while the transrectal, transvaginal, or transvesical routes provide good forward views of the upper abdominal structures. In an *in vivo* study by Voermans *et al*[34] involving 12 swine, transgastric peritoneoscopy was found to be inferior to laparoscopy in detecting simulated peritoneal metastasis, in particular for those located in the liver. In another study, they also found that both transgastric and transcolonic routes provided similar degrees of visualization and access efficacy to the liver and the peritoneal cavity[35]. More studies are required to determine the best access route for performing a particular abdominal NOTES procedure, and it is likely that the preference is governed by the nature of the procedures.

GASTRIC (LUMENAL) CLOSURE AND DEVELOPMENT OF SUTURING AND ANASTOMOTIC DEVICES

The ability to achieve secure lumenal closure is pivotal to the success of NOTES. Postoperative leakage is not only associated with morbidity and mortality, but it also bears potential legal and social consequences. Most of the novel designs of NOTES instruments published in the literature have attempted to address this issue. Technological advances in lumenal closure can be divided into four groups, namely the clipping, stitching, stapling, and occluding systems (Table 2).

Clipping systems

Jumbo endoclips *vs* over-the-scope clips: The jumbo endoclip was the earliest described method for lumenal closure[20]. It is achieved by applying clips to approximate the mucosal edges of an opening[20,36,37]. However, the technique can sometimes be difficult, especially if the luminal opening is too large to allow application of the jaws of the clips for mucosal apposition[38]. The fact that only the mucosal edges (and perhaps some submucosa) are recruited in these types of clips renders the security of approximation in large defects very much in doubt[36].

Table 2 Summary of devices for luminal closure in natural orifice transluminal endoscopic surgery

Mechanism of closure	Device name	Outcomes from comparative studies?	Tested in a survival study?
Clipping	Jumbo clips	Yes	Yes
	Over-the-scope clips	Yes	Yes
Stitching	Eagle claw	No	Yes
	T-tags	Yes	Yes
	Loop anchor purse-string	Yes	No
	G-prox needle	Yes	No
	Flexible endostitch	Yes	No
Stapling	SurgASSIST	Yes	No
Occluding	Nitinol cardiac occluder	No	Yes

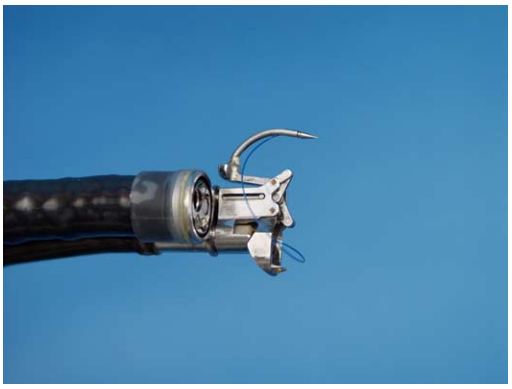

Figure 2 The Eagle claw.

Jumbo endoclips *vs* over-the-scope clips (OTSC)'s are nitinol clips that return to their original shape after release and allow approximation of large defects similarly to a surgical clamp[38]. The additional use of a twin grasper that allows inversion of the seromuscular layers has been shown to enhance approximation of all layers of the bowel wall. Randomized animal studies comparing OTSC's and hand-sewn closures have shown comparable leak pressures[39-41]. In another survival study, endoclip closure was found to be associated with significantly higher risk of leakage as compared to OTSC[42].

Stitching systems

A number of stitching systems have been developed, with none being commercially produced. These include the Eagle claw, T-tags, loop anchor purse-string, G-prox needle, and the Flexible endostitch.

Eagle claw: The Eagle claw (Olympus Medical Systems, Tokyo, Japan) was first developed by the Apollo group as an endoscopic suturing device to simulate surgical plication for hemostasis of bleeding peptic ulcers (Figure 2)[43]. The device consists of an opposing jaw that opposes tissue on closing and allows passage of a mounted 3O nylon stitch with a detachable needle. A metal pusher then

Figure 3 Appearance of the T-tags 1 wk after placement for repair of a colonic perforation in an animal model (Courtesy of Professor A Fritscher-Ravens).

tightens the stitch at the two edges of the stomach wall. In our survival study using the swine model, 10 gastrotomies were successfully closed after bilateral fallopian tubal ligation, and none of the animals suffered from suture line leakages upon post-mortem after 2 wk[44]. The device is now under development by Apollo Endosurgery and a newer version of the device has been shown to allow both interrupted and running suture placement[45].

T-tags: T-tags were first proposed by Fritscher-Ravens *et al*[46] in 2003 and is a device consisting of a series of double tags that are deployed by a transmural needle puncture through the two sides of the gastrotomy. The double tags are then tightened and locked, allowing opposition of the edges of the muscle wall (Figure 3). Other groups have also reported devices with similar design[27,47,48]. The device has been shown to produce fluid- and air-tight closures in the porcine model, and full thickness healing was observed. However, a few complications have been reported, including inadvertent injury to surrounding organs during transmural puncture of the needle[27,47].

Loop anchor purse-string: This is a variation of the T-tags where the anchors (loop anchors) are modified by adding a small metal wire loop to the crosspiece[49]. These anchors are then loaded onto a needle and deployed by using an inner stylet. To achieve gastrotomy closure, a transmural puncture is performed at the edges of the gastrotomy and anchors are deployed sequentially. The stitch is then tightened by pulling on the free ends of the suture and this leads to a purse-string closure of the defect. The device has been shown to achieve significantly higher leak pressures than endoclips in an *ex vivo* model.

G-Prox needle: G-prox has an operating mechanism similar to the T-tags. Closure of an enterotomy is achieved by puncturing the two edges of a defect with a 19-gauge needle, after which two pre-loaded expandable baskets connected by a non-absorbable suture are released[50]. One end of the suture is then tightened and this causes approximation of the baskets and closure of the defect. The device has been shown to create closures comparable to hand sewn sutures in an *ex vivo* model.

Flexible endostitch: The Flexible endostitch (Covidien, North Haven, USA) was adapted from a rigid laparoscopic version of the device. The jaws of the device holds a double-ended needle attached to a suture thread. The needle is toggled back and forth between the two jaws of the device to create a running suture[51]. In the *in vitro* model, it has been shown to produce leak pressures comparable to that of hand sewn sutures.

Stapling systems

SurgASSIST: Long before the advent of NOTES, stapling systems were shown to be reliable methods of creating anastomosis and closure of enterotomy in both open and laparoscopic surgery[52]. Flexible stapling systems based on the same technology should theoretically produce a low rate of leakage comparable to their rigid counterparts. SurgASSIST is a mechanically driven flexible linear stapler available from Power Medical Interventions (Langhorne, Pennsylvania, USA), which has been recently acquired by Covidien. The device has an automated firing system that aligns and approximates the staple arms and creates four linear rows of staples with closure of the enterotomy[53]. The problem with the device, however, is the difficulty in navigating the two staple jaws into a correct position before closure. Nevertheless, the device has been shown to produce burst pressures comparable to running sutures[36].

Occluding systems

Nitinol cardiac occluder: This occluder was originally designed for closure of atrial septal defects and was proposed to be a possible alternative method for closure of a gastrotomy. Animal survival studies have shown that prolonged closures up to 6 wk were possible with no evidence of leakage[54]. Results from comparative studies, however, are still lacking.

DEVELOPMENT OF A MULTITASKING PLATFORM

It is generally agreed that a multitasking flexible endoscope-based platform designated for NOTES is essential for replication of complex laparoscopic surgical manoeuvres, including dissection and suturing. This has spurred the development of a number of different platforms including the EndoSAMURAI (Olympus Corp, Tokyo, Japan) (Figure 4), the Anubis (Karl Storz, Tuttlingen, Germany), the Direct Drive Endoscopic system (DDES) (Boston Scientific, Massachusetts, USA), and the TransPort™ Multi-lumen Operating Platform (USGI medical, California, USA)[55-57]. The aim of these platforms is to provide a flexible, yet stable, system through which NOTES procedures can be performed universally through any of the transluminal approaches. Furthermore, these systems should provide a stable image of the operating field comparable to that in laparoscopic surgery

Figure 4 The EndoSAMURAI (Courtesy of Olympus Co., Tokyo, Japan).

and be independent of the movements of the working arms. More importantly, ergonomic user interfaces are available to control the movements of the arms (some of them capable of five degrees of freedom).

In a bench top simulation setting, both the EndoSAMURAI and the DDES have been shown to significantly enhance performance times and accuracy in complex surgical tasks as compared to using the double-channelled endoscope[55,56]. Twelve participants, who included experienced surgeons, medical students, and research assistants, were able to complete a suture using the EndoSAMURAI. The DDES system was also shown to allow performance of complex tasks, such as cutting, grasping, suturing, and knot tying[57]. An added advantage of DDES is that it can be operated by a single operator. Performance data of the other multitasking platforms, however, are still lacking and outcomes from human studies are still awaited.

DEVELOPMENT DEDICATED INSTRUMENTS

Flexible instruments and hemostatic appliances

Another obstacle to performing NOTES in a flexible system is the inferior properties of the endoscopic forceps or coagulation devices currently available when compared to their laparoscopic counterparts. In a recent study comparing the use of monopolar forceps, endoscopic suturing, and argon plasma coagulation in controlling bleeding from a major arterial branch, argon plasma coagulation was shown to be the quickest modality in achieving hemostasis[58]. In another study, the use of novel flexible bipolar forceps was shown to be comparable to laparoscopic bipolar forceps in stopping bleeding from blood vessels ranging from 1.5 to 6 mm in diameter. Delayed bleeding was observed in 3% of the blood vessels when blood pressure was raised to more than 200 mmHg for 10 min[59]. The development of other flexible instruments has also been announced but their performance data are still pending[60].

HUMAN NOTES PROCEDURES

Despite the tremendous amounts of research being directed towards NOTES, reports of human NOTES procedures are still limited. The majority of the publications were case series or single case reports, and only one study was comparative (Table 2)[2-16]. The most reported human NOTES procedure was a cholecystectomy and these procedures were performed *via* the transvaginal or transgastric routes[2,3,10-13]. NOTES peritoneoscopy, sleeve gastrectomy, sigmoidectomy, and nephrectomy have also been reported[4-9,14-16]. The NOTES appendectomy performed in India have been widely cited as a personal communications, but published data is still being awaited.

In fact, most NOTES cholecystectomies reported to date are hybrid procedures[2,3,5,10-13]. A 2 to 5 mm transumbilical port was first inserted for insufflation of pneumoperitoneum and also to allow for monitoring of the procedure. Most studies achieved transluminal access *via* the transvaginal route but the transgastric approach has also been described. In transvaginal cholecystectomy, a posterior colpotomy was made under direct laparoscopic view through the umbilical port and one to two trocars were inserted[10]. Both a flexible and an ultra-long rigid system have been used to perform the procedure. Retraction of the gallbladder was achieved by the umbilical port or additional transvaginal ports. In cases where a flexible endoscope was used, dissection was performed using instruments inserted through channels of the endoscope and clipping of the cystic artery and duct were done with either endoscopic hemoclips or surgical clips through the transumbilical or transvaginal trocars. In cases where a rigid system was used, the procedure was performed in a manner similar to traditional laparoscopic cholecystectomy, using ultra-long rigid instruments introduced through the transvaginal trocars.

In the human series describing NOTES cholecystectomy (Table 1), three out of 86 operations were unsuccessful and none required conversion. These three patients suffered from severe pelvic adhesions that prevented transvaginal insertion of trocars. The mean time to completion of the operation ranged from 51 to 135 min and no major complications were reported. In the largest series including 68 patients, the patients were also interviewed at 3 to 10 mo after surgery and none of them had abdominal or gynecological complaints in relation to sexual intercourse[9].

In the only human NOTES comparative study, transgastric peritoneoscopy was compared to diagnostic laparoscopy in evaluating patients with a pancreatic mass. Transgastric peritoneoscopy confirmed the decision to proceed to open laparotomy in nine out of ten patients, and the procedure was found to be safe and feasible. However, the authors also commented that the accesses to the right lobe of the liver and right upper quadrant structures were inadequate endoscopically and that attempted biopsies were unsuccessful due to inability to reach these areas[5].

On the other hand, there have also been case reports describing transvaginal nephrectomy, sleeve gastrectomy, and sigmoidectomy[6,7,9,15,16]. All these procedures were hybrid procedures where a transumbilical port was inserted for monitoring and retraction of the tissues, while the transvaginal ports were used for dissection and retrieval of

the specimen. All procedures were successfully performed and none reported major complications.

COMMENTS

The NOTES white paper in 2005 identified a number of fundamental obstacles to implementation of NOTES in humans[1]. Since then, these issues have become key areas of rigorous research in the laboratory setting and many findings have been published in the literature. Of interest is that, with the exception of case series and reports of human NOTES described in this paper, all of the studies performed so far were in animals. It is obvious that the intrinsic differences in physiology and anatomy between animals and humans do have significant impacts on the outcomes of the procedures, and whether results obtained in animals can be replicated in humans remains uncertain. More importantly, implementation of NOTES in human is still severely impaired by the availability of reliable devices specific for the procedure. The majority of devices that were described in this review remain as prototypes that are available to only a few exclusive centers. This limits the ability of researchers to compare different devices and procedures, let alone document the safety profiles and efficacy over a large study population.

In terms of the methods of gaining transluminal accesses, several problems remain to be solved. Firstly, the optimum method of creating the transluminal enterotomy is still uncertain. To some extent, the type of procedure being performed governs the methods of creating the opening. The submucosal tunneling method might be more appropriate when access to a particular organ is required. Likewise, the optimal access organ that provides the best in-line positioning when performing NOTES procedures on a specific region within the abdominal cavity will need to be determined. These issues remain to be resolved in future studies.

The NOTES white paper also states that a closure device that allows 100% reliability is a must before NOTES could be more widely implemented in humans. Along this line, many novel closure devices have been developed over the years. However, none of the reports have included a sufficiently large sample size to determine the exact risk of leakage. Direct head-to-head comparison of these devices has only been performed in one *in vitro* study, and there is a paucity of literature concerning the difference in *in vivo* efficacy of these devices[52]. Without these data, it is unlikely that any of the manufacturers will agree to undergo clinical human trials.

Besides closure devices, another area with exciting development is the research on flexible endoscope based multitasking platforms and instruments. The EndoSAMURAI, DDES, and the TransPort Multi-lumen Operating Platform™ were developed with an aim to perform complex transluminal procedures[55-57]. At present, most of these devices are still cumbersome to use and have been tested only in an *in vitro* setting. Size, ease of introduction, maneuverability *in vivo*, and lack of tactile feedback are some of the problems of the current platforms, which need to be addressed before they can be put into use in human subjects. It is also not certain how well they actually perform in a surgical operation, when grasping, dissecting, ligating, and suturing movements are performed in conjunction.

For the above reasons, the emergences of human NOTES procedures have largely been based on rigid platforms. This has been made possible by the adoption of ultra-long laparoscopic instruments introduced transvaginally, which allows replication of the steps of a laparoscopic surgical procedure. This may well be an intermediate form of NOTES before more reliable and steady platforms become available. Thus far, the outcomes of these NOTES procedures using laparoscopic instruments have been encouraging, and results from comparative studies are eagerly awaited to determine whether NOTES can truly offer earlier recovery and improve cosmesis.

CONCLUSION

Significant advances have been made in recent years in addressing the key obstacles to safe performance and introduction of human NOTES. However, most studies to date are still largely experimental, and whether these performance data can be repeated in humans remains uncertain. On the other hand, reports of human NOTES procedures are beginning to emerge. These studies have demonstrated the feasibility of performing human NOTES using existing technology. Dedicated devices are still lacking to allow for pure NOTES. Whether NOTES can deliver the postulated benefits of earlier recovery and improved cosmesis has yet to be confirmed.

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ORIGINAL ARTICLE

Role of *CYP2E1* gene polymorphisms association with hepatitis risk in Northeast India

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Abstract

AIM: To investigate hepatitis virus, genetic and environmental factors, and their interactions in predisposing patients to liver diseases in Northeast India.

METHODS: A total of 104 jaundice patients and 124 community controls were included. Serological analysis was performed by routine enzyme-linked immunosorbent assay, and nucleic acid testing for hepatitis viruses was done by polymerase chain reaction (PCR), followed by PCR direct sequencing for viral genotyping. Cytochrome P450 2E1 (*CYP2E1*) polymorphism was studied by PCR-restriction fragment length polymorphism. Nitrite and volatile nitrosamines in indigenous foods consumed routinely by the Northeast Indian ethnic population were estimated by Griess's reagent and GC-MS, respectively.

RESULTS: Hepatitis A virus (HAV) infection was predominantly prevalent (36.5%) in our cohort, followed by hepatitis B virus (HBV), hepatitis E virus (HEV) and hepatitis C virus. HBV genotype D and HEV genotype 1 were the most dominant. *CYP2E1* c1/c2 genotype frequency was comparatively higher in alcoholic ($P < 0.0001$, OR = 30.5) and cryptogenic ($P = 0.014$, OR = 8.714) patients, and was associated with significantly higher hepatitis risk ($P = 0.0.007$, OR = 6.489). Mutant C allele of Cyp2E1 *Dra* I frequency was comparatively higher in HAV ($P = 0.006$), alcoholic ($P = 0.003$) and cryptogenic ($P = 0.014$) cases, and was associated with overall hepatitis risk ($P = 0.026$, OR = 5.083). Indigenous foods, Gundruk, Kharoli, betel leaf and nuts were found to have the highest nitrite content.

CONCLUSION: Apart from viral factors, *CYP2E1* polymorphism might be associated with increased risk of liver diseases in Northeast India. Indigenous foods that contain nitrite and nitrosamine might be an associated risk factor.

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Key words: Viral hepatitis; Cytochrome P450 2E1; Gene polymorphism; Nitrites; Nitrosamines

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INTRODUCTION

Application of molecular genetic techniques in human can-

cer risk assessment will likely emerge as a method of identifying subpopulations with different sensitivities to carcinogen exposure[1]. Liver diseases and cancer show a marked worldwide geographic and ethnic distribution[2]. Etiological factors that have been associated with liver diseases and cancer include hepatitis virus infection[3], liver flukes[4], aflatoxins[5], alcohol[6], smoking[6] and dietary nitroso compounds[7]. Differences in hepatitis B virus (HBV)[8] and hepatitis C virus (HCV)[9] genotypes are linked to various degrees of liver disease severity and rate of disease progression towards hepatocarcinogenesis. Unfortunately, there are no data available on these important aspects in liver disease patients from Northeast India, who are ethnically distinct from those in other parts of India, and have the incidence of cancer of various etiologies in the country, according to a survey done by the National Cancer Registry Program of the Indian Council for Medical Research (ICMR).

According to epidemiological studies, 90% of cancers are associated with environmental factors, including nitrosamines, which are acquired through tobacco smoke, vehicle exhaust and foodstuffs[10]. It has been shown that nitrite alone can cause cancer; however, an even more serious cause of concern is the well-documented potential of nitrites/nitrates to cause cancer through the formation of nitrosamines[11].

Cytochrome P450 2E1 (*CYP2E1*) is an N-nitrosodimethyl-amine demethylase that is expressed primarily in the liver. It takes part in the metabolism of drugs, but also activates many pre-carcinogens and pre-toxins[12]. Cyp2E1 activates N-nitrosamines, which are contained in tobacco smoke and foodstuffs[13] and several industrial[14] and endogenous carcinogens[15]. Cyp2E1 activity is mediated by various determinants, such as obesity, fasting and liver dysfunction, and by a number of environmental factors[16]. Cyp2E1 activity is accompanied by generation of a significant amount of an active oxygen form, which damages cell membranes and macromolecules and leads to formation of DNA adducts. Polymorphism in the Cyp2E1 gene is associated with malignancies of different cellular origins, including the liver[17]. *CYP2E1* polymorphism in the 5'regulatory region with C→T replacement at position -1019 and *Rsa* I restriction site loss (*CYP2E1*5B*) is one of the most important polymorphisms identified. Homozygous c2/c2 genotype is associated with a 10-fold increase in *CYP2E1* gene transcription[18]. Another important *CYP2E1* polymorphism is located in intron 6, revealed by *Dra* I and identified as C (minor) and D (common) alleles[19].

Here, we present the results from Guwahati (the capital of Assam, and hub and gateway of Northeast India) of a case-control study designed to explore the viral, environmental and genetic risk factors for liver diseases. The study was approved by the Institutional Biosafety and Ethics Committee.

MATERIALS AND METHODS

Blood samples were obtained from 104 acute hepatitis patients with clinical jaundice and liver disease during the non-rainy season, who were receiving care at the Central Hospital, NF Railway, Maligaon, Guwahati. One hundred and twenty-four sex, age and residence pair-matched community controls, with similar ethnicity and food habits were also recruited for this study. Cases and controls were evaluated on the basis of history (including their food, drinking, smoking and tobacco chewing habits), clinical examination, liver function profile, and serological test of hepatitis A, B, C and E using commercially available IgM enzyme-linked immunosorbent assay (ELISA) kits (therefore including acute cases).

Viral DNA isolation and genotyping

Viral DNA isolation of HBV for hepatitis B surface antigen (HBsAg)-positive cases was performed using the standard phenol-chloroform method using 150 μL of patient plasma, followed by ethanol precipitation. The viral DNA thus isolated was resuspended in an adequate amount of nuclease-free water. HBV genotyping was performed by multiplex polymerase chain reaction (PCR) using specific primers for each genotype (A-F) of HBV[20], and validated by sequencing of representative cases for the basal core promoter, precore and core region of HBV genome.

RNA extraction and HCV and hepatitis E virus genotyping

Viral RNA was extracted from 140 μL of serum with QIAamp@Viral RNA Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA pellets were reconstituted in 60 μL elution buffer and stored at -20℃ until use. One-tube nested reverse transcription PCR (RT-PCR) amplification was performed using specific primers for the conserved 5'UTR region as described earlier for genotyping of HCV[21]. Amplification of the specific 256-bp product was achieved for the anti-HCV-positive cases. Briefly, 10 μL RNA was mixed with 0.2 μL (20 pmol) of antisense primer, incubated for 1 min at 94℃ and 1 min at 56℃, and then stored on ice. Fifty microliters of the reaction mixture that contained 10 × reaction buffer, dNTPs (10 mmol/L), $MgCl_2$ (2.5 mmol/L), 20 pmol primers AS1 and S1, 0.5 U *Taq* Polymerase (New England Biolabs, Ipswich, MA, USA) and 2.5 μL (20 U/μL) MMuLV Reverse Transcriptase (New England Biolabs) was added to the pre-cooled RNA mix for one-step RT-PCR. The conditions were 60 min at 42℃ for reverse transcription, 2 min at 95℃ for denaturation of the RT, followed by 35 cycles of 30 s at 95℃, annealing for 30 s at 54℃, and extension for 30 s at 72℃. After the last cycle, a final extension was made at 72℃ for 7 min. The second round of PCR was performed with the same master mix that contained AS2 and S2 primers using 5 μL of the first product as a template under the same reaction conditions. Positive and negative controls were included in every PCR amplification experiment. This was followed by direct sequencing and comparison with the standard NCBI Genbank database. Hepatitis E virus (HEV) genotyping was performed by RT-PCR amplification using the primers for the HEV ORF1 region reported by Jilani *et al*[22], which gave a PCR-amplified product of 343 bp; followed by direct sequenc-

Table 1 Demographical, biochemical and serological profiles of liver disease patients

Parameter	HAV	HBV	HCV	HEV	HAV + HBV	Alcoholic	Cryptogenic
n	38	22	4	10	2	12	16
Male:female	22:16	16:6	2:2	6:4	2:0	11:1	12:4
Mean age (yr)	23 ± 16	40 ± 28	45 ± 8	38 ± 15	55	44 ± 5	43 ± 21
Mean SGOT	241 ± 168	333 ± 108	86 ± 46	778 ± 336	40 ± 5	323 ± 212	109 ± 66
Mean SGPT	265 ± 198	243 ± 148	73 ± 28	513 ± 366	39 ± 8	283 ± 155	85 ± 43

HAV: Hepatitis A virus; HBV: Hepatitis B virus; HEV: Hepatitis E virus; HCV: Hepatitis C virus; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase.

ing and comparison with the available genotype database for HEV from the NCBI Genbank database.

PCR-restriction fragment length polymorphism analysis of CYP2E1 gene polymorphism

*CYP2E1*5B* (5' flanking region, -1019 bp site) genotyping was performed by PCR-fragment length polymorphism (RFLP) analysis with the primers reported by Hayashi *et al*[23]; and *Rsa* I restriction enzyme. An allele with an *Rsa* I site (characterized by two bands of 360 and 50 bp on agarose gel electrophoresis) was defined as wild-type and designated c1, and an allele without this site, as a variant or rare type and designated c2 (characterized by presence of a single band at 410 bp)[24].

Dra I digestion detects a polymorphism in intron 6 of the *CYP2E1* gene. Genomic DNA was PCR-amplified with primers reported by Kato *et al*[25], which yielded a 995-bp fragment that was subjected to *Dra* I restriction enzyme digestion. Two *Dra* I restriction enzyme recognition sites exist in this amplified DNA sequence but only one is known to be polymorphic. The presence of the polymorphic *Dra* I restriction site yielded three fragments of 572, 302 and 121 bp (type D, major allele), whereas the absence of the polymorphic site was determined by the presence of 874-bp and 121-bp fragments (type C, minor allele)[26].

To improve the genotyping quality and validation, 20% of samples were re-genotyped by other laboratory personnel and results were reproducible with no discrepancy in genotyping. Genotyping of 10% of samples was confirmed by DNA sequencing.

Nitrite determination and analysis of volatile nitrosamines

Several indigenously prepared fermented food products and the raw material used to prepare them were short-listed and collected, based on a questionnaire of the food habits of jaundice patients and community controls enrolled in the present study. Nitrite estimation in fresh and fermented foods from Northeast India was done using the standard Griess's reagent method followed by spectrophotometric detection at 540 nm. The presence of volatile N-nitrosamines in fresh and fermented foods was done by GC-MS analysis following the protocol of Mitacek *et al*[27], followed by detection using a Hewlett-Packard Model 5890 GC coupled to a model 610 Thermal Energy Analyzer (TEA; Thermo Electron, Waltham, MA, USA).

Statistical analysis

Results were expressed as mean ± SD. Serum aspartate aminotransferase and alanine aminotransferase levels in each group were analyzed by student's *t* test. ORs were calculated using logistic regression. Statistical analysis was carried out for *CYP2E1* genotypes in liver disease and hepatitis subgroups (specific for viral hepatitis groups, and alcoholic and cryptogenic cases) and compared to community controls using SPSS version 13.0 software. An adjusted two-tailed *P* value (corrected) less than 0.05 at 95% CI was considered statistically significant.

RESULTS

Blood samples were obtained from patients with liver disease who were receiving care in a regional referral hospital in Guwahati. These patients had a median age of 41 ± 16 years and showed a male to female ratio of 2.15:1. The majority of the liver disease patients were male (71/104, 68.27%). The hepatitis virus infection spectrum analyzed based on IgM ELISA results was, hepatitis A virus (HAV, 38/104, 36.5%), HBV (22/104, 21.15%), HCV (4/104, 3.8%), HEV (10/104, 9.6%), and HAV-HBV co-infection (2/104, 1.92%). Others had alcoholic (12/104, 11.53%) and cryptogenic (16/104, 15.38%) liver disease etiology (Table 1).

Viral genotyping

Viral genotyping was performed for HBV, HCV and HEV samples. HBV genotyping was performed by multiplex PCR. HBV genotype D (13/22, 59.1%) was the most prevalent in the HBV-positive cases, followed by HBV genotype A (4/22, 18.2%), mixed genotype A + D (4/12, 18.2%) and genotype C (1/22, 4.5%) (Figures 1A and 2A). A few of the randomly selected genotyped samples were cross-checked and validated by direct sequencing of the core region of HBV followed by phylogenetic analysis.

HCV genotype was determined by direct sequencing of the PCR amplicon generated from the conserved 5'UTR region of the HCV genome. The nucleotides that were sequenced by direct sequencing were aligned using ClustalW, and version 1.6 of the tree view program from Expasy (POWER) was then used to construct an uprooted phylogenetic tree (Figures 1B and 2B). After comparison with known sequences from the NCBI Genbank database, the distribution of the genotypes based on four isolated

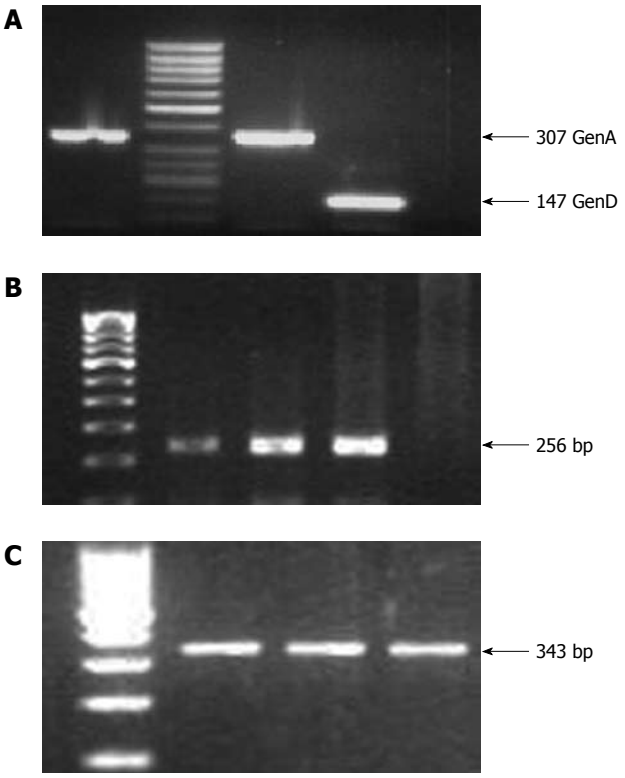

Figure 1 Polymerase chain reaction amplification results. A: Hepatitis B virus (HBV) genotyping results, where an amplicon of 307 bp represents HBV genotype A whereas an amplicon of 147 bp represents HBV genotype D; B: Hepatitis C virus (HCV) amplicon of 256 bp for the 5'UTR region; C: Hepatitis E virus (HEV) amplicon of 343 bp for the ORF1 region. The HCV and HEV amplicons were purified by gel extraction and subjected to direct sequencing for genotype determination on comparison with the standard NCBI representative sequences for HCV and HEV.

HCVs were found to be one each from genotypes 4 (*asm-hcv*-4), 3 (*asm-hcv*-3), 2 (*asm-hcv*-1) and 6 (*asm-hcv*-2).

HEV genotype was determined by amplification of the ORF1 region and subjecting the amplified product to direct sequencing, and then comparing the sequences with the standard NCBI Genbank sequences for HEV. HEV genotype 1 was the only genotype found in our cohort.

PCR-RFLP analysis of CYP2E1 gene polymorphism

The distribution of *CYP2E1*5B* c1c1, c1c2 and c2/c2 genotypes in liver disease cases were 90.38%, 9.62% and 0%, respectively compared to 98.39%, 1.61% and 0% in healthy controls (Figure 3). The *CYP2E1*6* DD, DC and CC genotype frequencies in liver disease cases were 92.3%, 3.85% and 3.85%, respectively, compared to 98.39%, 1.61% and 0% in healthy controls (Tables 2 and 3). Cyp2E1 *Rsa* I genotype distributions were consistent with Hardy-Weinberg equilibrium, but Cyp2E1 *Dra* I genotype was only consistent for the control population. The c1/c2 variant genotype was significantly more prevalent in alcoholic [$P < 0.0001$, OR = 30.5 (4.835-192.418)] and cryptogenic hepatitis [$P = 0.014$, OR = 8.714 (1.137-66.784)] cases. The prevalence of mutant C allele of *Dra* I was also predominant in alcoholic [$P = 0.003$, OR = 12.220 (1.550-96.035)] and cryptogenic hepatitis [$P = 0.014$, OR = 8.714 (1.137-66.784)] cases.

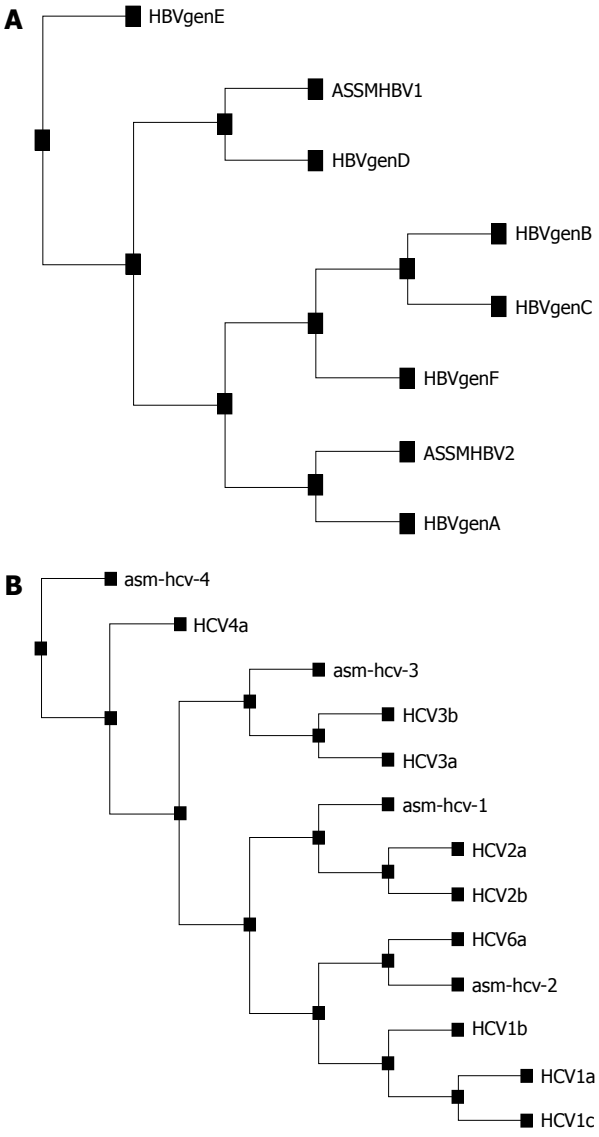

Figure 2 Phylogenetic analysis using the Expasy software tool. A: Randomly sequenced hepatitis B virus (HBV) cases representing HBV genotype D (*ASSMHBV1*) and A (*ASSMHBV2*) by multiplex polymerase chain reaction genotyping, therefore confirming and validating our genotyping results; B: Hepatitis C virus (HCV) genotype base on direct sequencing of 5'UTR for isolate from Guwahati.

Nitrite determination and analysis of volatile nitrosamines

To study the correlation of environmental factors with severity of liver disease, nitrite concentration in various fermented food products widely used in the households of Assam and Northeast India were estimated using Griess reagent. The concentration of nitrites in raw and fermented food products produced by indigenously developed fermentation techniques, as well as a few common supplementary food products consumed routinely, are shown in Figure 4 and Table 4. The highest amount of nitrite was found in mustard (fresh and fermented), followed by another fermented food product, Gundruk (fermented radish leaf) and betel leaf (commonly known as Pan, and taken in combination with betel nut).

Based on the case histories of the liver disease patients, which included their food habits, as well as considering

Table 2 Distribution of Cytochrome P450 2E1 genotype in hepatitis cases compared to controls

	Number of individuals (% of group)			Less common allele frequency	χ^2 value (*P* value)	OR (95% CI)
	Homozygous-more common allele	Heterozygous	Homozygous-less common allele			
Rsa I polymorphism	c1/c1	c1/c2	c2/c2			
Controls (*n* = 124)	122 (98.39)	2 (1.61)	0	1.61	ref.	
Hepatitis (*n* = 104)	94 (90.39)	10 (9.71)	0	9.61	0.007	6.489 (1.389-30.326)
Dra I polymorphism	DD	DC	CC			
Controls (*n* = 124)	122 (98.39)	2 (1.61)	0	1.61	ref.	
Hepatitis (*n* = 104)	96 (92.31)	4 (3.84)	4 (3.84)	7.69	0.026	5.083 (1.055-24.492)

Data represented as number of subjects showing respective genotype (%); $P < 0.05$ was considered to be statistically significant, control group was considered as reference group.

Table 3 Detail distribution of Cytochrome P450 2E1 genotypes in different underlying etiology of hepatitis

	Number of individuals (% of group)			Less common allele frequency	χ^2 value (*P* value)	OR (95% CI)
	Homozygous-more common allele	Heterozygous	Homozygous-less common allele			
Rsa I polymorphism	c1/c1	c1/c2	c2/c2			
Controls (*n* = 124)	122 (98.4)	2 (1.6)	0	1.6	ref.	
HAV (*n* = 38)	36 (94.7)	2 (5.3)	0	5.3	0.160	5.229 (0.840-32.537)
HBV (*n* = 22)	20 (90.9)	2 (9.1)	0	9.1	0.080	2.905 (0.252-33.484)
HCV (*n* = 4)	4 (100)	0 (0)	0	0	0.798	0.968 (0.938-1.0)
HEV (*n* = 10)	10 (100)	0 (0)	0	0	0.686	0.924 (0.880-0.971)
HAV + HBV (*n* = 2)	2 (100)	0 (0)	0	0	0.856	0.984 (0.962-1.006)
Alcoholic (*n* = 12)	8 (66.66)	4 (33.33)	0	33.33	< 0.0001	30.500 (4.835-192.418)
Cryptogenic (*n* = 16)	14 (87.5)	2 (12.5)	0	12.5	0.014	8.714 (1.137-66.784)
Dra I polymorphism	DD	DC	CC			
Controls (*n* = 124)	122 (98.4)	2 (1.6)	0	1.6	ref.	
HAV (*n* = 38)	34 (89.5)	2 (5.25)	2 (5.25)	10.5	0.006	7.176 (1.260-40.863)
HBV (*n* = 22)	22 (100)	0	0	0	0.514	0.847 (0.790-0.908)
HCV (*n* = 4)	4 (100)	0	0	0	0.798	0.968 (0.938-1.0)
HEV (*n* = 10)	10 (100)	0	0	0	0.164	0.924 (0.880-0.971)
HAV + HBV (*n* = 2)	2 (100)	0	0	0	0.856	0.984 (0.962-1.006)
Alcoholic (*n* = 12)	10 (83.3)	2 (16.7)	0	16.7	0.003	12.220 (1.550-96.035)
Cryptogenic (*n* = 16)	14 (87.5)	0	2 (12.5)	12.5	0.014	8.714 (1.137-66.784)

Data represented as number of subjects showing respective genotype (%); $P < 0.05$ was considered to be statistically significant, control group was considered as reference group. HAV: Hepatitis A virus; HEV: Hepatitis E virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

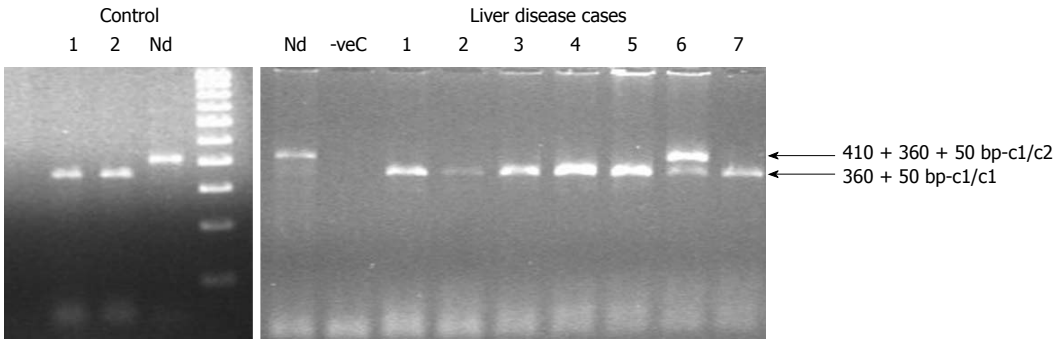

Figure 3 Polymerase chain reaction-restriction fragment length polymorphism results for Cytochrome P450 2E1 *rsa* I genotyping for the control and liver disease cases. Lane 1 and 2 in the control section and lane 1-5 and 7 of the liver disease section represents c1/c1 genotype (360 + 50 bp); whereas lane 6 of the liver disease section repre-sents c1/c2 genotype (410 + 360 + 50 bp). Nd: Non-digested samples of 410 bp.

the food habits of the general Northeast Indian population, we analyzed raw and fermented bamboo shoots and fish (including dried fish) for the presence of volatile N-nitrosamines by GC-MS. The GC-MS/TEA analysis revealed the presence of detectable amounts of N-nitrosamines in raw fish (data not shown), whereas there was

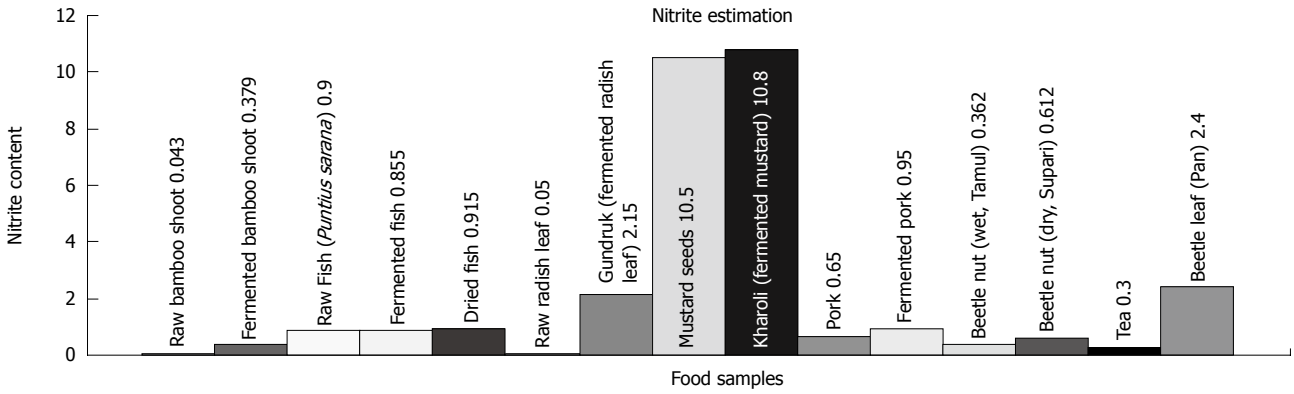

Figure 4 Presence of nitrite in (μg/mL) in different food products routinely consumed in Northeast India.

Table 4 Nitrite estimation in food products routinely consumed in Northeast India

Sl. No.	Name of foodstuff	Amount of nitrite present (μg/mL)
1	Raw bamboo shoot	0.043
2	Fermented bamboo shoot	0.379
3	Raw fish (*Puntius sarana*)	0.900
4	Fermented fish	0.855
5	Dried fish	0.915
6	Raw radish leaf	0.050
7	Gundruk (fermented radish leaf)	2.150
8	Mustard seeds	10.500
9	Kharoli (fermented mustard)	10.800
10	Pork	0.650
11	Fermented pork	0.950
12	Beetle nut (wet, Tamul)	0.362
13	Beetle nut (dry, Supari)	0.612
14	Tea	0.300
15	Betel leaf (Pan)	2.400

no detectable amount of N-nitrosamines in fermented and dry fish or raw and fermented bamboo shoots.

DISCUSSION

Along with age, sex and viral factors, alterations in host genetic factors and environmental factors are also considered to be important in the development of liver disease, but unfortunately, there are very limited data available on these important aspects in liver disease patients from Northeast India, who are ethnically distinct from those in other parts of the country. More importantly, the population in the northeast region is vulnerable to cancer of different etiology, as shown by the ICMR National Cancer Registry Program of. In this study, we determined the prevalence of systemic hepatotropic viruses and their genotypes, Cyp2E1 polymorphism, and the presence of dietary toxic environmental carcinogens such as nitrites and nitrosamines in fresh and fermented food from Northeast India, and evaluated the role that they might play in the severity of the liver disease or its predisposition.

The highest prevalence of fecal-oral infection occurs in regions where low standards of sanitation promote virus transmission[28]. In most industrialized nations, where hepatitis A is no longer considered a childhood disease, infections with HAV are increasingly contracted by adults[29]. Despite the high prevalence of antibody in highly endemic populations, the virus perpetuates in the region due to its high physical stability. In our study, HAV infection was found in 36.5% of the population and most of the infected individuals were children and young people. This was followed by HBV infection in 21.2% of the study population, which is relatively on the higher side compared to other reports from different parts of India[30-33]. This is a major concern because HBV infection is associated with a high rate of hepatocellular carcinoma[34]. HBV genotype D was the most predominant genotype in our cohort, which is contrary to a report from the sister state in Northeast India, Arunachal Pradesh[35].

HEV and HCV infection was found in 9.6% and 3.8% of the cases, respectively. Viral hepatitis is a major public health problem in India, which is hyperendemic for HAV and HEV. HEV is also the major cause of sporadic adult acute viral hepatitis and acute liver failure, and many epidemics of HEV have already been reported in India. HCV infection in India has a population prevalence of around 1%, and occurs predominantly through transfusion and the use of unsterile glass syringes. HCV genotypes 2 and 3 are found in 60%-80% of the population[36]. Our results showed the prevalence of different HCV genotypes, namely, 2, 3, 4 and 6, which warrants further study, including large cohort populations from all Northeast Indian states. Here, to the best of our knowledge, we reported the presence of HEV genotype 1 in Northeast India for the first time, which is similar to reports published from other parts of India.

CYP2E1 enzymes belong to the phase I group of drug-metabolizing enzymes that are involved in the metabolic activation and detoxification of various potential genotoxic compounds. *CYP2E1* is involved in metabolism of more than 80 low-molecular-weight, hydrophobic, toxicologically dangerous compounds and contributes to activation of many pro-carcinogens and several drugs to highly reactive metabolites[14]. Hepatic *CYP2E1* has been shown to activate various carcinogens, therefore, there has been interest in whether certain *CYP2E1* polymorphisms

might predispose to liver diseases and cancer[37]. The functional polymorphism in these genes exhibits inter-individual variations in susceptibility towards various diseases and differences in therapeutic response. The variant sequences of these genes differ considerably between ethnic groups. Therefore, the objective of the study was to assess the prevalence of *CYP2E1* gene variants in healthy volunteers and compare them with the liver disease patients from Guwahati.

The most important polymorphisms identified in 5' regulatory region with C→T replacement in position -1019 and *rsa* I restriction site loss (*CYP2E1*5B*) (77, 25). Variant *c*2 allele is expressed *in vitro* at a higher rate compared to wild-type, and homozygous *c*2/*c*2 genotype is associated with a 10-fold increase in *CYP2E1* gene transcription. The functional significance of *CYP2E1*5B* polymorphism might be due to its localization in presumed binding sites for hepatic transcription factor, hepatocyte nuclear factor-1[18,23]. Rare *c*2 allele frequency constitutes 24%-30% for Asian populations[25], 2%-3% for Caucasians[23], 0.3%-7% for Afro-Americans[23,38], 15% for Mexican Americans[39], and 18% for Taiwanese[40]. The *Dra* I polymorphism is also associated with altered activity of *CYP2E1*, although *Dra* I is located in intron 6 and is not thought to affect transcription of the gene[19].

Our study showed that the prevalence of mutant C1/C2 Cyp2E1 *Rsa* I allele and the mutant C allele of *Dra* I was significantly higher in liver disease patients. The presence of mutant Cyp2E1 *Rsa* I allele ($P = 0.007$, OR = 6.489 at 95% CI: 1.389-30.326) and mutant C allele of *Dra* I ($P = 0.026$, OR = 5.083 at 95% CI: 1.055-24.492) was significantly associated with hepatitis risk in Northeast Indian patients.

The prevalence of the *c*1/*c*2 genotypes was lower than that reported in other Asian countries, but amongst the highest reported in the Indian population[41]. The serum glutamic oxaloacetic transaminase (SGOT) levels were also significantly higher in HAV cases that contained wild-type Cyp2E1 *Dra* I allele ($P = 0.019$). The prevalence of mutant *Dra* I allele among patients with viral hepatitis was found to be significantly more only in cases of HAV infection ($P = 0.006$). The presence of underlying mutant *Dra* I allele might play a role in liver damage caused by acute HAV infection, but this also augments more indebt studies to conclude on the molecular interactions influenced by HAV on *CYP2E1* genes functionality or activity.

Induction of cytochrome P450 2E1 by ethanol is believed to be one of the central pathways by which ethanol generates a state of oxidative stress and causes hepatotoxicity. Hepatic *CYP2E1* enzyme activity is significantly higher in alcoholic patients with liver disease than in those without signs of liver disease[42]. In our study, Cyp2E1 *c*1/*c*2 ($P < 0.0001$) and mutant *Dra* I ($P = 0.003$) allele was significantly associated with alcoholic liver disease. Mutant *c*1/*c*2 and *Dra* I ($P = 0.014$) was also found to be associated with cryptogenic hepatitis in liver disease patients from Northeast India.

There is a concern to maintain the levels of nitrite as low as possible because of suspected adverse effects on oxygenation of the blood, and/or indirect carcinogenic effects, through formation of nitrosamines. Nitrites have been known to cause cancer directly. Although there is little correlation between nitrate/nitrite and nitrosamine content of food, nitrates and nitrites are agents in endogenous nitrosamine formation in the gastrointestinal tract[11]. Therefore, the presence of high nitrite concentration in raw and fermented mustard, radish and betel leaf and nut is also a high risk factor for adverse health effects, along with genetic and viral factors. Addition of nitrite-containing salts for storage of some dried fish products and fermented pork also adversely affects the quality of the food. The European Commission Scientific Committee for Food (document 111/5611/95) has recommended that nitrate and nitrite should be limited to an acceptable daily intake of 0.06 mg/kg. Therefore, ingestion of the above food products that contain high nitrite concentrations is a high risk factor, especially for children.

Case-control studies have suggested that exposure to exogenous and possibly endogenous nitrosamines in food or tobacco in betel nut and cigarettes plays a role in the development of liver disease and cancer. There is evidence that endogenous nitrosation of areca nut alkaloids can occur in animals and humans, and areca-nut-derived nitrosamines, including 3-(methylnitrosamino) propionitrile, have been detected in the saliva of betel quid chewers which is a common practice in Guwahati and throughout Northeast India. Epidemiological data have linked the use of areca nut with other cancers such as liver cancer[43]. The presence of volatile nitrosamines (N-diethylnitrosamine and N-dimethylnitrosamine) in raw fish has been detected using the protocol followed by Mitacek *et al*[27]. The presence of volatile nitrosamines could be an indication of increasing pollution of the River Brahmaputra, which is one of the life lines of Northeast India, and its tributaries, from where the fish *Puntius sarana* is caught and fermented and dried. Our results is of grave importance as case-control studies conducted in Thailand have implicated traditional lifestyle and especially consumption of fermented-style fish and fermented vegetables[44]. Fortunately, the nitrosamine levels were undetectable in fermented and dried fish, contrary to what has been reported in other countries[27]. The non-detection of nitrosamines in fermented fish, irrespective of its presence in raw fish, could be attributed to the activities of lactic acid bacteria during fermentation.

To conclude, the diversity of etiological factors associated with liver disease burden in Northeast India is enormous with respect to the high prevalence of certain hepatitis viruses, such as HBV, as well as the various HBV and HCV genotypes found in our study cohort. Moreover, strict vigilance and upgrading of overall hygiene standards is mandatory to investigate epidemics of HAV or HEV, which are also prevalent in Northeast India. *CYP2E1* polymorphism is supposedly associated with the risk of liver disease, especially in non-viral hepatitis patients, and the presence of higher nitrite concentration in fermented dietary products in Northeast India, and nitrosamines in *Areca catechu* (betel nut) and raw fish, have clinical signifi-

cance, because these environmental factors can act as additional risk factors in liver disease susceptibility, by virtue of the gene-environment interaction.

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COMMENTS

Background

Molecular epidemiology of risk factors such as viral (e.g. hepatitis A, B, C and E viruses), host genetic [e.g. Cytochrome P450 2E1 (*CYP2E1*) gene polymorphism] and environmental factors such as toxic components in food and alcohol, is important for liver disease assessment, which might help to identify subpopulations with different sensitivities and predisposition to different grades or severity of liver disease. Scanty or no data are available on the above aspects from Northeast India, which has an ethnically distinct and different population from the rest of the country. The authors present the results of a case-control study from Guwahati (the capital of Assam, and hub and gateway of Northeast India), which was designed to explore the viral, environmental and genetic risk factors for liver diseases.

Research frontiers

The authors identified patient subgroups by serological profiling based on standard enzyme-linked immunosorbent assay techniques, followed by a molecular-genotyping-based approach using polymerase chain reaction-restriction fragment length polymorphism direct sequencing for identifying hepatitis B, C and E genotypes, as well as *CYP2E1* polymorphisms. Biochemical assays (Griess's method) and GC-MS were used for analyzing and quantifying nitrites and nitrosoamines.

Innovations and breakthroughs

For the first time, all three critical parameters, that is, viral, host genetic and environmental risk factors were evaluated in a case-control study from Northeast India. The study analyzed hepatitis virus genotypes, the role of *CYP2E1* polymorphisms, and the presence of toxic carcinogenic components in routinely consumed indigenous food products of Northeast India.

Applications

The molecular genotyping data on viral hepatitis could be useful for clinicians because viral genotypes have been shown to influence disease progression and antiviral therapies, and therefore, will helpful for clinical interventions and patient care. *CYP2E1* genotyping data are useful as a prognostic marker for assessing the predisposition of patients towards liver disease. The safety aspects of the food products exclusively consumed in Northeast India were elucidated. Information about this is important for the public in Northeastern states of India.

Peer review

The results of this paper are interesting and well presented.

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ORIGINAL ARTICLE

A new index for non-invasive assessment of liver fibrosis

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Abstract

AIM: To construct and evaluate a new non-invasive fibrosis index for assessment of the stage of liver fibrosis.

METHODS: A new fibrosis index (Fibro-Stiffness index) was developed in 165 of 285 patients with chronic hepatitis C, and was validated in the other 120 patients where liver biopsy was performed. Its usefulness was compared with liver stiffness (LS) measured by FibroScan, the aminotransferase-to-platelet ratio index, the Forns index and the FibroIndex.

RESULTS: The Fibro-Stiffness index consists of LS, platelet count and prothrombin time. The values of the Fibro-Stiffness index differed significantly between neighboring fibrosis stages except F0-F1. The area under the receiver operating characteristics curves of the Fibro-Stiffness index for prediction of F $\geqslant$ 2 (0.90), F $\geqslant$ 3 (0.90) and F = 4 (0.92) in the estimation group and those for F $\geqslant$ 3 (0.93) and F = 4 (0.97) in the validation group were the highest among the 5 methods examined. The accuracy of the Fibro-Stiffness index had highest values for F $\geqslant$ 2, F $\geqslant$ 3 and F = 4 in both the estimation and validation groups. The diagnostic performance for F = 4 was improved by a combination of the Fibro-Stiffness index with serum hyaluronic acid level.

CONCLUSION: The Fibro-Stiffness index was constructed and validated. It showed superior diagnostic performance to other indices for F $\geqslant$ 2, 3 and 4.

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Key words: Non-invasive fibrosis index; Fibro-Stiffness index; Chronic hepatitis C; Liver stiffness; Liver fibrosis

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INTRODUCTION

The stage of liver fibrosis is important in the clinical

management of chronic hepatitis C, since the treatment and prognosis of chronic hepatitis depend on the fibrosis stage[1]. In chronic viral hepatitis, the presence of significant fibrosis (F ⩾ 2) indicates the need for antiviral therapies, and the outcome of therapy should be assessed by the improvement in fibrosis stage. Furthermore, the risk of hepatocellar carcinoma or bleeding from esophageal varices is high in patients with advanced fibrosis[2,3]. Liver biopsy is the gold standard for the assessment of fibrosis stage in chronic hepatitis. However, liver biopsy is an invasive and expensive procedure, and its accuracy is sometimes questionable because of sampling errors, inadequate specimens and the subjectivity of diagnosis[4,5].

Non-invasive assessment of liver fibrosis is a major objective that has been encouraging many approaches, such as routine laboratory tests and serum markers of fibrosis[6-12]. The aminotransferase-to-platelet ratio index (APRI)[11], the Forns index[6], the FibroTest[7] and the FibroIndex[12] have been proposed for use as non-invasive fibrosis indices. Transient elastography with the use of a new apparatus, FibroScan (EchoSens, Paris, France) for measurement of liver stiffness (LS) has been developed[13]. LS measured by FibroScan has been reported to correlate with stage of fibrosis in various liver diseases[13-24]. It was used for assessing the effect of treatment in chronic hepatitis C[25].

In the present study, we developed a new fibrosis index, the Fibro-Stiffness index, consisting of LS, platelet count and prothrombin time from 165 patients with chronic hepatitis C (estimation group) to improve the diagnostic efficacy of LS. We also tried a combination of Fibro-Stiffness index and routinely available laboratory tests to improve its diagnostic performance. These results in the estimation group were validated in 120 patients with chronic hepatitis C (validation group).

MATERIALS AND METHODS

Patients

In 285 consecutive patients with chronic hepatitis C virus infection, liver biopsy was performed at Fujita Health University Hospital from July 2004 to February 2009 (Table 1).

From July 2004 to September 2007, 165 of these patients (estimation group) were used to develop the Fibro-Stiffness index. From October 2007 to February 2009, the other 120 patients (validation group) were used to validate the diagnostic performance of the Fibro-Stiffness index. The usefulness of the Fibro-Stiffness index was compared with LS, the APRI, the Forns index and the FibroIndex.

Clinical data were collected within 3 d of liver biopsy. Sections were stained with hematoxylin-eosin stain and Azan stain. Liver biopsy specimens were assessed by 2 hepatologists (Yoshioka K and Kawabe N). When fibrosis stages evaluated by 2 hepatologists differed, the higher fibrosis stage was adopted. Fibrosis stage, determined according to the METAVIR score, was classified as F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

Liver stiffness measurement

LS measurement by transient elastography was performed with FibroScan (EchoSens, Paris, France) within a week of liver biopsy. FibroScan is equipped with a probe including an ultrasonic transducer and a vibrator. A vibration of mild amplitude and low frequency is transmitted from the vibrator placed on the body surface toward the liver through the intercostal space. The vibration induces an elastic shear wave that propagates through the liver tissue. The pulse-echo ultrasound acquisitions follow the propagation of the shear wave and determine its velocity. The velocity is directly related to tissue stiffness; the harder the tissue, the faster the shear wave propagates. LS is calculated from velocity and expressed in kilopascals (kPa). Ten successful acquisitions were performed on each measurement, and the median value was adopted as representative of LS.

Statistical analysis

The end point was the discrimination between F0 and F1-4, between F0-1 and F2-4, between F0-2 and F3-4 and between F0-3 and F4, using a combination of LS and relevant biochemical or hematological variables. Variables that correlated significantly with fibrosis stage in the estimation group were identified by univariate analyses (analysis of variance). Then the independent predictors of fibrosis stage were assessed by multiple regression analysis (ordinal logistic regression). A predictive index was constructed by modeling the values of the independent variables and their coefficient of regression. The difference of fibrosis indices between neighboring fibrosis stages were estimated by the Tukey-Kramer test. The optimal discriminate cut-off values of each fibrosis index were assessed from the area under the receiver operating characteristics (ROC) curves (AUCs). The optimal discriminating cut-off values were determined at the maximum total of sensitivity and specificity. The statistical analysis was performed by JMP® (SAS Institute, Cary, NC, USA).

RESULTS

Development of the Fibro-Stiffness Index

LS, platelet count, prothrombin time, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, γ globulin, total cholesterol and hyaluronic acid were significantly correlated with fibrosis stage in the estimation group (Table 2). Among these variables, LS ($P < 0.0001$), platelet count ($P = 0.0408$), and prothrombin time ($P = 0.0066$) were identified as independent predictors of fibrosis stage by multiple regression analysis (Table 3). By multiple regression analysis, the estimated values of LS, platelet count and prothrombin time were calculated as -0.2662, 0.0749 and 0.0560, respectively. The optimum intercept was also calculated as 5.7710. Thus the Fibro-Stiffness index was constructed with these 3 variables: Fibro-Stiffness index = 5.7710 - 0.2662 [LS (kPa)] + 0.0749 [platelet count ($\times 10^4$/mL)] + 0.0560 [prothrombin time (%)].

Table 1 Characteristics of the 165 patients in the estimation group and the 120 patients in the validation group (mean ± SD)

	All patients (n = 285)	Estimation group (n = 165)	Validation group (n = 120)	P-value
Male gender, n (%)	149 (52.3)	92 (55.8)	57 (47.5)	NS
Age (yr)	52.4 ± 13.3	53.2 ± 12.6	51.5 ± 14.2	NS
Liver stiffness (kPa)	9.99 ± 6.99	10.29 ± 7.33	9.58 ± 6.51	NS
Platelet count (× 10^4/mL)	16.54 ± 5.28	16.53 ± 5.41	16.57 ± 5.13	NS
Prothrombin time (%)	9.35 ± 11.3	92.4 ± 10.2	95.1 ± 12.6	NS
AST (IU/L)	52.5 ± 34.0	53.0 ± 34.6	51.8 ± 33.4	NS
ALT (IU/L)	70.8 ± 52.6	72.4 ± 54.9	68.7 ± 49.4	NS
Total protein (g/dL)	7.81 ± 0.52	7.79 ± 0.49	7.85 ± 0.57	NS
Albumin (g/dL)	4.31 ± 0.34 (n = 283)	4.31 ± 0.31 (n = 163)	4.31 ± 0.38	NS
γ-GTP (IU/L)	59.8 ± 62.0	58.1 ± 57.9	62.1 ± 67.5	NS
γ-globulin (g/dL)	1.57 ± 0.41 (n = 276)	1.54 ± 0.38 (n = 146)	16.1 ± 0.44	NS
Total cholesterol (mg/dL)	178.1 ± 31.9	177.2 ± 31.1	179.5 ± 33.0	NS
Hyaluronic acid (ng/mL)	104.1 ± 128.3 (n = 281)	114.5 ± 140.0 (n = 161)	90.0 ± 109.3	NS
Fibrosis stage, n (%)				
F0	28 (9.8)	14 (8.5)	14 (11.7)	
F1	85 (29.8)	52 (31.5)	33 (27.5)	
F2	82 (28.8)	42 (25.5)	40 (33.3)	
F3	53 (18.6)	33 (20.0)	20 (16.7)	
F4	37 (13.0)	24 (14.5)	13 (10.8)	

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; NS: Not significant.

Table 2 Variables associated with fibrosis stage in the estimation group (165 patients) in univariate analysis (mean ± SD)

	F0 (n = 14)	F1 (n = 52)	F2 (n = 42)	F3 (n = 33)	F4 (n = 24)	P-value
Liver stiffness (kPa)	5.58 ± 1.96	5.76 ± 1.91	9.02 ± 4.11	13.40 ± 4.95	20.77 ± 10.81	< 0.0001
Platelet count (× 10^4/mL)	20.80 ± 4.77	18.79 ± 5.19	15.66 ± 4.54	15.72 ± 5.58	11.75 ± 2.63	< 0.0001
Prothrombin time (%)	101.8 ± 10.3	96.1 ± 7.9	92.5 ± 7.9	88.7 ± 10.5	83.6 ± 9.6	< 0.0001
AST (IU/L)	30.3 ± 17.3	40.1 ± 23.7	46.9 ± 31.8	73.2 ± 40.3	77.0 ± 33.3	< 0.0001
ALT (IU/L)	44.4 ± 34.2	58.1 ± 47.1	62.5 ± 42.9	103.0 ± 72.9	94.6 ± 47.9	< 0.0001
Total protein (g/dL)	7.61 ± 0.30	7.78 ± 0.58	7.74 ± 0.44	7.83 ± 0.44	7.91 ± 0.48	0.4459
Albumin (g/dL)	4.52 ± 0.26 (n = 13)	4.44 ± 0.23 (n = 51)	4.32 ± 0.32	4.15 ± 0.24	4.12 ± 0.38	< 0.0001
γ-GTP (IU/L)	40.07 ± 26.10	57.52 ± 84.73	49.29 ± 32.91	75.79 ± 47.91	61.25 ± 40.46	0.2409
γ-globulin (g/dL)	1.22 ± 0.25 (n = 12)	1.44 ± 0.32 (n = 45)	1.52 ± 0.33 (n = 36)	1.62 ± 0.35 (n = 30)	1.83 ± 0.43 (n = 23)	< 0.0001
Total cholesterol (mg/dL)	179.5 ± 29.4	186.7 ± 31.5	173.8 ± 32.0	176.4 ± 26.8	162.3 ± 30.4	0.0251
Hyaluronic acid (ng/mL)	59.6 ± 83.1	49.64 ± 41.1 (n = 50)	110.3 ± 113.3	136.0 ± 138.4	266.6 ± 217.9 (n = 23)	< 0.0001

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase.

Table 3 Multiple regression predicting liver fibrosis stage with liver stiffness and laboratory data in the estimation group

	Estimated value	Standard error	χ^2	P-value
Liver stiffness (n = 165)	-0.2661602	0.0481713	30.53	< 0.0001
Platelet count (n = 165)	0.0748652	0.0366051	4.18	0.0408
Prothrombin time (n = 165)	0.0560460	0.0206514	7.37	0.0066
AST (n = 165)	-0.0093853	0.0100069	0.88	0.3483
ALT (n = 165)	0.0007594	0.0058897	0.02	0.8974
Albumin (n = 163)	-1.1130330	0.7353340	2.29	0.1301
γ-globulin (n = 146)	-0.6349751	0.5201549	1.49	0.2222
Total cholesterol (n = 165)	0.0011393	0.0057519	0.04	0.8430
Hyaluronic acid (n = 161)	-0.0019629	0.0017064	1.32	0.2500

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Comparison of Fibro-Stiffness index with LS, the APRI, the Forns index and the FibroIndex in the estimation group

Fibro-Stiffness index was compared with LS, the APRI, the Forns index and the FibroIndex in the estimation group (Figure 1). The values of Fibro-Stiffness index and LS significantly differed between neighboring fibrosis stages except F0-F1 (Figure 1A and B). The APRI did not significantly differ between any neighboring stages (Figure 1C). The Forns index significantly differed only between F1 and F2 (Figure 1D). The FibroIndex significantly differed between F1 and F2 and between F3 and F4 (Figure 1E).

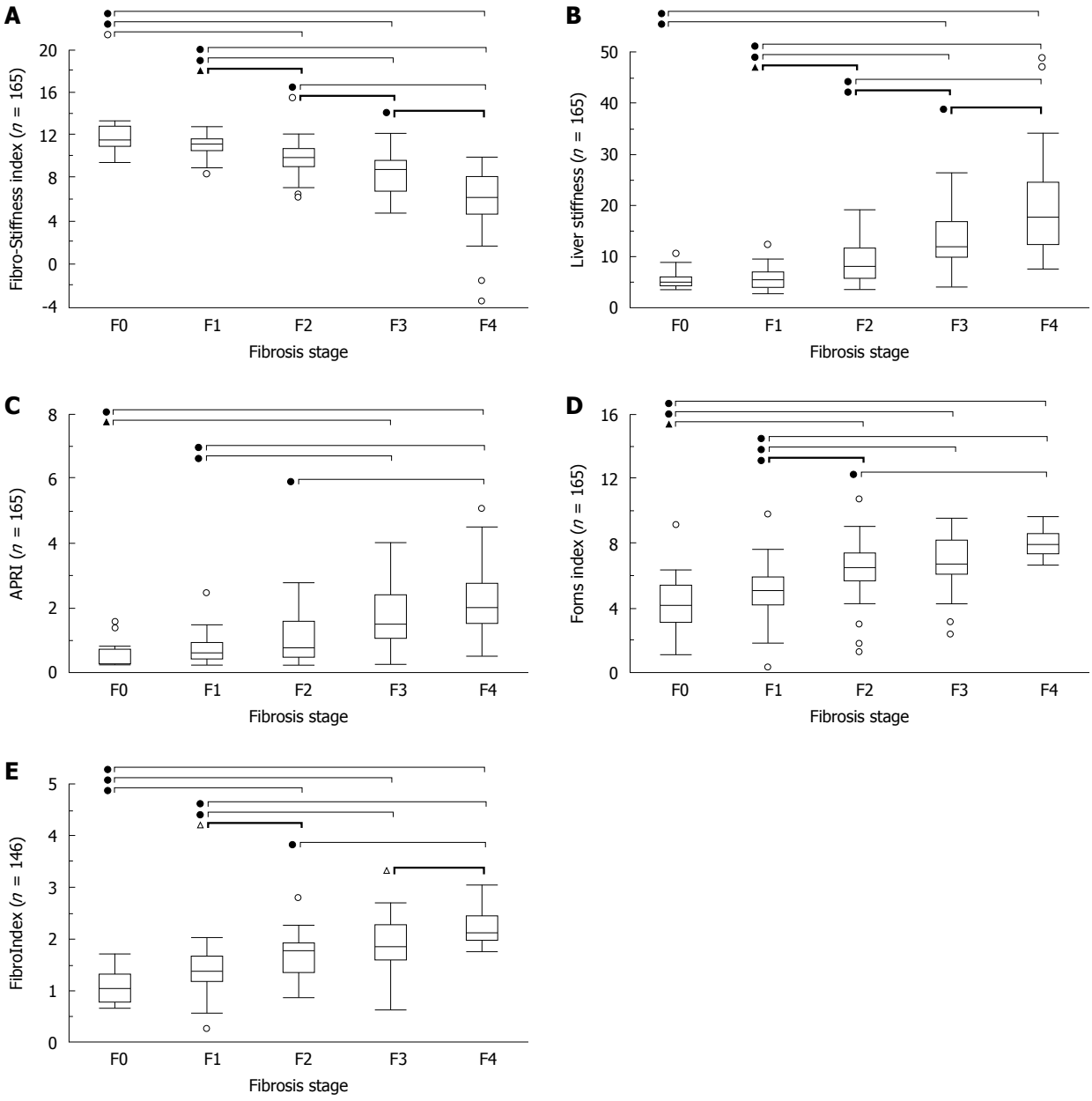

Figure 1 Correlation between 5 noninvasive methods for assessment of fibrosis and stage of fibrosis in the estimation group. A: Fibro-Stiffness index: ρ = -0.7626, *P* < 0.0001; B: Liver stiffness measured by FibroScan: ρ = -0.7340, *P* < 0.0001; C: Aspartate aminotransferase-to-platelet ratio index (APRI): ρ = 0.6008, *P* < 0.0001; D: Forns index: ρ = 0.6175, *P* < 0.0001; E: FibroIndex: ρ = 0.6496, *P* < 0.0001. The top and bottom of each box represent the 25th and 75th percentiles, giving the interquartile range. The line through the box indicates the median, and the error bars indicate the 10th and 90th percentiles. The closed circles, the closed triangles, the open circles and the open triangles indicate *P*-values of 0.001, 0.005, 0.01 and 0.05, respectively.

ROC analysis for comparison of diagnostic performance of the Fibro-Stiffness index with LS, the APRI, the Forns index and the FibroIndex in the estimation group

The ROC analysis of the Fibro-Stiffness index, LS, the APRI, the Forns index and the FibroIndex was performed to discriminate between fibrosis stages (Table 4). The AUC of the Fibro-Stiffness index was the highest for discriminating F ≥ 2, F ≥ 3 and F = 4 among the 5 examined methods. The AUC of the FibroIndex was the highest for discriminating F ≥ 1.

Optimal discriminating cut-off values of the Fibro-Stiffness index, LS, the APRI, the Forns index and the FibroIndex were determined by ROC analysis. The cut-off values of the Fibro-Stiffness index for F ≥ 1, F ≥ 2, F ≥ 3 and F = 4 were 11.09, 10.12, 9.87 and 8.51, respectively (Table 4). The diagnostic performance was assessed by sensitivity, specificity, accuracy, positive and negative predictive values, and likelihood ratio. Regarding accuracy, the values of the Fibro-Stiffness index for F ≥ 2, F ≥ 3 and F = 4 were the highest among the 5 examined methods. The value of the APRI was the highest for F ≥ 1.

Improvement of diagnostic performance by combination of the Fibro-Stiffness index with laboratory tests

The negative predictive value for F ≥ 1 and the positive

Table 4 Assessment of liver fibrosis stages classification by liver fibrosis indices

	AUCs (95% CI)	Optimal cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio
F ≥ 1 (F0 *vs* F1-2-3-4)								
Fibro-Stiffness index	0.82 (0.715-0.928)	11.07	77.5	78.6	77.6	97.5	24.4	3.62
Fibro-Stiffness index and AST	Non	11.07 and 51 (IU/L)	85.4	78.6	84.8	97.7	33.3	3.99
Liver stiffness	0.77 (0.666-0.867)	6.6 (kPa)	64.2	85.7	66.1	97.8	18.2	4.49
APRI	0.81 (0.680-0.934)	0.44	88.1	64.3	86.1	96.4	33.3	2.47
Forns index	0.77 (0.640-0.908)	4.72	82.1	71.4	81.2	96.9	27.0	2.87
FibroIndex	0.85 (0.757-0.932)	1.19	82.1	75.0	81.5	97.3	27.3	3.28
F ≥ 2 (F0-1 *vs* F2-3-4)								
Fibro-Stiffness index	0.90 (0.847-0.943)	10.12	78.8	89.4	83.0	91.8	73.8	7.43
Liver stiffness	0.88 (0.826-0.929)	7.1 (kPa)	80.8	80.3	80.6	86.0	73.6	4.10
APRI	0.78 (0.714-0.851)	1.06	64.6	84.8	72.7	86.5	61.5	4.27
Forns index	0.82 (0.757-0.890)	6.22	76.8	83.3	79.4	87.4	70.5	4.61
FibroIndex	0.82 (0.754-0.888)	17.00	74.2	80.7	76.7	85.7	66.7	3.84
F ≥ 3 (F0-2 *vs* F3-4)								
Fibro-Stiffness index	0.90 (0.851-0.953)	9.87	93.0	79.6	84.2	70.7	96.7	4.56
Liver stiffness	0.90 (0.856-0.952)	9.6 (kPa)	87.7	82.4	84.2	72.5	92.7	4.98
APRI	0.84 (0.767-0.904)	1.13	80.7	80.6	80.6	68.7	88.8	4.15
Forns index	0.80 (0.732-0.874)	6.36	82.5	70.4	74.5	59.5	88.4	2.78
FibroIndex	0.85 (0.749-0.901)	1.85	73.6	80.6	81.4	68.4	84.3	3.80
F = 4 (F0-1-2-3 *vs* F4)								
Fibro-Stiffness index	0.92 (0.871-0.965)	8.51	91.7	83.7	84.8	48.9	98.3	5.62
Fibro-Stiffness index and HA	Non	8.51 and 68 (ng/mL)	91.3	87.9	88.4	55.3	98.4	7.26
Liver stiffness	0.90 (0.844-0.957)	11.6 (kPa)	91.7	78.0	80.0	41.5	98.2	4.17
APRI	0.84 (0.759-0.915)	1.30	91.7	74.5	77.0	37.9	98.1	3.59
Forns index	0.87 (0.816-0.923)	7.07	95.8	75.9	78.8	40.4	99.1	3.97
FibroIndex	0.89 (0.830-0.943)	1.90	91.3	78.0	80.1	43.8	98.0	4.16

AUCs: Area under the receiver operating characteristics; CI: Confidence interval; HA: Hyaluronic acid; AST: Aspartate aminotransferase; APRI: Aminotransferase-to-platelet ratio index.

predictive value for F4 with the Fibro-Stiffness index were rather low. Thus a combination of the Fibro-Stiffness index with AST, ALT, albumin, γ globulin, total cholesterol and hyaluronic acid, which were correlated with fibrosis stages and not included in the Fibro-Stiffness index was examined to improve diagnostic performance in each fibrosis stage. Optimal discriminating cut-off values of these laboratory tests for F ≥ 1 and F = 4 were calculated by ROC analysis (not shown). In F ≥ 1, the best combination for improvement of diagnostic performance was the Fibro-Stiffness index ≤ 10.09 or AST ≥ 51 IU/L. The negative predictive value for F ≥ 1 was improved by this combination compared to the Fibro-Stiffness index alone, although it was same as that of the APRI (Table 4). The combination of the Fibro-Stiffness index ≤ 8.51 and serum hyaluronic acid ≥ 68 ng/mL was the best combination for F = 4. The negative predictive value for F4 was improved by this combination, and was the highest among the 6 examined methods.

Validation of performance of the Fibro-Stiffness index, its combination with AST for F ≥ 1, and its combination with hyaluronic acid for F4

The results in the estimation group were validated in the validation group of 120 patients with chronic hepatitis C (Table 5). The AUC of the Fibro-Stiffness index was the highest for F ≥ 3 and F = 4 among the 5 examined methods. The AUC of the FibroIndex was the highest for F ≥ 1 and that of LS was the highest for F ≥ 2.

The accuracy of the Fibro-Stiffness index for F ≥ 3 and F = 4 was 86.7% and 85.8%, respectively, similar to the values in estimation group, and the highest value among all the 5 methods. For F ≥ 1, the accuracy of the FibroIndex was the highest. For F ≥ 2, the accuracy of LS was the highest.

The combination of the Fibro-Stiffness index and AST for F ≥ 1 improved the negative predictive value, although it was lower than that of the FibroIndex, and the accuracy was lower than those of the APRI and the FibroIndex. The combination of the Fibro-Stiffness index and hyaluronic acid for F = 4 improved the positive predictive value, and its accuracy and positive predictive value were the highest among all the 6 examined methods.

DISCUSSION

In the present study, we constructed a new fibrosis index for non-invasive assessment of liver fibrosis, the Fibro-Stiffness index, using LS, platelet count and prothrombin time. LS measured by FibroScan has been reported to correlate with stage of liver fibrosis in various liver diseases[13-24]. Previous studies also confirmed that platelet count and prothrombin time also correlated with stage of liver fibrosis[6,11,26-29]. A decrease in the platelet count is caused by splenomegaly and reduced production of thrombopoietin, accompanied by the advance of liver fibrosis. Prolongation of prothrombin time is caused by reduced production of coagulation factors by the liver

Table 5 Validation of liver fibrosis stages classification by liver fibrosis indices

	AUCs (95% CI)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio
F ≥ 1 (F0 *vs* F1-2-3-4)							
Fibro-Stiffness index	0.80 (0.712-0.878)	73.6	78.6	74.2	94.0	27.7	3.52
Fibro-Stiffness index and AST	Non	78.3	78.6	78.3	96.5	32.4	3.64
Liver stiffness	0.82 (0.727-0.907)	63.2	85.7	65.8	97.1	23.5	4.42
APRI	0.80 (0.704-0.899)	87.7	35.7	81.7	91.2	27.8	1.36
Forns index	0.77 (0.659-0.880)	72.4	71.4	72.3	95.0	25.6	2.53
FibroIndex	0.83 (0.723-0.937)	89.6	50.5	85.8	93.1	38.9	1.79
F ≥ 2 (F0-1 *vs* F2-3-4)							
Fibro-Stiffness index	0.82 (0.750-0.898)	65.7	96.6	76.7	90.6	63.8	10.31
Liver stiffness	0.85 (0.787-0.920)	76.7	83.0	79.2	87.5	69.6	4.51
APRI	0.82 (0.741-0.893)	50.7	89.4	65.8	88.1	53.8	4.76
Forns index	0.78 (0.700-0.864)	58.3	80.9	67.2	82.4	55.9	3.05
FibroIndex	0.79 (0.713-0.875)	69.5	83.0	74.2	86.2	62.9	4.02
F ≥ 3 (F0-2 *vs* F3-4)							
Fibro-Stiffness index	0.93 (0.876-0.986)	93.9	83.9	86.7	68.9	97.3	5.84
Liver stiffness	0.92 (0.871-0.977)	81.8	82.8	82.5	64.3	92.3	4.75
APRI	0.83 (0.753-0.922)	75.8	81.6	80.0	61.0	89.9	4.12
Forns index	0.84 (0.759-0.912	78.1	71.3	73.1	50.0	90.0	2.72
FibroIndex	0.85 (0.772-0.928)	76.6	80.5	80.0	60.5	90.9	4.03
F = 4 (F0-1-2-3 *vs* F4)							
Fibro-Stiffness index	0.97 (0.934-0.997)	100	84.1	85.8	43.3	100	6.29
Fibro-Stiffness index and HA	Non	100	86.9	88.3	48.1	100	7.63
Liver stiffness	0.97 (0.942-0.999)	100	83.2	85.0	41.9	100	5.94
APRI	0.92 (0.867-0.972)	100	75.7	78.3	33.3	100	4.12
Forns index	0.88 (0.798-0.968)	83.3	72.9	73.9	25.6	97.5	3.07
FibroIndex	0.92 (0.874-0.971)	100	77.6	80.0	35.0	100	4.46

AUCs: Area under the receiver operating characteristics; CI: Confidence interval; HA: Hyaluronic acid; AST: Aspartate aminotransferase; APRI: Aminotransferase-to-platelet ratio index.

with advanced fibrosis. The Fibro-Stiffness index, which combines these 3 factors, was shown to be a highly accurate index to estimate fibrosis stage in chronic hepatitis C.

So far, several non-invasive fibrosis indices such as the APRI[11], the Forns index[6], the FibroIndex[12], and the FibroTest[7] have been developed. The Fibro-Stiffness index showed its superior correlation with fibrosis stage compared with the APRI, the Forns index and the FibroIndex. The Fibro-Stiffness index and LS showed a significant difference between neighboring fibrosis stages except between F0 and F1 in the estimation group. The AUC of the Fibro-Stiffness index was the highest among the 5 examined methods for F ≥ 2, F ≥ 3 and F = 4 in the estimation group, and for F ≥ 3 and F = 4 in the validation group. The AUCs of the APRI, the Forns index and the FibroIndex for predicting F4 in the present study were similar to the values reported in their respective original manuscripts (APRI, 0.88; Forns index, 0.81; FibroIndex, 0.86)[6,11]. Therefore, the results of the present study can be considered to be appropriate. The superiority of the Fibro-Stiffness index was further demonstrated by the accuracy values. The accuracy of the Fibro-Stiffness index was highest for F ≥ 2, F ≥ 3 and F = 4 in both the estimation group and validation group.

Although the Fibro-Stiffness index was shown to be a highly accurate index, the positive predictive value was rather low for F4. A combination of the Fibro-Stiffness index and hyaluronic acid was shown to improve the diagnostic performance. Serum hyaluronic acid has been reported to be useful for diagnosis of liver fibrosis and cirrhosis[8,30]. In the estimation group and in the validation group, both the accuracy and positive predictive value of the combination of the Fibro-Stiffness index and hyaluronic acid were higher than those of the Fibro-Stiffness index alone, and were the highest among all the 6 examined methods. The fact that a combination of the Fibro-Stiffness index and hyaluronic acid enables us to diagnose F4 with a sensitivity of 91%-100% and positive predictive value of 48%-57% is important, because the risk of hepatocellar carcinoma or bleeding from esophageal varices is high in patients with F4[2,3].

For predicting F ≥ 1, the Fibro-Stiffness index was inferior to the other fibrosis indices in terms of sensitivity, accuracy and negative predictive value. The combination of Fibro-Stiffness index with AST improved sensitivity, accuracy and negative predictive value in both the estimation group and the validation group. However, the combination of Fibro-Stiffness index with AST was still inferior to the APRI in the estimation group, and inferior to the FibroIndex and the APRI in the validation group. Further investigation is necessary to improve the diagnostic efficacy of the Fibro-Stiffness index for F ≥ 1.

In chronic viral hepatitis, the presence of significant fibrosis (F ≥ 2) indicates the need for antiviral therapies. The Fibro-Stiffness index showed a highly accurate diagnostic performance for F ≥ 2 in both the estimation group and validation group. Thus the patients with a Fibro-Stiffness index of ≥ 10.12 which indicate F ≥ 2

will be candidates for liver biopsy or interferon treatment.

In conclusion, a new fibrosis index for non-invasive assessment of liver fibrosis, the Fibro-Stiffness index, was constructed using LS measured by FibroScan, platelet count and prothrombin time and was validated. The Fibro-Stiffness index demonstrated superior diagnostic performance to LS alone, the APRI, the Forns index and the FibroIndex for F $\geqslant$ 2, F $\geqslant$ 3 and F = 4. The diagnostic performance of the Fibro-Stiffness index for F4 was further improved by combination with hyaluronic acid levels.

COMMENTS

Background

The stage of liver fibrosis is important for clinical management of chronic hepatitis C, since the treatment and prognosis of chronic hepatitis depend on the fibrosis stage. Liver biopsy is the gold standard for the assessment of fibrosis stage. However, it is an invasive and expensive procedure, and its accuracy is sometimes questionable.

Research frontiers

A number of non-invasive fibrosis indices, such as the aminotransferase-to-platelet ratio index (APRI), the Forns index and the FibroIndex have been proposed for assessment of liver fibrosis. Transient elastography with the use of a new apparatus, FibroScan, for measurement of liver stiffness (LS) was developed. LS has been reported to correlate with liver fibrosis in various liver diseases. So far no fibrosis indices incorporating LS have been reported. In the present study, we developed a new non-invasive fibrosis index, the Fibro-Stiffness index, which incorporated LS.

Innovations and breakthroughs

The Fibro-Stiffness index consists of LS, platelet count and prothrombin time. In the present study, its usefulness was compared with LS, the APRI, the Forns index and the FibroIndex. The diagnostic performance of the Fibro-Stiffness index was superior to other indices. Furthermore, the diagnostic performance of the Fibro-Stiffness index for F4 was further improved by combination with hyaluronic acid.

Applications

Using the Fibro-Stiffness index, it is possible to assess the stage of liver fibrosis of patients with chronic hepatitis C non-invasively, accurately and quantitatively. Therefore, the Fibro-Stiffness index is useful not only for the diagnosis of stage of liver fibrosis but also for the assessment of regression of liver fibrosis by interferon treatment in patients with chronic hepatitis C.

Terminology

Fibro-Stiffness index: a new non-invasive fibrosis index which we developed in the present study and consists of LS, platelet count and prothrombin time. Its diagnostic performance is superior to other indices.

Peer review

The authors proposed a novel index for non-invasive assessment of hepatic fibrosis. Its reliability was validated on another group of patients. I think the index is clinically useful and significant.

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BRIEF ARTICLE

Helicobacter species and common gut bacterial DNA in gallbladder with cholecystitis

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Abstract

AIM: To analyze the association between *Helicobacter* spp. and some common gut bacteria in patients with cholecystitis.

METHODS: A nested-polymerase chain reaction (PCR), specific to 16S rRNA of *Helicobacter* spp. was performed on paraffin-embedded gallbladder samples of 100 cholecystitis and 102 control cases. The samples were also analyzed for some common gut bacteria by PCR. Positive samples were sequenced for species identification.

RESULTS: *Helicobacter* DNA was found in seven out of 100 cases of acute and chronic cholecystitis. Sequence analysis displayed *Helicobacter pullorum* (*H. pullorum*) in six cases and *Helicobacter pylori* in one; *H. pullorum* was only found in cases with metaplasia. Control samples were negative for *Helicobacter* spp. and some common gut bacteria. There was a significant difference (P = 0.007) between cholecystitis and control samples for *Helicobacter* DNA.

CONCLUSION: A possible relationship was detected between *Helicobacter* DNA and cholecystitis. Further serological and immunohistochemical studies are needed to support these data.

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Key words: *Helicobacter*; Gallbladder; Cholecystitis; 16S rRNA; Polymerase chain reaction

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INTRODUCTION

The most well-known member of the *Helicobacter* genus, *Helicobacter pylori* (*H. pylori*), is classified as a type 1 carcinogen[1], and infects the human stomach and causes gastritis, peptic ulcer disease and gastric cancer. Besides *H. pylori*, the genus *Helicobacter* contains more than 25 species[2], many of which cause extragastric diseases in humans and animals[3-10]. These are named enterohepatic *Helicobacter* species (EHS) or EHS and colonize the hepatobiliary tract of humans, and include *Helicobacter hepaticus* (*H. hepaticus*), *Helicobacter bilis* (*H. bilis*), *Helicobacter rappini* (*H. rappini*), *Helicobacter ganmani* (*H. ganmani*) and *Helicobacter pullorum* (*H. pullorum*). Several of these EHS are associated with the pathogenesis of chronic biliary disorders, such as cholecystitis, cholelithiasis, gallbladder carcinoma and bile tract carcinoma and some liver diseases, such as primary

sclerosing cholangitis, primary biliary cirrhosis and hepatocellular carcinoma[11-14]. Moreover, chronic pancreatitis and pancreatic cancer, as well as inflammatory bowel diseases in humans have also been reported to be positive for EHS in various polymerase chain reaction (PCR)-based studies[15-17].

Chronic cholecystitis is the most prevalent disease in various populations in industrialized countries[18]. During the 20 years from 1965-1969 to 1985-1989, the mortality from gallbladder cancer increased by 30% in Sweden. However, not all high-risk European countries showed such an increase and the mortality decreased in some countries[19]. Chronic cholecystitis is commonly associated with gallstone disease[20] and some studies have shown that cholecystitis and gallstones can cause epithelial hyperplasia of the gallbladder mucosa or cancer, and various bacterial genomes have been detected in gallbladder carcinoma tissue[21]. Moreover, a recent study has shown that *H. pylori* can damage human gallbladder epithelial cells *in vitro*, and could be the key factor that leads to clinical cholecystitis[22]. Some studies have revealed the presence of bile-resistant EHS in the gallbladder mucosa and in gallstones. It has been shown that the presence of *H. pylori* and EHS in bile might represent a risk factor for bile stone formation[4,23-26]. One study has clearly demonstrated the presence of a mixed bacterial population in gallstones[4]. *Salmonella typhi* is another bacterial pathogen of the biliary tree in human gallstones and gallbladder cancer[27,28]. *Salmonella* biofilm has been shown on human gallstones[29]. Moreover, *Campylobacter* spp. have also been detected in bile and epithelial samples in cholecystolithiasis[30].

H. pylori, *H. pullorum* and *H. bilis* have been isolated from humans with gallbladder disease such as cholecystitis, cholelithiasis[9,31,32], gallbladder carcinoma and bile tract carcinoma[33]. A possible relationship between chronic cholecystitis and *Helicobacter* DNA has been shown by some investigators[9,31,32,34] but, as far as we are aware, there has been no study published on Scandinavian patients with cholecystitis. Therefore, we examined the relationship between *Helicobacter* spp. and some common gut bacteria in Swedish patients with cholecystitis.

MATERIALS AND METHODS

Patients and histological methods

We re-examined the gallbladders from 100 cholecystitis patients from 2006-2007 (mean age: 48 years; range: 20-84 years; 35 male, 65 female) and 102 control patients (mean age: 58 years; range: 11-85 years; 54 male, 48 female) from 1999 to 2009, taken from the files of the Department of Pathology, Lund University Hospital. Of the 100 cholecystitis samples, 50 were acute (mean age: 55 years; range: 23-81 years; 22 male, 28 female), and 50 were chronic (mean age: 44 years; range: 20-84 years; 13 male, 37 female). Among the 50 patients with acute cholecystitis, 34 cases (median age: 56 years; range: 23-79 years; 15 male, 19 female) were without metaplasia and 16 (median age: 54 years; range: 36-81 years; 7 male, 9 female) had metaplasia. Among the 50 patients with chronic cholecystitis, 27 cases (median age: 45 years; range 20-84 years; 8 male, 19 female) were without metaplasia and 23 (median age: 42 years; range: 20-71 years; 5 male, 18 female) had metaplasia. As control samples, we used 18 normal gallbladders from patients with pancreatic malignancies reported elsewhere[17], and 84 consecutive patients with normal gallbladders from 1999 to 2009 (median age: 61 years; range: 11-85 years; 44 male, 40 female). There was no metaplasia in these gallbladders. The diagnosis was: six hepatocellular carcinoma, 40 liver metastases (mainly colorectal), four intestinal carcinoids, three liver carcinoid metastases, seven focal nodal hyperplasias, three bile duct cysts, one gallbladder adenoma, three splenomegalies, two pancreatic neuroendocrine malignancies, one benign pancreatic cyst, one adrenal carcinoma, and 13 normal gallbladders with no other diagnosis.

Two to five sections were taken from each case, and one section from the ductus cysticus. Sections that showed mucosal metaplasia were stained with Alcian blue-periodic acid Schiff (AB-PAS), pH 2.5, and Warthin-Starry silver stain for *Helicobacter* spp. One section was immunostained with anti-*H. pylori* antibody (DAKO, Glostrup, Denmark; diluted 1:300) according to Apostolov *et al*[9]. Mucosa was cut from the paraffin blocks with the tip of a scalpel by careful comparison with the slides. Areas with gastric metaplasia, if present, were included in the samples. The Research Ethics Committee at Lund University approved this study (permit number 588/2006).

DNA extraction

DNA was extracted from approximately 5 mg of each paraffin-embedded gallbladder tissue sample. To ascertain that epithelium was included, two pieces, each of 2-3 mg, were taken from each case. Paraffin-embedded gallbladder samples were de-embedded as previously described[10]. Gallbladder tissue samples were de-embedded by heating at 60℃ for 10 min, followed by washing in xylene for 2 × 5 min. The specimens were rehydrated through graded ethanol (99% and 95% for 2 × 5 min and 70% for 5 min), and finally washed for 5 min in double-distilled water. DNA was extracted by a QIAamp DNA Mini Kit tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracts (200 μL total volume) were combined, and 5 μL of the mixtures was analyzed by PCR.

Helicobacter-specific PCR

DNA extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA) using a semi-nested PCR assay specific for *Helicobacter* 16S rDNA, as previously described[11], using primers 1F (5'CTATGACGGGTATCCGGC3'), 1R (5'CTCACGACACGAGCTGAC3') and 2R (5'TCGCCTTCGCAATGAGTATT3'). Primers 1F and 1R were used in the first step, whereas primers 1F and 2R were used in the second step. The reaction mixture of the first step (25 μL) contained 0.5 μmol/L each primer (1F and 1R), 0.8 mmol/L

each dNTP (Amersham Biosciences, Uppsala, Sweden), 1 × chelating buffer, 2.5 mmol/L $MgCl_2$, 0.05% casein, 0.05% formamid, 1.25 U r*Tth* DNA polymerase (Applied Biosystems), and 5 μL extracted DNA. *H. pylori* (CCUG 17874) was used as a positive control in all PCR reactions. The amplification conditions for the first step were 94℃ for 2 min; 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s; and finally 72℃ for 5 min. The reaction mixture of the second step (25 μL) contained 0.5 μmol/L each primer (1F and 2R), 0.2 mmol/L each dNTP, 1 × buffer Ⅱ, 2.5 mmol/L $MgCl_2$, 1.0 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and 2 μL 10 × diluted PCR product from the first step. The 416-bp PCR products were visualized by 1.3% agarose gel electrophoresis.

Amplification of non-Helicobacter bacteria

Enterobacteriaceae-, *Bacterioides-Prevotella* group- *and Enterococcus*-specific PCRs were performed. The reaction mixture and amplification conditions, except for annealing temperatures, for non-*Helicobacter* PCR assays were the same as in the first step of the semi-nested *Helicobacter* PCR. The annealing temperatures and primers used for detection of Enterobacteriaceae, *Bacterioides-Prevotella* group and *Enterococcus* were as described before[11]. Primers Eco1457F (5'CATTGACGTTACCCGCAGAAGAAGC3') and Eco1652R (5'CTCTACGAGACTCAAGCTTGC3') were used to amplify Enterobacteriaceae and primers Ent1F (5'TACTGACAAACCATTCATGATG3') and Ent2R (5'AACTTCGTCACCAACGCGAAC3') were used to amplify *Enterococcus,* whereas primers Bac303F (5'GAAGGTCCCCCACATTG3') and Bac708R (5'CAATCGGAGTTCTTCGTG3') were used to amplify the *Bacteroides-Prevotella* group. As positive controls, *Escherichia coli* (CCUG 17620), *Bacteroides fragilis* (CCUG 4856), and *Enterococcus faecalis* (CCUG 9997) were used in all PCR reactions. The 112-bp PCR product of *Enterococcus,* 418-bp product of *Bacteroides* and 195-bp product of Enterobacteriaceae were visualized by 1.3% agarose gel electrophoresis.

DNA sequence analysis

Helicobacter-specific PCR products were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. DNA sequence reactions were performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems), as described by Tolia *et al*[10]. Products of the sequence reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis.

Statistical analysis

Statistical analyses were done by χ^2 and Fisher's exact tests. $P < 0.05$ was considered to be significant.

RESULTS

Histology

Little metaplasia was detected in the sections and only a few glands or a few cells displayed gastric (antrum) metaplasia and/or acid mucin. Acid or neutral mucins were often seen only in parts of the epithelial cell cytoplasm. The AB-PAS staining method for metaplasia revealed among the chronic cases three with only gastric metaplasia (neutral mucosubstances), five with only non-gastric metaplasia (acid mucosubstances), and 15 with both types. For acute cholecystitis, these figures were two, five and nine, respectively (Table 1). The two types of metaplasia are displayed in Figure 1. Whartin-Starry staining and immunohistochemistry for *H. pylori* were negative in all studied specimens. The *H. pylori*-positive specimen was from a case of acute cholecystitis with extensive necrosis, but with a small area of preserved epithelium without metaplasia, from which the sample was taken.

Table 1 Number of cases with metaplasia in patients with cholecystitis

	Acute	Chronic
Gastric	2	3
Non-gastric	5	5
Both	9	15
None	34	27
Total	50	50

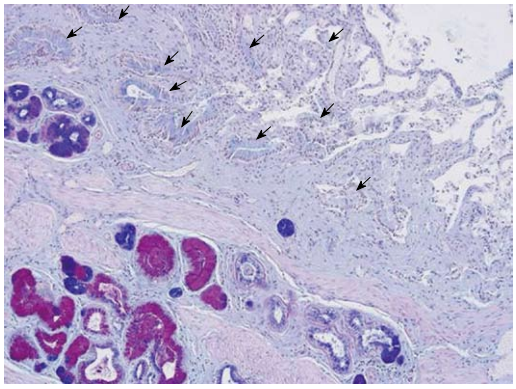

Figure 1 Histological section of ductus cysticus from a patient with chronic cholecystitis. Low-power view displaying antrum-type (red) and intestinal-type (blue) mucous metaplasia in glands. The ductus lumen is seen in the opposite corner of the photo with folds of the mucosa layer covered by epithelium without metaplasia, arrows (no intense color). Alcian blue-periodic acid Schiff staining.

Helicobacter-specific PCR assay and sequencing results

Using the *Helicobacter*-specific PCR assay and agarose electrophoresis, *Helicobacter* DNA was detected in 7/100 of gallbladder specimens of patients with cholecystitis. There were 4/50 (8%) and 3/50 (6%) samples positive for *Helicobacter* spp. among acute and chronic cholecystitis patients, respectively. Six samples showed 98-99% sequence similarity to *H. pullorum* and one to *H. pylori* (Table 2). *H. pullorum* was only found in cases with metaplasia, in six out of 39, as compared to none out of 61 without metaplasia. The difference was statistically significant ($P = 0.002$). All control samples were negative for *Helicobacter* spp. The difference between *Helicobacter* DNA prevalence in gallbladder

Table 2 Prevalence of *Helicobacter* spp. and some common gut bacteria *n* (%)

Patient group	*Helicobacter* PCR	Gut bacteria PCR	Sequencing results (No. of samples)
Acute cholecystitis	4/50 (8)	0/50 (0)	*H. pylori* (1) *H. pullorum* (3)
Chronic cholecystitis	3/50 (6)	0/50 (0)	*H. pullorum* (3)
Controls	0/102 (0)	0/102 (0)	-

Results are shown as the number of positive patients and the number of all patients in the group followed by the percentage in parenthesis. PCR: Polymerase chain reaction; *H. pylori*: *Helicobacter pylori*; *H. pullorum*: *Helicobacter pullorum*.

Table 3 Prevalence of *Helicobacter* DNA in cholecystitis mucosa in different studies from various geographical regions

Region	Prevalence (%)	Patients (*n*)	Ref.
Germany	2	1/57	Bohr *et al*[35] 2007
Japan	12-13	2/16	Murata *et al*[36] 2004
	27	4/15	Fukuda *et al*[37] 2002
China	27.2	22/81	Chen *et al*[34] 2007
Chile	39	9/23	Fox *et al*[31] 1998
Ukraine	73	16/22	Apostolov *et al*[9] 2005

of cholecystitis patients and controls was also significant ($P = 0.007$).

PCR and sequence detection of bacterial DNA other than Helicobacter

None of the tested patients' samples with acute and chronic cholecystitis and control samples was positive using the *Bacteroides*-, Enterobacteriaceae- and *Enterococcus*-specific PCR assays.

DISCUSSION

Helicobacter DNA was found in 7% of cholecystitis mucosa (8% acute, 6% chronic cholecystitis); none of the control samples was positive for *Helicobacter*. There are several reports on the presence of *Helicobacter* DNA in cholecystitis mucosa (Table 3). The studies in Germany, China and Japan with a prevalence of 2%-27% were more similar to our study[34-37] than was the study in Chile (39% prevalence)[31]. However, in a study from Ukraine (73%) the prevalence was much higher than in our study[9].

Six samples (three from acute and three from chronic cholecystitis) were positive for *H. pullorum*. Fox *et al*[31] have reported a link between EHS infections and chronic cholecystitis. *H. bilis* was the most common but *H. pullorum* was also reported[31]. Apostolov *et al*[9] have developed a first generation of enzyme immunoassays and immunoblotting to serodiagnose EHS infections in mice and humans. *H. pullorum* was found in 18% of patients with hepatitis C virus by immunohistochemistry in one of our previous studies[38]. However, *H. pullorum* is most commonly seen in poultry[39]. There is most likely a zoonotic transmission between humans and chickens by undercooked chicken.

One sample with a similar sequence to *H. pylori* was detected. Other studies on gallbladders or gallstones from patients with cholecystitis and cholelithiasis have shown the presence of *H. pylori*[9,32,40]. Other *Helicobacter* species have also been detected in different studies such as, *H. rappini*, *H. ganmani*[35] and *H. hepaticus*[41].

Kawaguchi *et al*[42] were the first to demonstrate *Helicobacter* spp. in cholecystits mucosa that displayed gastric metaplasia. Metaplasia was seen in all cases of cholecystitis in a Chilean study[31], in 15% of cases in a British study[43], and in 14% of cases in a Ukrainian study[9]. In the British study, no *Helicobacter* was found by immunostaining. Our results confirm the importance of gastric metaplasia for detection of *Helicobacter* DNA. Misra *et al*[40] have detected *Helicobacter* only in areas with gastric metaplasia, with a prevalence of 45%, but could not detect *Helicobacter* DNA in paraffin blocks or formalin-fixed mucosal tissue.

None of the gallbladder samples was positive for *Bacteroides* and *Enterococcus* spp. in our study. Enteric bacteria have been detected from gallstones and bile samples by culturing and PCR methods in some studies[44-47], but not by fluorescence *in situ* hybridization[48].

Apart from geographical differences, the variation in *H. pylori*, EHS and some gut pathogens between countries could be due to the use of different PCR methods. Our PCR technique was evaluated as a highly reliable method for genus level identification of *Helicobacter* spp.[49], and inhibitors that might influence the PCR results have been discussed in our other studies[50]. Moreover, some of the studies have used inappropriate control groups. We selected 102 normal control gallbladders from patients diagnosed with diseases other than cholecystitis.

In cholecystitis, *Helicobacter* DNA might preferentially or only be found in epithelial cells or on their surface, thus, much care has to be taken when selection the samples from the paraffin blocks.

In conclusion, several *Helicobacter* spp. infect a range of hosts (most probably, certain species are pathogens in some animals and humans). Divergent results might be due to different geographical areas, different PCR methods, using different control groups or lack of control groups, and sampling from different areas of the biopsy. The present study shows the possible relationship between *Helicobacter* spp. and cholecystitis in Swedish patients. Further studies are needed to determine the possible role of EHS and other pathogens in biliary tract infections and the possible relationship to various hepatobiliary malignancies such as cholangiocarcinoma.

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COMMENTS

Background

Helicobacter genus has nearly 25 species and many of them cause extragastric diseases in humans and animals.

Research frontiers

Helicobacter DNA in gallbladder mucosa has been reported with different prevalence and is associated with several biliary tract diseases, but there are still doubts about the relationship between enterohepatic *Helicobacter* species (EHS), *Helicobacter pylori* and hepatobiliary diseases.

Innovations and breakthroughs

Recent reports have highlighted the presence of *Helicobacter* in the biliary tract in different regions. However, this is believed to be the first study to report the possible relationship between chronic cholecystitis in Scandinavian patients.

Applications

By understanding the relationship between *Helicobacter* and cholecystitis, this study could represent a future strategy for further pathological studies of patients with cholecystitis.

Terminology

EHS are species in the genus *Helicobacter* that colonize the hepatobiliary tract and can cause extragastric diseases in humans or in animals.

Peer review

The authors have tackled a newly developing area of interest to many researchers. The work is a contribution to the study of the association between *Helicobacter* spp. and some common gut bacteria in patients with cholecystitis. They concluded that there is a possible relationship between *Helicobacter* DNA and cholecystitis, and recommended further serological and immunohistochemical studies to support their data.

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BRIEF ARTICLE

Tumor budding predicts response to anti-EGFR therapies in metastatic colorectal cancer patients

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Abstract

AIM: To investigate whether the evaluation of tumor budding can complement K-RAS analysis to improve the individualized prediction of response to anti-epidermal growth factor receptor based therapies in metastatic colorectal cancer (mCRC) patients.

METHODS: Forty-three patients with mCRC treated with cetuximab or panitumumab were entered into this study. According to the Response Evaluation Criteria in Solid Tumors criteria, 30 patients had stable or progressive disease (non-responsive), while 13 patients had a partial response. Tumor buds were evaluated from whole tissue sections stained for pan-cytokeratin, evaluated in the densest region using a 40 × objective and "high-grade" tumor budding was defined as 15 buds/high-power field.

RESULTS: Tumor buds and K-RAS mutation both correctly classified 68% of patients. All patients with K-RAS mutation (n = 7) or high-grade tumor budding (n = 11) were non-responsive, of which 4 patients had both features. All 13 partial responders were K-RAS wild-type with low-grade tumor budding. Combined, the predictive value of K-RAS and tumor budding was 80%. Additionally, high-grade tumor budding was significantly related to worse progression-free survival [HR (95% CI): 2.8 (1.3-6.0, P = 0.008)].

CONCLUSION: If confirmed in larger cohorts, the addition of tumor budding to K-RAS analysis may represent an effective approach for individualized patient management in the metastatic setting.

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Key words: Anti-epidermal growth factor receptor therapy; Colorectal cancer; K-RAS; Prognosis; Tumor budding

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INTRODUCTION

Between 40%-50% of all patients with colorectal cancer are diagnosed with metastatic colorectal cancer (mCRC)[1]. Overall 5-year survival rates for these patients are still less than 10% despite improvements in treatment and com-

bined systemic chemotherapies. Monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) such as cetuximab and panitumumab have recently been approved for the treatment of mCRC patients, however, response rates in general vary from 10%-20%[2,3]. Several molecular and protein biomarkers are currently being intensively investigated for their potential predictive value including K-RAS, B-RAF, PIK3CA and PTEN. Although recent randomized clinical trials have not been unanimous concerning the predictive value of K-RAS on outcome, the vast majority of studies to date do support a lack of responsiveness in patients with mutation[4-9]. These data have led the American Society of Clinical Oncology, Food and Drug Administration and European Medicines Agency to recommend that patients with mCRC be tested for K-RAS gene mutation before administration of EGFR-targeted therapies[10]. It is, however, clear that not all patients with wild-type K-RAS tumors achieve a response to anti-EGFR therapies and the results concerning other genetic alterations are not conclusive, suggesting that continued efforts on predictive biomarkers are warranted.

In colorectal cancer, tumor buds, defined as dedifferentiated single cells or clusters of up to 5 cells at the invasive tumor front, are considered the histological hallmark of epithelial mesenchymal transition and are thought to be responsible for the subsequent steps in invasion and metastasis[11]. Although tumor buds can be observed using regular hematoxylin and eosin (HE) slides, evaluation is facilitated by pan-cytokeratin stains[12]. Tumor budding has consistently been linked to higher tumor grade, vascular and lymphatic invasion and is highly predictive of both lymph node and distant metastasis stage[13-25]. Moreover, most studies confirm that high-grade tumor budding is an independent prognostic factor and recognized as such by the American Joint Committee on Cancer and International Union against Cancer (AJCC/UICC)[26]. In addition, we have previously shown that tumor budding is not related to mutation of K-RAS, leading to the hypothesis that this histomorphological feature could perhaps be used to complement the assessment of response in mCRC patients treated with anti-EGFR-based therapies[27].

Therefore, the aim of this study was to evaluate the predictive and prognostic value of tumor budding and determine its complementary value to K-RAS gene status in mCRC patients treated with cetuximab or panitumumab-based regimens.

MATERIALS AND METHODS

Patients and specimen characteristics

Forty-three consecutive patients with histologically confirmed mCRC treated at the Oncology Institute of Southern Switzerland, Bellinzona, Switzerland with cetuximab or panitumumab-based regimens were entered into this retrospective study. Cetuximab was administered at a standard loading dose of 400 mg/m^2 over 2 h, followed by a weekly dose of 250 mg/m^2 over 1 h. Single agent panitumumab 6 mg/kg every 2 wk was administered to 2 patients who were refractory to oxaliplatin- and irinotecan- based regimens. With the exception of 2 patients who received cetuximab as frontline therapy, the others had failed at least one prior chemotherapy regimen. For those who progressed on irinotecan-based regimens, cetuximab was administered in combination with these regimens given at the same dose and schedule. Therefore, patients were selected based on evidence that treatment outcome was attributable only to the administration of cetuximab or panitumumab. Treatment was continued until progressive disease (PD) or toxicity occurred. Response was assessed every 6-8 wk by means of computerized tomodensitometry or nuclear magnetic resonance. The Response Evaluation Criteria in Solid Tumors were adopted for evaluation and objective tumor response was classified into complete response (CR), partial response (PR), stable disease (SD) and PD[28]. Accordingly, only patients who achieved either CR or PR were considered as responders.

Assay methods

K-RAS, B-RAF and PIK3CA mutational status: Formalin-fixed paraffin-embedded surgical resection specimens were available for all patients. We searched for point mutations in K-RAS exon 2 (including codons 12 and 13), *BRAF* exon 15 (including codon 600) and PIK3CA exons 9 and 20 (including codons 542, 545 and 1047). All samples were subjected to automated sequencing by ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA) and analysed with appropriate software (Applied Biosystems). Each sequence reaction was performed at least twice, starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

Epidermal growth factor receptor: fluorescent in situ hybridization

The *EGFR* gene status evaluation was performed by fluorescent *in situ* hybridization (FISH) on 3-μm thick tissue sections. Tissue sections were treated using Paraffin Pretreatment Kit Ⅱ (Abbott Molecular AG, Baar, Switzerland) according to the manufacturer›s instructions. Dual-colour FISH assay was performed using LSI EGFR/CEP7 probes (Vysis). The LSI EGFR probe is labelled in SpectrumOrange and covers an approximately 300 kb region that contains the entire *EGFR* gene at 7p12. The CEP7 probe, labelled in SpectrumGreen, hybridises to the α satellite DNA located at the centromere of chromosome 7 (7p11.1-q11.1). Target sections and probe were co-denatured at 75℃ for 5 min and allowed to hybridise overnight at 37℃. Post-hybridisation stringency wash was carried out in a water bath at 72℃ for 5 min. After washing twice and drying at room temperature for 10 min, slides were mounted with 4'6-diamidino-2-phenylindole (DAPI Ⅱ, Abbott Molecular). Fluorescent *in situ* hybridization signals were evaluated with a Zeiss Axioscope equipped with single and triple band pass filters. Images for documentation were captured using an AxioCam camera and processed using the AxioVision system. Patients

showing two of chromosome 7 in the vast majority of cells were classified as eusomic. Patients with an aberrant number of chromosome 7, defined as more than 4 in at least 50% of cells, were classified as markedly polysomic. Patients with a ratio more than 3 between the *EGFR* gene and chromosome 7 centromere signals in at least 10% of cells were classified as having *EGFR* gene amplification[29].

Immunohistochemistry

Immunohistochemistry staining was performed for both CK22 (an epithelial cell marker facilitating the visualization of tumor buds) and PTEN. Paraffin-embedded tissue blocks were cut at 3 μm. Whole tissue sections were de-waxed and re-hydrated in dH_2O. Following pressure cooker-mediated antigen retrieval in 0.001 mol/L ethylenediaminetetraacetic acid pH 8.0, endogenous peroxidase activity was blocked using 0.5% H_2O_2. Sections were incubated with 10% normal goat serum for 20 min. After incubation with primary antibody (PTEN Ab-4, Neomarkers, Fremont, CA, USA; 1:50 and CK22 polyclonal, Genetex, Inc, 1:100), sections were incubated with HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) for 30 min at room temperature, immersed in 3-amino-9-ethylcarbazole+substrate-chromogen (DakoCytomation) for 30 min, and counterstained with haematoxylin. PTEN protein expression was detected mainly at the cytoplasmic level, although occasional nuclear positivity was present. PTEN negative tumors were those showing a dramatic reduction or absence of immunostaining in at least 50% of cells, as compared with the internal control. The evaluations were performed without knowledge of clinical data or the results of other analyses.

Assessment of tumor budding

Tumor budding was defined as dedifferentiated single cells or clusters of < 5 cells at the invasive tumor front. In all cases, the tumor invasive front was scanned at low power using a 5 × objective lens and the region of densest tumor budding was identified. The number of tumor buds within this region was counted using a 40 × objective lens. Evaluation was performed blinded to clinical endpoints. Inter-observer agreement was assessed between independent observers (Lugli A, Vlajnic T, Zlobec I). Discordant cases were discussed until agreement was reached. High-grade tumor budding was defined as 15 tumor buds/HPF.

Study design

The study was designed as a retrospective analysis. The main objective was to correlate response to anti-EGFR-based therapies with pathological and molecular findings. The secondary endpoint was represented by the correlation of tumor budding with progression-free survival (PFS) and overall survival.

Statistical analysis

Threshold values for determining high-grade *vs* low-grade tumor budding were assessed using receiver operating characteristic curve analysis with 100- bootstrapped replications of the data. The sensitivity, specificity, positive predictive value and negative predictive value (NPV) for high-grade tumor budding, EGFR amplification or copy number gain, K-RAS, B-RAF, PIK3CA and loss of PTEN as well as their association with response were evaluated by simple logistic regression analysis. Inter-observer variability of tumor budding (low-grade/high-grade) was assessed by the κ statistic and by investigating the percentage of concordance between independent observers. Univariate and multivariable PFS time differences stratified by tumor budding and after adjustment for K-RAS mutational status were evaluated using simple and multiple Cox regression analysis, respectively, after verification of the proportional hazards assumption. The Kaplan-Meier method was used to illustrate PFS time differences by tumor budding grade. Fisher's Exact test was used to determine the association of tumor budding for response in subgroup analysis. Finally, classification and regression tree analysis (CART) methods were used to determine the features best predicting response to treatment[30]. The CART trees were fitted using DTREG statistical software. To assess the amount of overfitting, 100 10-fold cross-validation experiments were performed[31]. In each of those 100 experiments, the data set was randomly split into 10 smaller data sets and a pruning method was used to choose the best number of nodes for the original tree pruned with respect to 90% of the data according to the misclassification rate for the other 10% of the data. To resolve uncertainty in assessing the optimal number of terminal nodes for the full data set, we conducted a two-tailed Fisher's exact test to test for a relationship between tumor budding, K-RAS mutation and response to therapy. Given the significant association of both these features with response, CART analysis was performed for patients with low-grade tumor budding and K-RAS wild-type gene status only. A second CART analysis was performed conditioning only on K-RAS wild-type patients.

RESULTS

Patient characteristics

The present study analyzed forty-three patients, 26 men (60%) and 17 women (40%). Patient characteristics and response by treatment with anti-EGFR monoclonal antibodies are summarized in Table 1. Median survival time was 37.3 mo (range 3.6-180) and PFS time was 16.0 mo (range 1-171). Thirteen patients (30%) achieved PR after cetuximab- or panitumumab-based therapy.

Association of tumor budding with molecular features

The percentage of concordance between observers was 88% with a κ value of 0.6. Tumor budding and K-RAS gene status was evaluable in all cases. High-grade tumor budding occurred in 11 cases (25.6%) while low-grade tumor budding was found in the remaining 32 patients (74.4%). Tumor budding was not significantly associated with either EGFR status (P = 0.95), K-RAS (P = 0.43),

Table 1 Characteristics of metastatic colorectal cancer patients treated with anti-epidermal growth factor receptor therapy (*n* = 43) *n* (%)

Clinico-pathological feature	Frequency
Age (yr), median (range)	64 (26-82)
Gender	
Male	26 (60.5)
Female	17 (39.5)
Response	
Progressive disease	19 (44.2)
Partial response	13 (30.2)
Stable disease	11 (25.6)
EGFR	
No Amplification/gene copy number gain	4 (10.5)
Amplification/gene copy number gain	34 (89.5)
K-RAS	
Wild-type	32 (74.4)
Mutation	11 (25.6)
B-RAF	
Wild-type	38 (88.4)
Mutation	5 (11.6)
PIK3CA	
Wild-type	41 (95.4)
Mutation	2 (4.7)
PTEN	
Negative	12 (27.9)
Positive	31 (72.1)
Overall survival time (mo), median (range)	37.3 (3.6-180)
Progression-free survival (mo), median (range)	16.0 (1-171)
Number of tumor buds, median (range)	9.0 (1-44)
Tumor budding	
High	11 (25.6)
Low	32 (74.4)

EGFR: Epidermal growth factor receptor.

B-RAF (P = 0.598), PIK3CA (P = 0.451) or PTEN expression (P = 0.241) (data not shown).

Association of tumor budding with response

The predictive ability of each feature for response is shown in Table 2. High-grade tumor budding was significantly associated with no objective response (P = 0.011). In fact, all patients with PR had low-grade tumor budding (sensitivity 100%) and all patients with high-grade tumor budding were PD or SD (negative predictive value, NPV: 100%). The overall accuracy of tumor budding for response was 68.3%.

K-RAS gene status was evaluated in all 43 patients and 11 (25.6%) were identified as mutated while the remaining 32 cases (74.4%) were wild-type. A significant association of K-RAS mutation with no objective response was observed (P = 0.011). Moreover, all patients achieving PR were K-RAS wild-type (sensitivity 100%) while K-RAS mutated cases were all non-responders (NPV 100%). As for tumor budding, the overall accuracy of K-RAS for response was 68.3%.

Of the 19 patients with wild-type K-RAS and no response, high-grade tumor budding was able to identify an additional 7 non-responder patients (Table 3). Together, the combined overall accuracy of tumor budding and K-RAS increased from 68.3% to 80% with a sensitivity of 100% for PR and an improvement in specificity to 72.1% with only 12/43 cases in this series misclassified with these two parameters alone.

Algorithm for patient classification using tumor budding, EGFR, K-RAS, B-RAF, PIK3CA and PTEN

Since the predictive accuracy for response using tumor budding combined with K-RAS mutation was 80%, the classification of wild-type K-RAS/low-grade tumor budding patients was further investigated using the remaining molecular parameters and analyzed by CART (Figure 1A). For the remaining 25 patients, negative expression of PTEN occurred in 6 cases and 5/6 (83%) were not responders. Of the remaining 16 patients with positive PTEN expression and available EGFR status, all cases with amplification or copy number gain (n = 13, three were not evaluable for EGFR gene status) had a PR. PIK3CA and B-RAF gene status did not contribute predictive information in this setting which included tumor budding. Moreover, of the 43 patients, 4 cases were misclassified, leading to 90.7% of patients being classified into appropriate response groups.

In order to compare the performance of this algorithm conditioned on K-RAS and tumor budding to an algorithm conditioned only on K-RAS, we performed a second CART analysis to classify patients with wild-type K-RAS gene status into response groups using the remaining molecular features, as described above (Figure 1B). Using this approach, PTEN expression, followed by B-RAF mutation and EGFR amplification or copy number gain were included in the analysis. PIK3CA was not a predictive factor here, most likely due to the low frequency (n = 2) of patients with mutation in this cohort. Seven of the 43 patients were incorrectly classified leading to an overall classification rate of 83.7%.

An overview of the predictive accuracies of K-RAS, tumor budding, K-RAS plus tumor budding, as well as the two algorithms including and excluding tumor budding is presented in Figure 2. In particular, the accuracy of either tumor budding alone or K-RAS analysis alone was 68.3%. This value improved to 80% when analyzing the combined accuracy of budding with K-RAS gene status. The predictive ability of a 4-panel combination of features including K-RAS/PTEN/B-RAF/EGFR was 83.7%. Among the features evaluated, the combined analysis of K-RAS/tumor budding/PTEN/EGFR demonstrated an overall accuracy of 90.7% for response to anti-EGFR agents.

Tumor budding, K-RAS and PFS

When evaluating PFS, high-grade tumor budding was significantly linked to an increased relative risk [HR (95% CI): 2.8 (1.3-6.0), P = 0.008] (Figure 3). In addition, when evaluating both tumor budding and K-RAS mutation status in multivariable analysis, high-grade tumor budding maintained its negative effect on clinical outcome [HR (95% CI): 2.78 (1.3-6.0), P = 0.022],

Table 2 Predictive ability of each feature for partial response

Feature	Total No. of patients	No. of correctly predicted PR	No. of correctly predicted PD + SD	No. of PD + SD predicted as PR	No. of PR predicted as PD + SD	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	*P*-value
Budding (≤ 15 cells)	43	13	11	19	0	100	40.6	37	100	68.3	0.011
EGFR (no AMP/CNG)	38	10	4	24	0	100	29.4	14	100	57.1	0.556
K-RAS (wild-type)	43	13	11	19	0	100	40.6	37	100	68.3	0.011
B-RAF (wild-type)	43	13	5	25	0	100	34.2	17	100	58.3	0.301
PIK3CA (wild-type)	43	13	2	28	0	100	31.7	7	100	53.3	1.0
PTEN (positive)	43	12	11	19	1	91.7	38.7	37	92.3	64.5	0.07

PD: Progressive disease; SD: Stable disease; PR: Partial response; EGFR: Epidermal growth factor receptor; PPV: Positive predictive value; NPV: Negative predictive value; AMP/CNG: Amplification or copy number gain.

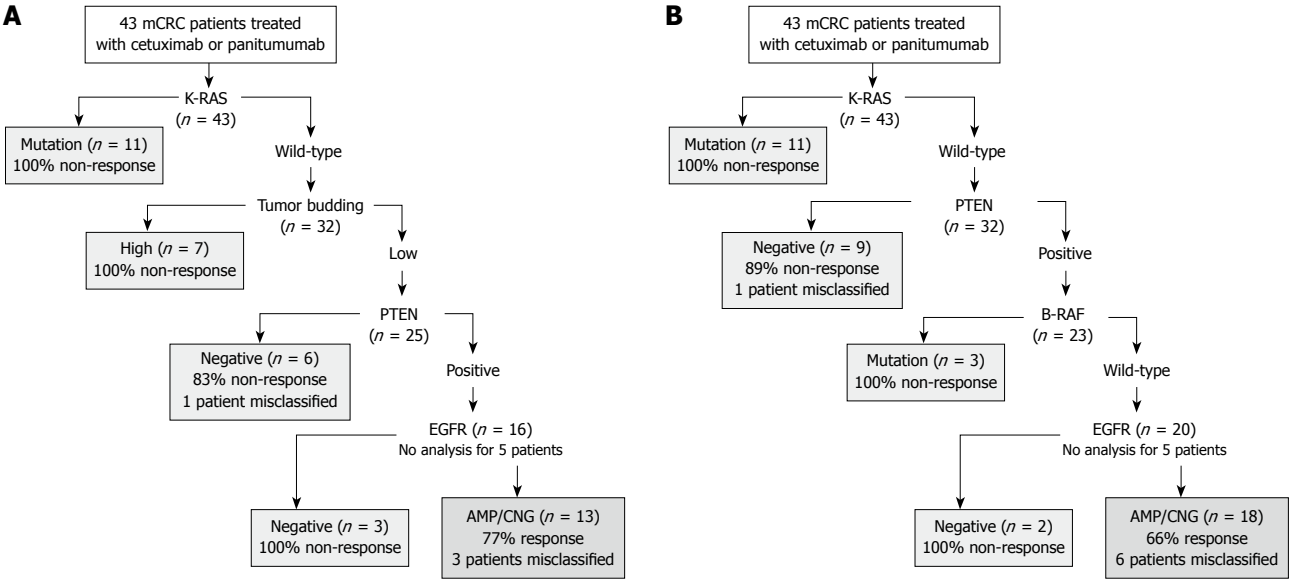

Figure 1 Algorithm illustrates the classification of patients into response groups. Non-response: Patients with progressive disease or stable disease. A: Classification and regression tree (CART) analysis was performed for patients with K-RAS wild-type/low tumor budding. CART identified a significant contribution of PTEN and epidermal growth factor receptor (EGFR) to the classification of responsive and non-responsive patients. Thirty-nine patients correctly classified (90.7%); B: CART analysis performed for patients with K-RAS wild-type tumors identifying a significant contribution of PTEN, B-RAF and EGFR to the classification of responder and non-responder patients. Thirty-six patients correctly classified (83.7%). AMP/CNG: Amplification or copy number gain; mCRC: Metastatic colorectal cancer.

while K-RAS was not linked to PFS [HR (95% CI): 1.54 (0.8-3.1), $P = 0.236$].

DISCUSSION

The aim of this study was to determine whether tumor budding is a predictive or prognostic factor in mCRC patients treated with anti-EGFR-based therapies. Our results show that high-grade tumor budding predicts non-response in these patients and in combination with K-RAS mutation may correctly predict response with 80% accuracy. Additionally, high-grade tumor budding was found to lead to unfavourable PFS also in a K-RAS-independent manner.

We found no association between high-grade tumor budding and K-RAS gene mutation in this series of mCRC patients. Using two entirely independent cohorts of 88 and 117 patients, respectively, we have previously shown this lack of association between K-RAS and tumor budding, although mutation in codon 12 and 13 was observed in 38.6% of all high-grade tumor budders[27,32]. Our findings here using a third independent cohort are in agreement with these results. In contrast, Prall and Oswald documented in 95 sporadic CRC patients, a significant association between mutation and tumor budding, and moreover, independently of invasion growth patterns[20]. Our differing results may be explained by the types of tumor specimens used (paraffin-embedded *vs* fresh frozen), differences in molecular analysis (DNA sequencing *vs* PCR-RFLP) and notably by the choice of methods of evaluation (tumor buds only *vs* tumor buds plus cytoplasmic pseudo-fragments).

We document here a significant association between high-grade tumor budding and a lack of objective response in patients with mCRC treated with anti-EGFR therapies. Tumor budding has been significantly related to unfavourable clinical and histopathological features including higher tumor grade, vascular invasion, lymph node

Table 3 Tumor budding and K-RAS followed by PTEN, epidermal growth factor receptor, B-RAF and PIK3CA stratified by response group

Response	K-RAS	Tumor budding	PTEN	EGFR (AMP/CNG)	B-RAF	PIK3CA
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	MUT	WT
NR	WT	Low	POS	No	WT	WT
NR	WT	Low	POS	No	WT	WT
NR	WT	Low	POS	Yes	WT	WT
NR	WT	Low	POS	Yes	WT	WT
NR	WT	Low	POS	Yes	WT	WT
NR	WT	Low	POS	NE	MUT	WT
NR	WT	Low	POS	No	MUT	MUT
NR[1]	WT	High	NEG	No	WT	WT
NR[1]	WT	High	NEG	Yes	WT	WT
NR[1]	WT	High	NEG	Yes	MUT	MUT
NR[1]	WT	High	POS	Yes	WT	WT
NR[1]	WT	High	POS	Yes	WT	WT
NR[1]	WT	High	POS	Yes	WT	WT
NR[1]	WT	High	POS	Yes	MUT	WT
NR[1]	MUT	Low	NEG	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	High	NEG	Yes	WT	WT
NR[1]	MUT	High	NEG	Yes	WT	WT
NR[1]	MUT	High	POS	No	WT	WT
NR[1]	MUT	High	POS	Yes	WT	WT
PR[1]	WT	Low	NEG	NE	WT	WT
PR[1]	WT	Low	POS	NE	WT	WT
PR[1]	WT	Low	POS	NE	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT

[1]Cases which were correctly predicted using K-RAS and tumor budding. EGFR: Epidermal growth factor receptor; AMP/CNG: Amplification or copy number gain; MUT: Mutation; NE: Not evaluable; NEG: Negative; NR: Non response; POS: Positive; PR: Partial response; WT: Wld-type.

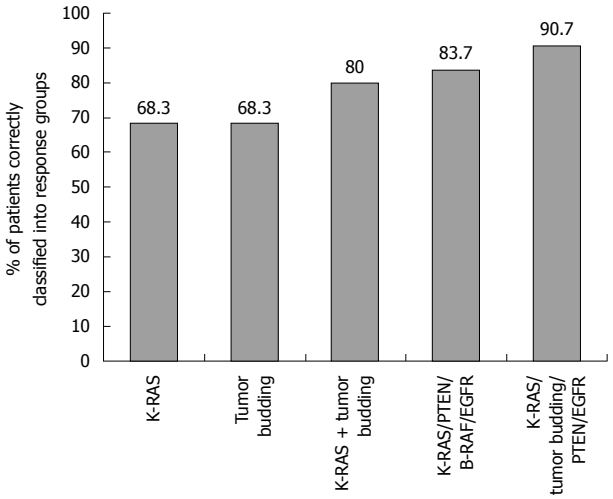

Figure 2 Overview of classification rates for various combinations of features. EGFR: Epidermal growth factor receptor.

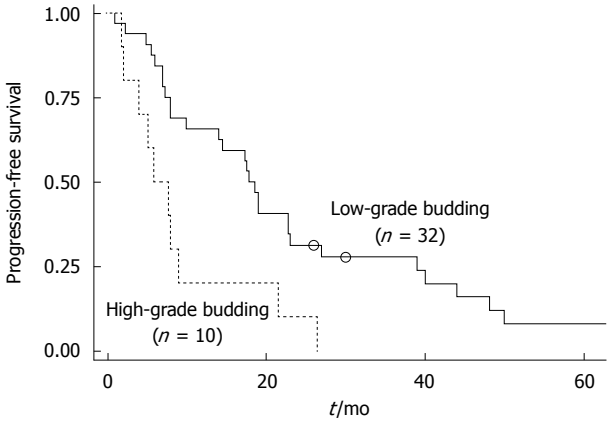

Figure 3 Kaplan-Meier survival curve showing the unfavourable progression-free survival of patients with high-grade tumor budding.

metastasis, distant metastasis, local recurrence, and poorer overall and disease-specific survival time independently of TNM stage[13-25]. Additionally, tumor budding is inversely related to dense peritumoral lymphocytic inflammation at the invasive front suggesting that the pro-budding phenotype may be tempered by specific immune responses[33]. We have recently reported that a high ratio of CD8+/tumor buds in non-metastatic CRC was found to be a more important prognostic factor than either CD8+ T-lymphocytes or tumor budding alone[27]. Although we evaluated CD8+ cells in these 43 specimens and their ratio with tumor budding, we did not find any predictive or prognostic value of CD8+ in this series, suggesting that the immune response may not play a role in conferring response in these treated, metastatic patients (data not shown). On the other hand, high-grade tumor budding was not only associated with non-response to anti-EGFR therapies but all patients with this unfavourable feature were non-responsive. In addition, we found a significantly shorter PFS in patients with high-grade tumor budding independently of K-RAS, supporting the predictive and prognostic effect of this histomorphological feature among this cohort of patients.

An association between K-RAS gene mutation and lack of response to anti-EGFR therapies has been consistently described[5,7,8,34]. Indeed, K-RAS mutational investigations are now routinely performed in molecular pathology laboratories and recommended for patients with mCRC to determine their potential benefit from anti-EGFR therapies[10]. In our study, all patients with a K-RAS mutation were non-responsive to therapy. Nonetheless, 7 patients with wild-type K-RAS were also found to be non-responders and all of these had high-grade tumor budding. In fact, all patients with an objective response to therapy

had simultaneous wild-type K-RAS and low-grade tumor budding, thereby improving the predictive accuracy for response from 68% for each biomarker alone to 80% when assessed in combination.

It is recognized that a subgroup of mCRC patients with wild-type K-RAS do not respond to anti-EGFR agents[35]. In this study, although all responders were indeed those with wild-type K-RAS and low-grade tumor budding, a considerable proportion of patients, namely 12/43 (27.9%) found with this constellation had PD or SD after treatment. In this setting, we found that loss of PTEN expression could accurately identify 83% of non-responsive cases and that *EGFR* amplification or copy number gain in PTEN-positive tumors correctly predicted 77% of responders, findings which are in line with numerous reports concerning the predictive value of these markers[36-38]. Together, 90.7% (39/43) of patients were correctly classified into response groups using these four features. Mutations in B-RAF and PIK3CA mutations have also been found to lead to non-response in mCRC patients[39-41]. Indeed, in this study, cases with either mutation were found to be patients who did not respond to therapy. However, after accounting for K-RAS and tumor budding only 3 B-RAF mutations were observed and 1 PIK3CA mutation was found, therefore the low frequency of these events may have led to their exclusion from the predictive algorithm.

Our study is constrained by several factors, the most important limitation being the sample size. To our knowledge, this is the first study evaluating tumor budding as a potential predictive or prognostic factor in mCRC patients treated with cetuximab or panitumumab, nonetheless these results need to be validated in larger cohorts. Secondly, although tumor budding is considered an additional prognostic factor by the AJCC/UICC, it has not been incorporated into standard pathological routine due to the absence of standardized methods of evaluation. Our cut-off score to define high-grade tumor budding was determined using a 40 × high-power field and found to be reproducible between independent pathologists. Not only was the threshold of 15 tumor buds defined using a valid cut-point determination method and tested using re-sampling methods, but resembles the definition of high-grade tumor budding used by Prall *et al*[19] to define the optimal threshold value for predicting metastatic disease in CRC patients (25 tumor buds observed in the densest region using a 20 × objective lens). Despite these limitations, our study is innovative, in that it appears to be the first evidence suggesting that a histomorphological feature, namely tumor budding, is both a predictive and prognostic factor in patients with mCRC treated with anti-EGFR-based therapies. Moreover, the combined analysis of K-RAS gene status and tumor budding may accurately predict both responders and non-responders with up to 80% accuracy.

These preliminary results suggest that tumor budding evaluated using pan-cytokeratin stains improves the individualized prediction of outcome in combination with K-RAS mutation for mCRC patients treated with anti-EGFR therapies. These findings warrant further investigation in large prospective studies.

COMMENTS

Background

Tumor budding is a histological feature which has consistently been linked to higher tumor grade, vascular and lymphatic invasion and is predictive of both lymph node and distant metastasis stage. Most studies confirm that high-grade tumor budding is an independent prognostic factor and recognized as such by the American Joint Committee on Cancer and International Union against Cancer. In addition, tumor budding does not appear to be related to mutation of K-RAS, leading to the hypothesis that this histomorphological feature could perhaps be used to complement the assessment of response in metastatic colorectal cancer (mCRC) patients treated with anti-epidermal growth factor receptor (EGFR)-based therapies.

Research frontiers

Monoclonal antibodies targeting the EGFR such as cetuximab and panitumumab have been recently approved for the treatment of mCRC patients, however, response rates in general vary from 10%-20%. Several molecular and protein biomarkers are being investigated as predictive factors of response including K-RAS, B-RAF, PIK3CA and PTEN. The vast majority of studies to date do support a lack of responsiveness in patients with mutation of K-RAS. It is, however, clear that not all patients with wild-type K-RAS tumors achieve a response to anti-EGFR therapies and the results concerning other genetic alterations are not conclusive, suggesting that continued efforts on predictive biomarkers are warranted.

Innovations and breakthroughs

The results show that high-grade tumor budding predicts non-response in mCRC patients who receive anti-EGFR therapies. In combination, K-RAS mutation status and tumor budding together can correctly predict response with 80% accuracy. Additionally, high-grade tumor budding was found to lead to unfavourable progression-free survival also in a K-RAS-independent manner. This study appears to be the first to show that a histomorphological feature, namely tumor budding, may be a predictive factor of response in mCRC patients treated with anti-EGFR therapy.

Applications

These preliminary results suggest that tumor budding evaluated using pan-cytokeratin stains improves the individualized prediction of outcome in combination with K-RAS mutation for mCRC patients treated with anti-EGFR therapies. These findings warrant further investigation in large prospective studies.

Terminology

Tumor budding is considered the histological hallmark of Epithelial Mesenchymal Transition. Tumor buds are defined as dedifferentiated single cells/small clusters at the invasive front of colorectal cancer.

Peer review

This is a well written and presented manuscript. The data are of major importance to the clinicians. The authors studied over more than 40 human samples and made a direct link between molecular expression, prognosis and treatment.

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BRIEF ARTICLE

Liver stiffness measurements in patients with HBV *vs* HCV chronic hepatitis: A comparative study

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Abstract

AIM: To assess the values of liver stiffness (LS) in patients with hepatitis B virus (HBV) chronic hepatitis and to compare them with those in patients with hepatitis C virus (HCV) chronic hepatitis.

METHODS: The study included 140 patients with HBV chronic hepatitis, and 317 patients with HCV chronic hepatitis, in which LS was measured (FibroScan®-Echosens®) and liver biopsy was performed in the same session (assessed according to the Metavir score).

RESULTS: According to the Metavir score of the 140 HBV patients: one had F0, 32 had F1, 67 had F2, 33 had F3 and 7 had F4. Of the 317 HCV patients: 5 had F0, 34 had F1, 146 had F2, 93 had F3 and 39 had F4. For the same severity of fibrosis, the mean values of LS in HBV patients were similar to those in HCV patients: F1, 6.5 ± 1.9 kPa *vs* 5.8 ± 2.1 kPa (P = 0.0889); F2, 7.1 ± 2 kPa *vs* 6.9 ± 2.5 kPa (P = 0.3369); F3, 9.1 ± 3.6 kPa *vs* 9.9 ± 5 kPa (P = 0.7038); F4, 19.8 ± 8.6 kPa *vs* 17.3 ± 6.1 kPa (P = 0.6574). A significant direct correlation between LS measurements and fibrosis was found in HCV patients (Spearman's r = 0.578, P < 0.0001), as well as in HBV patients (r = 0.408, P < 0.0001). The correlation was more significant in HCV than in HBV patients (Fisher's Z-test, Z = 2.210, P = 0.0271).

CONCLUSION: In our group, the mean values of LS in patients with chronic B hepatitis were similar to those in patients with chronic HCV hepatitis, for the same stage of fibrosis. Also, LS was correlated with the severity of fibrosis both in HBV and HCV chronic hepatitis patients.

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Key words: Chronic B hepatitis; Chronic C hepatitis; Fibrosis; Transient elastography; Liver biopsy

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INTRODUCTION

The non-invasive assessment of fibrosis in chronic hepatitis, especially of viral etiology, is accepted more and more, partially replacing liver biopsy (LB) in some countries[1]. Guidelines from France[1] recommend that the first-line test for untreated patients with hepatitis C virus (HCV)

chronic hepatitis, with no comorbities, should be a non-invasive procedure (either FibroTest® or FibroScan®).

The non-invasive methods used for the evaluation of chronic hepatitis are: serum markers (the best known is FibroTest-ActiTest - a biochemical test which uses 6 serum biomarkers, correlated with the age and gender of the patient in a mathematical formula)[2-5]; transient elastography (TE) (FibroScan®)[6,7]; SonoElastography (Real-Time Tissue Elastography)[8-11] and magnetic resonance imaging elastography[12,13].

Recent meta-analyses[7,8] have tried to assess the practical value of TE for the evaluation of patients with chronic hepatitis. Many studies were published regarding the value of TE for evaluation of patients with HCV chronic hepatitis, but only a few studies in patients with chronic hepatitis B virus (HBV) infection. On the other hand, published data showed discordant results regarding liver stiffness (LS) in patients with HBV and HCV chronic hepatitis[14,15].

The aim of our study was to determine whether the values of LS evaluated by means of TE (FibroScan®) were similar for the same degree of fibrosis (evaluated by means of LB), in patients with chronic HBV and HCV hepatitis.

MATERIALS AND METHODS

Patients

Our study included a total of 457 successive patients, 140 with HBV chronic hepatitis and 317 with HCV chronic hepatitis. All the patients were referred to our department during a 2-year period (January 2008 to December 2009) for hepatitis assessment (according to the guidelines valid in Romania in that period, LB was mandatory for fibrosis staging). LS was evaluated in all patients by means of FibroScan, and LB was performed in the same session during the standard of care evaluation of patients with chronic hepatitis. The inclusion criteria were: (1) HCV chronic hepatitis: patients with positive anti-HCV antibodies for at least 6 mo, with or without cytolysis; detectable viral load by polymerase chain reaction (PCR); pathological lesions of chronic hepatitis demonstrated by LB; no signs of decompensated liver disease (actual or history of jaundice, ascites); and (2) HBV chronic hepatitis: patients with positive HBsAg for at least 6 mo, with or without cytolysis; positive or negative HBeAg; HBV DNA > 2000 IU/mL (> 10000 copies/mL) by PCR; pathological lesions of chronic hepatitis demonstrated by LB; no signs of decompensated liver disease (actual or history of jaundice, ascites).

TE

TE was performed in all 457 patients with the FibroScan® (Echosens®, Paris, France) by 3 experienced physicians (each having performed more than 1000 TE examinations). In each patient, 10 valid measurements were performed, after which a median value of LS was obtained, measured in kilopascals (kPa). Only patients in which LS measurements had a success rate of at least 60%, with an interquartile range (IQR) < 30%, were included in our study. The success rate was calculated as the ratio of the number of successful acquisitions over the total number of acquisitions. IQR is the difference between the 75th percentile and the 25th percentile, essentially the range of the middle 50% of the data.

LB

Echo-assisted LB was performed in all 457 patients, using Menghini type modified needles, 1.4 and 1.6 mm in diameter. Only LB fragments of at least 2 cm, including at least 8 portal tracts, were considered adequate for the pathological interpretation. All the LBs were assessed according to the Metavir score, by a senior pathologist. Fibrosis was staged on a 0-4 scale: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa extending into lobules; F3, numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4, cirrhosis.

Statistical analysis

For a statistical analysis of quantitative variables, the mean and standard deviation were calculated. Two-way ANOVA test and *t*-tests were performed, to compare mean values of LS in various fibrosis subgroups in HBV *vs* HCV patients. To compare correlations, Fisher's *Z* test was used (hypotheses about the value of the population correlation coefficient ϱ between variables X and Y can be tested using the Fisher transformation applied to the sample correlation coefficient r)[16]. The diagnostic performance of LS measurements was assessed using receiver operating characteristics (ROC) curves. ROC curves were used for the detection of significant fibrosis (F $\geqslant$ 2 Metavir) and severe fibrosis (F $\geqslant$ 3 Metavir). Optimal cut-off values for LS measurements were chosen to maximize the sum of sensitivity and specificity. The statistical analysis was performed using Microsoft Excel 2007, GraphPad Prism 5 and MedCalc programs.

RESULTS

Patients

The subgroup of HBV patients consisted of 140 subjects (31 women, 109 men; mean age 39.2 ± 12.8 years). According to the Metavir scoring system, one had F0, 32 had F1, 67 had F2, 33 had F3 and 7 had F4.

The subgroup of HCV patients consisted of 317 subjects (213 women, 104 men; mean age 49.7 ± 10.2 years). According to the Metavir scoring system, 5 had F0, 34 had F1, 146 had F2, 93 had F3 and 39 had F4.

LS measurements by TE

The mean values of LS in HBV patients were not statistically significantly different from those of HCV patients for the same degree of fibrosis (Table 1).

A significant direct correlation of LS measurements with fibrosis was found to exist in HCV patients (Spearman's correlation coefficient $r = 0.578$, $P < 0.0001$), as

Table 1 Mean values of liver stiffness according to fibrosis stage in patients with hepatitis B virus *vs* hepatitis C virus chronic hepatitis

Category	Hepatitis B virus		Hepatitis C virus		*P*
	Cases	Mean values of LS (kPa)	Cases	Mean values of LS (kPa)	
Total cases	140	8.1 ± 4.2	317	8.9 ± 5.2	0.395 (NS)
F = 0	1	7.4	5	5.2 ± 0.7	-
F = 1	32	6.5 ± 1.9	34	5.8 ± 2.1	0.0889 (NS)
F = 2	67	7.1 ± 2	146	6.9 ± 2.5	0.3369 (NS)
F = 3	33	9.1 ± 3.6	93	9.9 ± 5	0.7038 (NS)
F = 4	7	19.8 ± 8.6	39	17.3 ± 6.1	0.6574 (NS)

F: Fibrosis; LS: Liver stiffness; NS: Not statistically significant.

Table 2 Predictive value of liver stiffness for the presence of significant fibrosis (F2), severe fibrosis (F3) and cirrhosis (F4) in hepatitis B virus *vs* hepatitis C virus patients

Parameter	Hepatitis B virus			Hepatitis C virus			All		
	F2	F3	F4	F2	F3	F4	F2	F3	F4
AUROC	0.658	0.753	0.974	0.750	0.797	0.933	0.712	0.786	0.943
Cut-off (kPa)	7	8.8	13.6	6.8	8.6	13.3	6.9	8.7	13.6
Sensitivity (%)	59	53	86	60	62	77	59	60	74
Specificity (%)	70	85	99	88	81	93	78	83	95
PPV (%)	86	58	78	97	71	61	93	68	64
NPV (%)	39	82	99	23	75	96	26	77	97

AUROC: Area under the receiver operating characteristics curve; PPV: Positive predictive value; NPV: Negative predictive value.

well as in HBV patients ($r = 0.408$, $P < 0.0001$). The correlation was more significant in HCV than in HBV patients (Fisher's *Z*-test, $Z = 2.210$, $P = 0.0271$).

The predictive values of LS measurements for the presence of significant fibrosis (F2), severe fibrosis (F3) and cirrhosis (F4) are presented in Table 2.

DISCUSSION

After a number of articles were published in France regarding the value of transient elastographic LS measurement in the evaluation of fibrosis in chronic hepatitis[17-22], numerous papers have been published in other countries[15,23-29] making this method a recognized test worldwide[30]. A meta-analysis published in 2008[30] proved that TE had an excellent diagnostic accuracy for the diagnosis of cirrhosis [mean area under the ROC (AUROC), 0.94 (95% CI: 0.93-0.95)]. However, a high variation of the AUROC was found regarding the diagnosis of significant fibrosis, dependent on the underlying liver disease [AUROC for significant fibrosis, 0.84 (95% CI: 0.82-0.86)].

The vast majority of studies assessing TE as compared to LB, were performed in patients with HCV chronic hepatitis[22,24,28,31,32]. At the same time, many studies were performed to evaluate this method in other chronic hepatopathies, such as nonalcoholic steatohepatitis, hemochromatosis and primary biliary cirrhosis[6,20,23,25].

Published studies regarding the value of LS measurement by means of TE in patients with HBV chronic hepatitis have shown conflicting results.

A Korean study performed by Seo *et al*[14] included 64 patients with chronic HBV hepatitis and 27 patients with chronic HCV hepatitis who underwent LB and TE in the same session (about two-thirds male; mean age 40 years, range 14-68 years). In that study, LS measurements were better correlated with the fibrosis score in patients with chronic HCV hepatitis than in those with chronic HBV hepatitis (0.773 *vs* 0.557, $P < 0.001$). The AUROC was larger in the group of patients with chronic HCV hepatitis (0.944, 0.982, and 0.958 for F $\geqslant$ 2, F $\geqslant$ 3, and F4, respectively) than in those with chronic HBV hepatitis (0.881, 0.863, and 0.850, respectively). The optimal cut-off values for F $\geqslant$ 2 and F $\geqslant$ 3 were similar for patients with chronic HCV hepatitis (7.05 and 11.4 kPa, respectively) and chronic HBV hepatitis (7.15 and 10.75 kPa, respectively). However, sensitivity and specificity were superior in patients with chronic HCV hepatitis. The conclusion of the study was that the efficacy of LS measurement for the assessment of liver fibrosis was superior in patients with chronic HCV hepatitis than in patients with chronic HBV hepatitis.

In a study performed by Ogawa *et al*[15] in 68 patients with chronic HBV hepatitis and 161 patients with chronic HCV hepatitis, the mean values of LS measurements were 3.5 kPa for F0, 6.4 kPa for F1, 9.5 kPa for F2, 11.4 kPa for F3, and 15.4 kPa for F4 in patients with chronic HBV infection, and 6.3 kPa for F0, 6.7 kPa for F1, 9.1 kPa for F2, 13.7 kPa for F3, and 26.4 kPa for F4 in those with chronic HCV infection. The values were significantly correlated with fibrosis stage for both groups of patients (HBV, $r = 0.559$, $P = 0.0093$, and HCV, $r = 0.686$, $P < 0.0001$). This study concluded that TE was an

Table 3 Cut-off values for different stages of fibrosis in patients with hepatitis B virus chronic hepatitis, proposed by various authors (kPa)

Fibrosis	Marcellin *et al*[17]	Chang *et al*[33]	Chan *et al*[34]	Kim *et al*[35]
F0	5.1	6.9	5.9	-
F1	6.0	12.2	5.9	9.1
F2	7.0	-	7.0	-
F3	12.8	24.8	8.8	-
F4	23.7	-	14.2	14.0

efficient and simple method for the evaluation of liver fibrosis in patients with chronic viral infection, both in HBV and HCV hepatitis.

Our study, performed on a large cohort of patients (457 subjects) aimed to find out if there were significant differences in LS in patients with HBV *vs* HCV chronic hepatitis for the same degree of fibrosis, as compared to the LB. LS measurement has a well established value for staging fibrosis in HCV chronic hepatitis, proved by 2 meta-analyses[7,30]. In patients with HBV chronic infection, data regarding LS measurement for fibrosis staging are conflicting. Why? One explanation could be that the necroinflammatory activity in HBV infection can vary with time, as well as the fact that fluctuations in aminotransferases can occur. Different studies have proposed various cut-off values for different stages of fibrosis, as seen in Table 3.

In our cohort of 140 chronic HBV infected patients, the mean values for F1, F2, F3 and F4 were: 6.5, 7.1, 9.1 and 19.8 kPa, respectively, similar to those obtained in the study performed by Marcellin. Also, we must bear in mind that only the Marcellin study was performed in a Caucasian population (such as ours), the others being performed in Asian populations. In our study, the sensitivity of TE for cirrhosis prediction was better in HBV than in HCV patients, but this finding needs further confirmation since the number of F4 patients in the HBV group was small (only 7) *vs* 39 in the HCV group.

Regarding the correlation between fibrosis and LS, a significant direct correlation of TE measurements with fibrosis was found to exist in HCV patients (Spearman's correlation coefficient $r = 0.578$, $P < 0.0001$), more significant than in HBV patients ($r = 0.408$, $P < 0.0001$) ($Z = 2.210$, $P = 0.0271$). Thus it is likely that the correlation between LS and fibrosis in HBV patients can be of use in clinical practice.

As mentioned earlier, high levels of aminotransferases can influence the LS values obtained by means of TE, so that LS measurements have to be interpreted in a biochemical context, otherwise there is a risk of overestimating the severity of fibrosis. Also this is why LS measurements are not performed in acute hepatitis or during alanine aminotransferase (ALT) flares in HBV chronic hepatitis[29,36]. In order to minimize the risk of overestimating fibrosis during ALT flares, Chan *et al*[34] calculated LS cut-off values for various stages of fibrosis considering also the aminotransferase levels. In this study, the LS cut-off value for F3 was 9 kPa in patients with normal ALT and 12 kPa in patients with ALT higher than 5 times the upper limit of normal. The cut-offs for cirrhosis were 12 kPa in patients with normal ALT and 13.4 kPa in those with high ALT.

In conclusion, in our study, LS measured by TE was correlated with the degree of fibrosis both in HBV and HCV patients, the correlation being more significant in HCV patients. Our data showed that there were no statistically significant differences between the mean values of LS in HBV and in HCV patients for the same degree of fibrosis.

COMMENTS

Background

Non-invasive methods for fibrosis assessment in chronic hepatitis, such as transient elastography (TE), are being accepted more and more, replacing the invasive methods, especially in hepatitis C virus (HCV) chronic hepatitis.

Research frontiers

Many studies have been published regarding the value of TE evaluation of patients with HCV chronic hepatitis, but only a few studies in chronic hepatitis B virus (HBV) infection, showing discordant results.

Innovations and breakthroughs

This research article determined if the authors can also use liver stiffness (LS) measurement by TE for the evaluation of patients with HBV chronic hepatitis, and concluded that LS is correlated with fibrosis in both HBV and HCV patients, and that there are no statistically significant differences between the mean LS values in HBV *vs* HCV patients, for the same degree of fibrosis. These findings are concordant with previous studies by Wang *et al*, Marcellin *et al*, and Ogawa *et al*, indicating that the diagnostic accuracy of LS is comparable in HBV and HCV infection related fibrosis.

Applications

This study showed that LS evaluated by means of TE was correlated with degree of fibrosis in both HBV and HCV patients and that there were no statistically significant differences between the mean values of LS in HBV *vs* HCV patients for the same degree of fibrosis, so the authors can also use this method for the evaluation of patients with HBV chronic hepatitis in daily practice.

Terminology

TE (FibroScan) is an ultrasound-based method that uses the transmission of low frequency vibrations to create an elastic shear wave that propagates into the liver, followed by the detection of wave propagation velocity, which is proportional to the tissue stiffness, with faster wave progression occurring through stiffer tissue.

Peer review

The authors present the data from their research on whether the accuracy of LS measurement in estimating liver fibrosis differs in people with chronic HCV or HBV infection. Although many reports on small or large populations exist on the same issue, the readers of the journal may find reading the data interesting.

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BRIEF ARTICLE

Erythropoietin ameliorates early ischemia-reperfusion injury following the Pringle maneuver

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Abstract

AIM: To investigate the protective effect of erythropoietin (Epo) against ischemia-reperfusion injury (IR/I) following the Pringle maneuver (PM), in comparison with conventional steroid administration in a prospective randomized trial.

METHODS: Patients were randomized by age, sex, diagnosis, and surgical method, and assigned to three groups: (1) A steroid group (STRD, n = 9) who received 100 mg of hydrocortisone before PM, and on postoperative days 1, 2 and 3, followed by tapering until postoperative day 7; (2) An EPO1 group (n = 10) who received 30 000 U of Epo before the PM and at the end of surgery; and (3) An EPO2 group (n = 8) who received 60 000 U of Epo before the PM. Hemoglobin (Hb), hematocrit (Ht), aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), lactate, interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α were measured before and just after (Day 0) surgery, and on postoperative days 1, 3, 7 and 14.

RESULTS: There were no increases in Hb and Ht in the EPO1 and EPO2 groups. AST was significantly lower in EPO1 than in STRD on Day 0 (P = 0.041), and lower in EPO1 than in STRD and EPO2 on Day 1 (P = 0.018). ALT was significantly lower in EPO1 than in STRD and EPO2 on Day 0 (P = 0.020) and Day 1 (P = 0.004). There were no significant inter-group differences in the levels of LDH and lactate. IL-6 was significantly lower in EPO1 than in STRD and EPO2 on Day 0 (P = 0.0036) and Day 1 (P = 0.0451). TNF-α was significantly lower in EPO1 than in STRD and EPO2 on Day 0 (P = 0.0006) and Day 1 (P < 0.0001). Furthermore, hospitalization was significantly shorter in EPO1 and EPO2 than in STRD.

CONCLUSION: Epo has greater potential than steroids to ameliorate IR/I after the PM. Epo at a dose of 30 000 U, administered before PM and just after surgery, yields better results.

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Key words: Erythropoietin; Hepatic resection; Pringle maneuver; Steroid; Prospective randomized study

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INTRODUCTION

The Pringle maneuver (PM) is a standard procedure used worldwide to reduce blood loss during hepatic resection. However, this procedure inevitably results in some degree of ischemia-reperfusion injury (IR/I). To ameliorate IR/I, several procedures have been used, such as steroid administration and ischemic preconditioning[1,2]. Although intravenous administration of steroids is widely used, its rationality has not been established, and major concerns have been raised regarding its possible side effects, such as infection, diabetes mellitus, and disturbance of wound healing[3].

Erythropoietin (Epo) is a hematopoietic peptide that has been used successfully for treatment of anemia in patients with end-stage renal disease. Recently, attention has been focused on the extra-hematopoietic effects of Epo, including an organ-protective effect against IR/I[4-6]. We have already reported the protective effect of Epo in a porcine liver IR/I model[7].

To elucidate and apply the protective effect of Epo against liver IR/I following the PM in a clinical setting, we performed a randomized prospective clinical trial.

MATERIALS AND METHODS

The study was performed in accordance with the principles of the Declaration of Helsinki, and was approved by the ethics committee of Dokkyo Medical University Hospital. Because Epo has been used successfully in a clinical setting for more than 15 years, we did not perform a phase I study.

Phase Ⅱ study

To determine the optimum dose, timing, and frequency of Epo administration, Epo was injected intravenously into groups of 3 patients who received each of the following doses: 6000 U (× 1), 12000 U (× 1), 18000 U (× 1), 24000 U (× 1), 30000 U (× 1), and 60000 U (× 1) at 5 min before the PM, or at 30000 U (× 2) at 5 min before the PM, and at the end of the operation. None of the patients in these groups showed any significant increase in red blood cell count or other side effects. We did not use doses of Epo exceeding 60000 U (× 1) or 30000 U (× 2), because experience with patients suffering from end-stage renal disease had shown that one or two injections of Epo did not increase hematopoiesis. Also, a study by Lipsic *et al*[8] had demonstrated the safety of a single injection of Epo at a dose of 60000 U, and this dose was the maximum for a single injection available at the time of the study. We decided to use Epo at a dose of 30000 U × 2 (at 5 min before the PM and at the end of the operation; EPO1 group), and 60000 U × 1 (at 5 min before the PM, EPO2 group).

Phase Ⅲ study

Patients treated with conventional steroids received 100 mg of hydrocortisone at 5 min before the PM, at the end of the operation, and on postoperative days 1, 2, and 3. Thus, the patients were allocated to 3 groups: a steroid group (STRD, $n = 9$), and EPO1 ($n = 10$) and EPO2 ($n = 8$) groups.

Study population and inclusion/exclusion criteria

Eligibility criteria for participation were elective primary liver surgery with written informed consent. Patients who were expected to undergo non-curative hepatectomy were excluded.

Randomization

Preoperative random allocation to the 3 groups was made on the basis of 4 parameters: age (< 65 or ⩾ 65 years), sex, indocyanine green excretion rate at 15 min (< 10% or ⩾ 10%), and disease diagnosis (hepatocellular carcinoma or others). The operative procedure was standardized so that hepatectomy was performed by a single surgeon (co-author Kubota K). The patients' clinical background factors are shown in Table 1.

Data collection

Blood samples were taken on admission (Pre), just after surgery (Day 0), and on postoperative days 1 (Day 1), 3 (Day 3), 7 (Day 7), and 14 (Day 14). Aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), lactate, hemoglobin (Hb), and hematocrit (Ht) were measured from each sample. AST, ALT, LDH, lactate, Hb, and Ht were measured at the central laboratory of our institution. Proinflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, were measured on Day 0 and Day 1. IL-6 was determined using a Human IL-6 Quantikine ELISA kit (R&D System, Minneapolis, MN) and TNF-α was determined using a Human TNF-α Quantikine ELISA kit (R&D System). All samples were measured in triplicate, in accordance with the manufacturer's recommendations.

Objectives

The primary objective of the study was to compare the protective effects of Epo and steroid against IR/I following the PM. The secondary objective was to elucidate the frequency of Epo-associated side effects.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 5.0 (Graphpad Software, La Jolla, CA). Comparisons between the two groups were made using Mann-Whitney *U* test. Kruskal-Wallis test with post-hoc test (Dunn's multiple comparison test) was used for comparisons between the conventional, EPO1, and EPO2 groups. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Figure 1 shows changes in Hb and Ht. During the periop-

Table 1 Patients' backgrounds

	STRD (n = 9)	EPO1 (n = 10)	EPO2 (n = 8)	P-value
Age (yr)	62.7 ± 10.6	62.1 ± 15.8	69.3 ± 7.8	0.395
Male/female	6/3	7/3	6/2	0.976
ICG R15 (%)	14.0 ± 8.8	10.7 ± 4.8	11.3 ± 5.2	0.854
Diagnosis (HCC/others)	6/3	6/4	4/4	0.890
Operation time (min)	322 ± 122	245 ± 83	299 ± 57	0.105
Pringle time (min)	43.9 ± 26.8	39.2 ± 21.2	53.8 ± 15.1	0.153
Operative blood loss (mL)	674 (70-3673)	469 (66-1900)	356 (228-1362)	0.386
Operative method (anatomical/ nonanatomical)	6/3	4/6	6/2	0.321

HCC: Hepatocellular carcinoma; STRD: Steroid group; EPO: Erythropoietin.

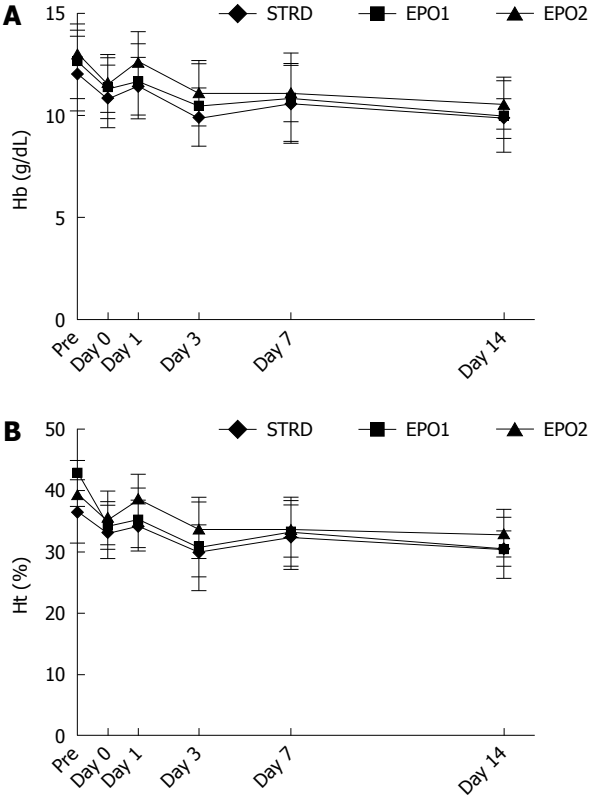

Figure 1 Changes in hemoglobin and hematocrit. Hemoglobin (Hb) (A) and hematocrit (Ht) (B) did not differ between the three groups until Day 14 (P > 0.05 for Hb and Ht, Kruskal-Wallis test). STRD: Steroid group; EPO: Erythropoietin.

erative period, there were no significant differences in Hb and Ht between the three groups, and no complications associated with the Epo treatments.

Figure 2 shows changes in the AST level at Pre, and on Days 0, 1, 3, 7 and 14. On Day 0, median values in the STRD, EPO1, and EPO2 groups were 409 (124-1481), 142 (34-552) and 205 (115-357) IU/L, respectively (P = 0.041, Kruskal-Wallis test). The AST level in the EPO1 group was significantly lower than that in the STRD group (P = 0.023). On Day 1, the corresponding median values were 275 (143.0-1389), 157 (55-552) and 342 (172-1361) IU/L, respectively (P = 0.018, Kruskal-Wallis test). The AST level in the EPO1 group was significantly lower than that in the STRD and EPO2 groups (P = 0.023 and 0.016, respectively). There were no significant inter-group differences in the AST level after Day 3.

Figure 3 shows changes in the ALT level at Pre, and on Days 0, 1, 3, 7 and 14. On Day 0, the median levels in the STRD, EPO1, and EPO2 groups were 351 (140-1390), 76 (20-319) and 141 (85-323) IU/L, respectively (P = 0.020, Kruskal-Wallis test). The ALT level in the EPO1 group was significantly lower than that in the STRD and EPO2 groups (P = 0.023 and 0.017, respectively). On Day 1, the corresponding median levels were 300 (110-1700), 112 (20-256) and 289 (200-1253) IU/L, respectively (P = 0.004, Kruskal-Wallis test). The AST level in the EPO1 group was significantly lower than those in the STRD and EPO2 groups (P = 0.008 and 0.001, respectively). There were no significant inter-group differences in the ALT level after Day 3.

Figure 4 shows the changes in LDH and lactate levels at Pre, and on Days 0 and 1. There were no significant differences in LDH or lactate levels between the three groups.

On Day 0, the median levels of the proinflammatory cytokine, IL-6, in the STRD, EPO1, and EPO2 groups were 300 (93-477), 155 (44-523) and 347 (300-414) pg/mL, respectively (P = 0.0036, Kruskal-Wallis test) (Figure 5A). The IL-6 level in the EPO1 group was significantly lower than that in the EPO2 group (P = 0.0037). On Day 1, the corresponding median levels of IL-6 were 129 (22-317), 75 (13-146) and 83 (56-240) pg/mL, respectively (P = 0.0451, Kruskal-Wallis test) (Figure 5B). The IL-6 level in the EPO1 group was significantly lower than that in the STRD group (P = 0.0185). On the other hand, on Day 0, the median levels of TNF-α in the STRD, EPO1, and EPO2 groups were 13.5 (10.1-21.0), 9.7 (5.0-11.0) and 8.0 (3.1-11.3) pg/mL, respectively (P = 0.0006, Kruskal-Wallis test) (Figure 6A), the levels in the EPO1 and EPO2 groups being significantly lower than that in the STRD group. On Day 1, the corresponding median levels of TNF-α were 17.7 (10.0-29.7), 4.6 (2.0-8.0) and 3.5 (1.4-6.0) pg/mL, respectively (P < 0.0001, Kruskal-Wallis test) (Figure 6B), the levels of TNF-α in the EPO1 and EPO2 groups being significantly lower than that in the STRD group.

Postoperative complications were observed in two cases in the STRD group, but no such cases occurred in the EPO1 and EPO2 groups (Table 2). The median periods

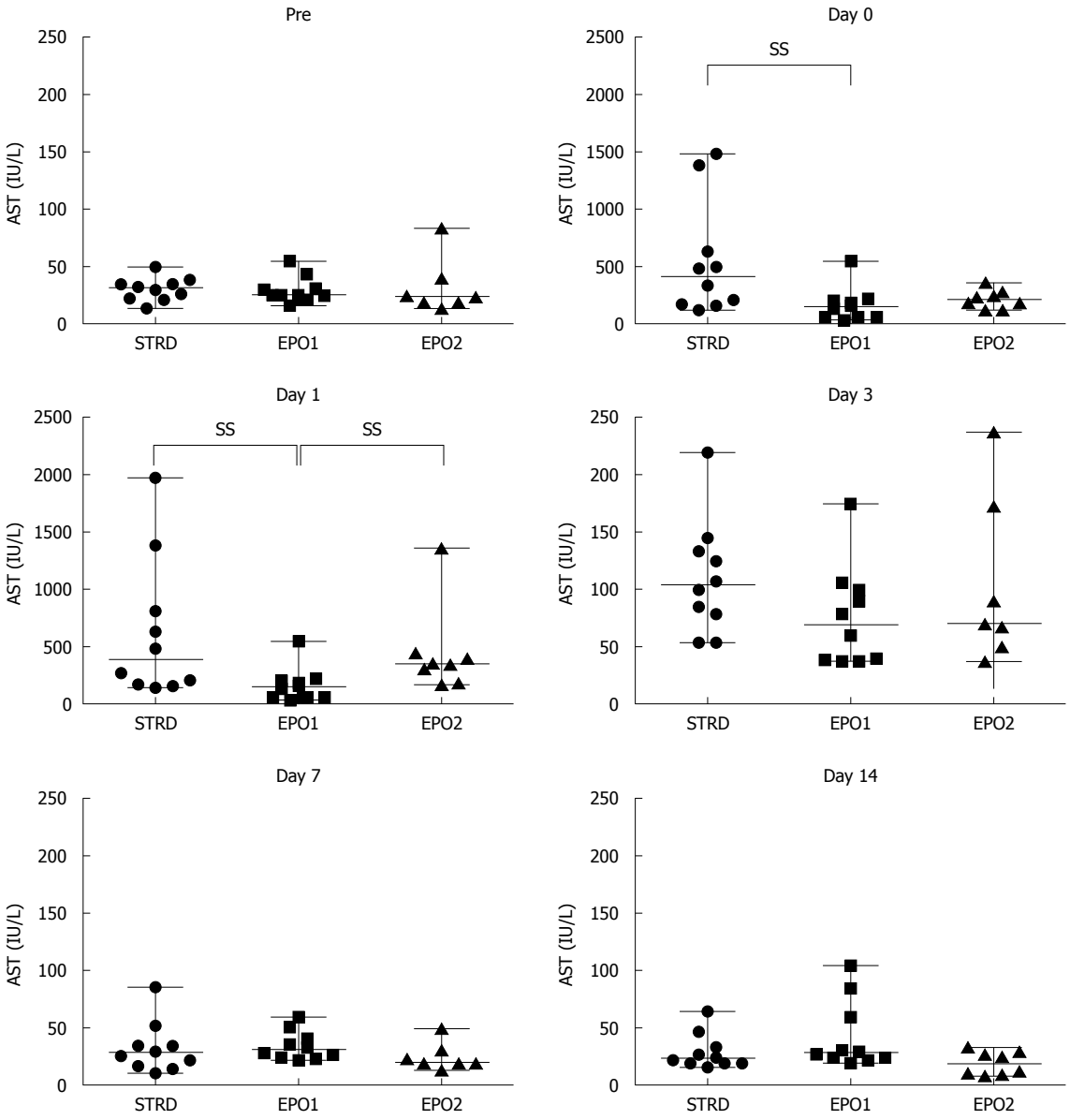

Figure 2 Changes in aspartate aminotransferase. The aspartate aminotransferase (AST) level in the erythropoietin (EPO)1 group was significantly lower than that in the steroid (STRD) group on Day 0 (P = 0.041, Kruskal-Wallis test), and also significantly lower than that in the STRD and EPO2 groups on Day 1 (P = 0.018, Kruskal-Wallis test). There were no significant differences in the AST level after Day 3. Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

Table 2 Postoperative complications

	Complication	Frequency (%)
STRD (n = 10)	n = 2 Prolonged ascites Pleural effusion	20
EPO1 (n = 10)	0	0
EPO2 (n = 8)	0	0

STRD: Steroid group; EPO: Erythropoietin.

of hospitalization in the STRD, EPO1, and EPO2 groups were 32 (10-74), 13 (8-77) and 16 (10-22) d, respectively (P = 0.0463, Kruskal-Wallis test) (Figure 7), this period being significantly longer in the STRD group than in the EPO1 and EPO2 groups.

DISCUSSION

Most patients undergoing hepatic resection have associated chronic hepatic disease and deterioration of hepatic functional reserve. A dilemma therefore arises in deciding a balance between the curativeness of hepatic resection and the likelihood of postoperative hepatic failure. There is a tendency for surgeons to aim at removing as large a portion of liver as possible to ensure that any disease, such as carcinoma, is resected completely. On the other hand, excessive removal of liver parenchyma results in a higher risk of postoperative hepatic failure. Amelioration of IR/I is therefore a crucial factor to consider in the prevention of liver function deterioration.

The present study demonstrated that Epo potently ameliorated IR/I following the PM. The AST level in

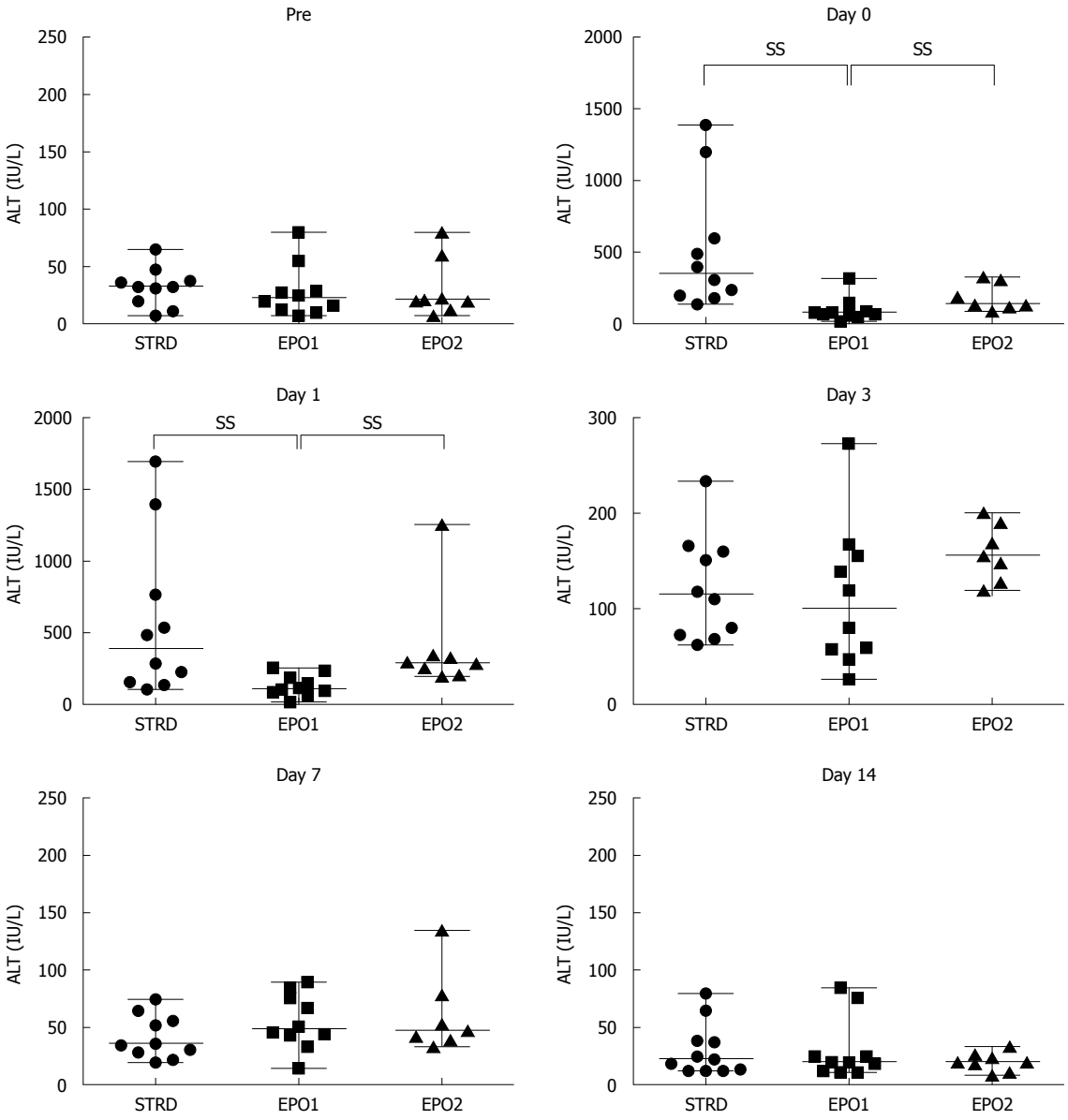

Figure 3 Changes in alanine transaminase. The alanine transaminase (ALT) level in the erythropoietin (EPO)1 group was significantly lower than that in the steroid (STRD) and EPO2 groups on Day 0 (P = 0.020, Kruskal-Wallis test) and Day 1 (P = 0.004, Kruskal-Wallis test). There were no significant differences in the ALT level after Day 3. Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

the EPO1 group was significantly lower than that in the STRD group on Day 0, and significantly lower than in the STRD and EPO2 groups on Day 1. The ALT level in the EPO1 group was significantly lower than in the STRD and EPO2 groups on both Day 0 and Day 1. There were no significant differences in AST and ALT levels between the three groups after Day 3. Our results demonstrated that the protective effect of Epo against IR/I was most pronounced at a very early stage after surgery, and furthermore, was stronger than that of steroids. During the very early post-surgical phase, there were no differences in LDH level, although the lactate level tended to be lower in the EPO1 group than in the STRD and EPO2 groups. The levels of proinflammatory cytokines, IL-6 and TNF-α, showed similar tendencies. The level of IL-6 in the EPO1 group was significantly lower than in the EPO2 and STRD groups. The level of TNF-α in the EPO1 and EPO2 groups was significantly lower than that in the STRD group.

The mechanism responsible for the protective effect of Epo against IR/I has been studied extensively by various groups, including our own[9-11]. As shown in this study, Epo protects organs and tissues from IR/I by inhibiting IR/I-induced cell apoptosis. Furthermore, Epo induces neovascularization and inhibits the secretion of acute inflammatory cytokines[4-6]. Epo binds to its specific cell-surface receptor (EpoR) and transduces signals to the nucleus[11]. The most important signaling pathways are the Jak2/STAT, Jak2/PI3K/AKT, and Jak2/MAPK/p38 pathways[12,13]. These signals activate anti-apoptotic genes, such as XIAP and BclxL, and suppress proapoptotic genes, such as BAD and GSK3β, resulting in inhibition of

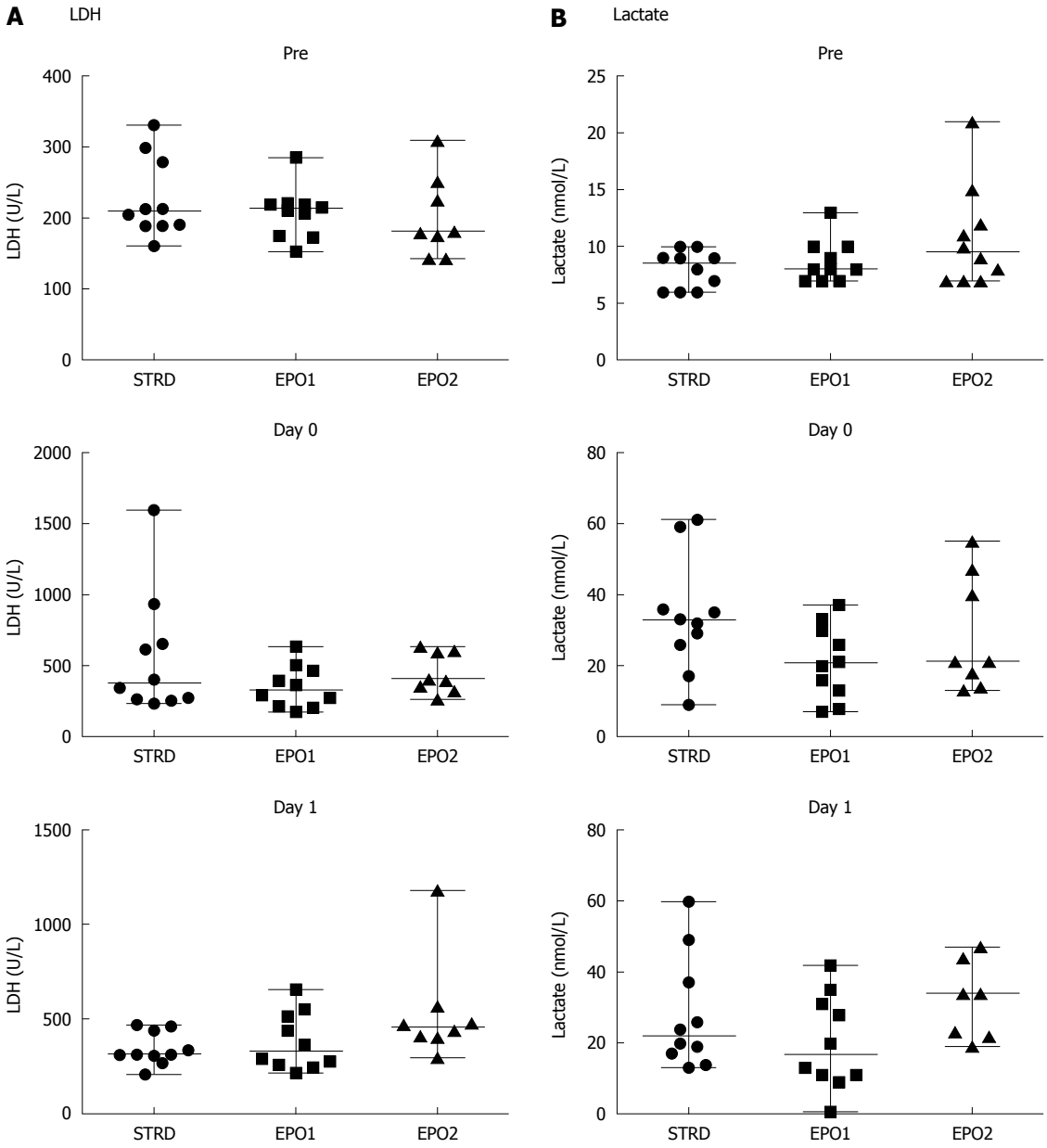

Figure 4 Lactate dehydrogenase (A) and lactate (B) levels at Pre, and on Day 0 and Day 1. There were no significant differences between the three groups in both lactate dehydrogenase (LDH) and lactate levels. Note that the scale of the Y-axis differs in each graph ($P > 0.05$ for hemoglobin and hematocrit, Kruskal-Wallis test). STRD: Steroid group; EPO: Erythropoietin.

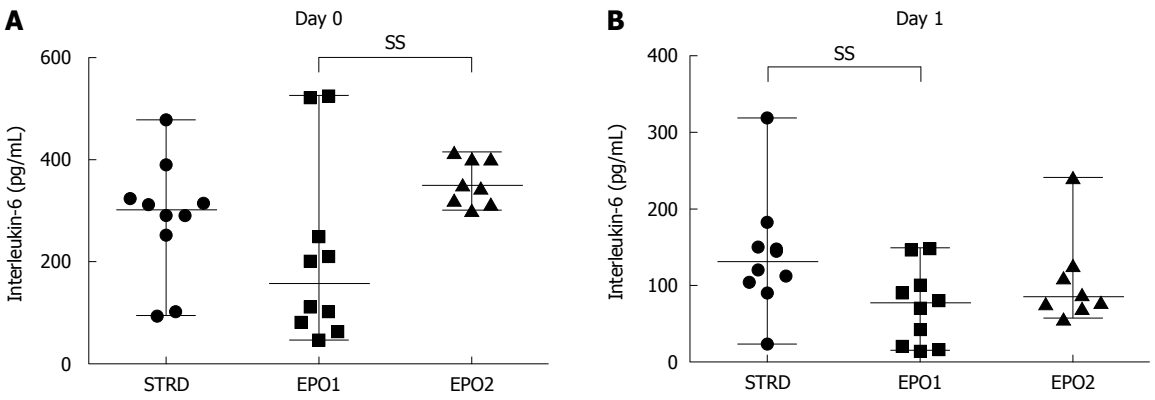

Figure 5 Interleukin-6 level on Day 0 (A) and Day 1 (B). The interleukin-6 level in the erythropoietin (EPO)1 group was significantly lower than that in the EPO2 group and the steroid (STRD) group on Day 0 ($P = 0.0036$, Kruskal-Wallis test). Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

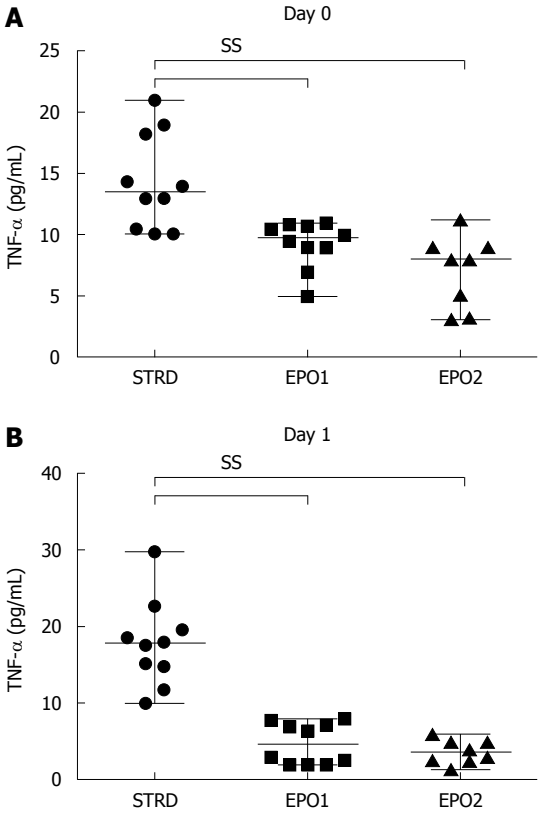

Figure 6 Tumor necrosis factor-α level on Day 0 (A) and Day 1 (B). Levels of tumor necrosis factor (TNF)-α in the erythropoietin (EPO)1 and EPO2 groups were significantly lower than that in the steroid (STRD) group on both Day 0 (P = 0.0006, Kruskal-Wallis test) and Day 1 (P < 0.0001, Kruskal-Wallis test). Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

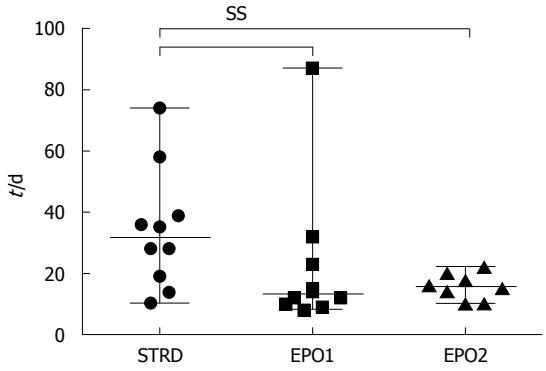

Figure 7 Length of hospitalization. Hospitalization in the steroid (STRD) group was significantly longer than that in the erythropoietin (EPO)1 and EPO2 groups (P = 0.0463, Kruskal-Wallis test). SS: Statistically significant.

apoptosis[10,14-17]. EpoR usually has a homodimeric structure, but Epo has been shown to bind to heterodimeric EpoR, consisting of EpoR and the β common receptor when exerting its extra-haematopoietic effect. This heterodimeric receptor is expressed in various organs, including the liver [18].

We employed two different dose regimes for Epo: EPO1 (30000 U × 2) and EPO2 (60000 U × 1). The results, in terms of AST, ALT, and IL-6 levels, indicated that EPO1 was more effective. In terms of the TNF-α level, the EPO2 regimen showed a stronger inhibitory effect than the EPO1 regimen, but not to a significant degree. Although the reason for this finding is unclear, previous studies have reported that Epo directly and indirectly inhibits the expression of IL-6 and TNF-α, and reduces their systemic levels[19-21]. The extra-hematopoietic effects of Epo have been studied in various animal models and clinical settings. These previous studies each employed different doses of Epo. The use of Epo in animal models has been reviewed by Sharples *et al*[13], Johnson *et al*[22], and Arcasoy[6]. In clinical studies, Ehrenreich *et al*[23] used Epo at a total dose of 100000 U for the first 3 d after acute cerebral stroke, noting that this was safe and well tolerated, and also used a dose of 40000 U for 3 mo to treat schizophrenia[9]. Lipsic *et al*[5] used a single bolus injection of Epo at a dose of 60000 U for patients with acute myocardial infarction, and did not observe any significant increase in Hb. Mocini *et al*[24] used a single injection of Epo at a dose of 40000 U before cardiac surgery, but did not observe any significant improvement or an increase in erythropoiesis. The results suggest that the optimal dose of Epo and the mode of administration may vary according to species and target organ. Our present findings indicate that 30000 U of Epo × 2, rather than 60000 U of Epo × 1, has a stronger inhibitory effect on IR/I following the PM, and that the doses and the mode of administration we employed did not affect haematopoiesis (Figure 1).

The period of hospitalization was significantly longer in the STRD group than in the EPO1 and EPO2 groups. This was a prospective study performed during a single period. Therefore, the patients who were treated with steroid stayed longer in hospital. The occurrence of complications in the STRD, EPO1, and EPO2 groups was 20%, 0% and 0%, respectively. In this study, we evaluated only grade Ⅱ complications by Clavien's classification[25]. Although the number of patients in each group was small and more studies are needed, the data suggest that Epo might reduce the rate of complications after hepatic resection.

We conclude that Epo has greater potential than steroids to ameliorate IR/I following the PM, and that Epo at a dose of 30000 U, given just before the PM and just after surgery, yields better results. This protective effect of Epo may be applicable not only in patients undergoing hepatic resection, but also for much more severe IR/I, such as that occurring in liver transplantation.

COMMENTS

Background

Ischemia-reperfusion injury (IR/I) is an obstacle encountered in various medical fields. Especially in liver surgery, IR/I after the Pringle maneuver (PM) hinders curability. Up to now, steroids have been used for amelioration of IR/I, but stronger and more effective drugs to prevent IR/I are needed.

Research frontiers

Erythropoietin (Epo) is a hematopoietic cytokine that has been used to treat anemia in patients with end-stage renal disease. Recently, extra-hematopoietic effects of Epo have been reported, including an organ-protective effect against IR/I. However, there have been no reports of the hepatoprotective effect of Epo in a clinical setting.

Innovations and breakthroughs

This study is the first prospective study to have confirmed the protective effect of Epo against IR/I after the PM, in comparison with steroid treatment. It was found that Epo strongly ameliorated IR/I after the PM, and that the effect was better than that of the steroid. Epo would therefore contribute to better preservation of liver function after liver surgery.

Applications

This study demonstrated that Epo shows promise for preventing IR/I after the PM. This finding extends its application not only to liver resection, but also liver transplantation, where amelioration of IR/I would improve survival.

Terminology

IR/I is a tissue injury occurring after ischemia-reperfusion. Organs are continuously perfused with blood, but once the blood supply is interrupted, organs sustain damage due to an anaerobic environment (ischemia). Restoration of the blood supply then causes further organ damage (reperfusion injury).

Peer review

This is an original study because it is the first prospective randomized study of its kind, although the number of patients in each group is small.

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BRIEF ARTICLE

mPGES-1 expression in non-cancerous liver tissue impacts on postoperative recurrence of HCC

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Abstract

AIM: To investigate whether microsomal prostaglandin E synthase-1 (mPGES-1) expression in hepatocellular carcinoma (HCC) and in non-cancerous liver affects HCC prognosis after hepatectomy.

METHODS: The relationship between patient clinical profiles, tumor factors, surgical determinants, and mPGES-1 expression and the recurrence-free survival rate were examined in 64 patients who underwent curative hepatectomy between March 2003 and December 2006.

RESULTS: The scores for mPGES-1 expression were higher in well differentiated and moderately differentiated HCC tissues than in poorly differentiated HCC tissues (well differentiated, 5.1 ± 2.7; moderately differentiated, 5.1 ± 1.7; poorly differentiated, 3.0 ± 1.8). In non-cancerous liver tissues, the mPGES-1 levels were higher in injured liver tissues than in normal tissues. Cirrhotic livers had higher mPGES-1 levels than livers with chronic hepatitis (normal livers, 3.3 ± 0.7; chronic hepatitic livers, 5.4 ± 1.9; cirrhotic livers, 6.4 ± 1.6). A univariate analysis revealed that the recurrence-free survival rate was significantly lower in patients with vascular invasion, a higher mPGES-1 level in non-cancerous liver tissue, a larger tumor diameter (≥ 5 cm), and a lower serum albumin level (≤ 3.7 g/dL). The mPGES-1 expression in HCC tissues did not correlate well with postoperative recurrence. A multivariate analysis demonstrated that the presence of vascular invasion and higher mPGES-1 levels were statistically significant independent predictors for early postoperative recurrence of HCC.

CONCLUSION: Increased mPGES-1 expression in non-cancerous liver tissues is closely associated with the early recurrence of HCC after curative resection.

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Key words: Curative resection; Hepatocellular carcinoma; Microsomal prostaglandin E synthase-1; Non-cancerous liver tissue; Recurrence-free survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cause of cancer death worldwide[1,2]. Hepatectomy is one of the best treatment modalities for HCC. Recent advances in surgical techniques and perioperative management have led to improved survival after curative resection. However, the rates of postoperative recurrence remain high (60%-80%)[3], and such recurrences can originate from intrahepatic metastases of the primary HCC and from the multicentric occurrence of new tumors[4]. With regard to the latter, many studies have reported a significant association between HCC development and underlying liver disease[5,6]. Therefore, HCC tumor factors as well as the underlying hepatic status should be carefully examined to predict tumor recurrence after curative resection and to choose optimal treatments.

A variety of malignant tumors in many visceral sites have appeared after chronic inflammation[7]. Clinical and biochemical evidence suggests that prostaglandin E_2 (PGE_2) produced at inflammation sites and its receptors play an important role in the development of malignant tumors, including HCC and other cancers[8,9]. The biosynthesis of PGE_2 from arachidonic acid requires two enzymatic activities that include cyclooxygenase (COX) and prostaglandin E synthase (PGES), which is the terminal enzyme for PGE_2 biosynthesis. Three PGES isoforms have been identified, including microsomal PGES-1 (mPGES-1), mPGES-2, and cytosolic PGES[10,11]. In particular, mPGES-1, an enzyme induced by pro-inflammatory stimuli, has received much attention[8,11]. Previous studies have indicated that mPGES-1 overexpression was associated with various types of cancer, including HCC[12,13]. Therefore, mPGES-1 may play an important role in HCC recurrence in the remnant liver tissue after curative resection for HCC.

The aim of the present study was to clarify whether mPGES-1 expression in HCC and non-cancerous liver tissues affects the clinical course of HCC patients undergoing curative resection.

MATERIALS AND METHODS

Patients and follow-up

Sixty-four consecutive patients (42 males and 22 females) underwent curative liver resection for HCC at the Division of Surgery, National Hospital Organization, Nagasaki Medical Center, between March 2003 and December 2006. In all cases, the diagnosis of HCC was confirmed by pathological examination of the resected specimens.

The inclusion criteria for the study were as follows: (1) the absence of extrahepatic metastasis; (2) curative resection defined as histological evidence of the complete removal of HCC tumors; and (3) no additional therapies or multi-modality treatment for HCC until the development of recurrence. Written informed consent was obtained from all the patients. They were regularly followed up at our outpatient clinic and were prospectively monitored for disease recurrence by serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), and ultrasonography or computed tomography every 3 mo. Suspected intra-hepatic recurrence was confirmed by hepatic angiography, and if necessary, by percutaneous needle biopsy. The follow-up period was at least 12 mo or until death in patients who died within 12 mo of their operation. The study was conducted in accordance with the Helsinki Declaration and the guidelines issued by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ethics Committee at National Hospital Organization, Nagasaki Medical Center.

Tissue samples

HCC tissues and non-cancerous liver tissues from the opposite liver lobe in which HCC developed were obtained. The tissues were frozen in liquid nitrogen and stored at -80℃ until use. For immunohistochemical analysis, the tissues were formalin-fixed and paraffin-embedded.

Histologically "normal" livers (free of hepatitis B or C viral infections and without any significant pathological abnormalities) were obtained from 7 patients with liver metastases from colorectal cancer.

Immunohistochemistry

For immunohistochemical analysis of the mPGES-1 protein, formalin-fixed and paraffin-embedded tissue blocks were cut into 4 μm-thick sections. The sections were deparaffinized in xylene and subsequently rehydrated in sequential ethanol (100%-70%). After washing 3 times with 10 mmol/L phosphate-buffered saline (PBS) (pH 7.4), antigen retrieval was performed by first heating in a microwave at 95℃ for 20 min, then by washing twice in PBS for 10 min. The sections were treated with peroxidase-blocking solution (DAKO Japan, Kyoto, Japan) for 5 min, and incubated with the primary antibody for 60 min at room temperature. The primary antibody used was a 1:100 dilution of a mPGES-1 polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA). A standardized two-step method with ENVISION plus (DAKO) was used for detection. The reaction products were visualized using diaminobenzidine as a chromogen (DAKO), and counterstained with Mayer's hematoxylin (DAKO). The specificity of the antibody was checked by the adsorption with corresponding blocking peptides (Cayman Chemical) using a 1:1 ratio of primary antibody to blocking peptide.

Scoring criteria for mPGES-1 expression

Two blinded investigators (MI and KN) evaluated the immunostained sections. To assess the mPGES-1 protein staining results, the cytoplasmic immunoreactive intensity was scored as previously described[14]. In summary, the staining intensity for mPGES-1 was scored in each specimen on a scale of 0-3, with 0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining (Figure 1). The staining intensity was evaluated for the maximum intensity among

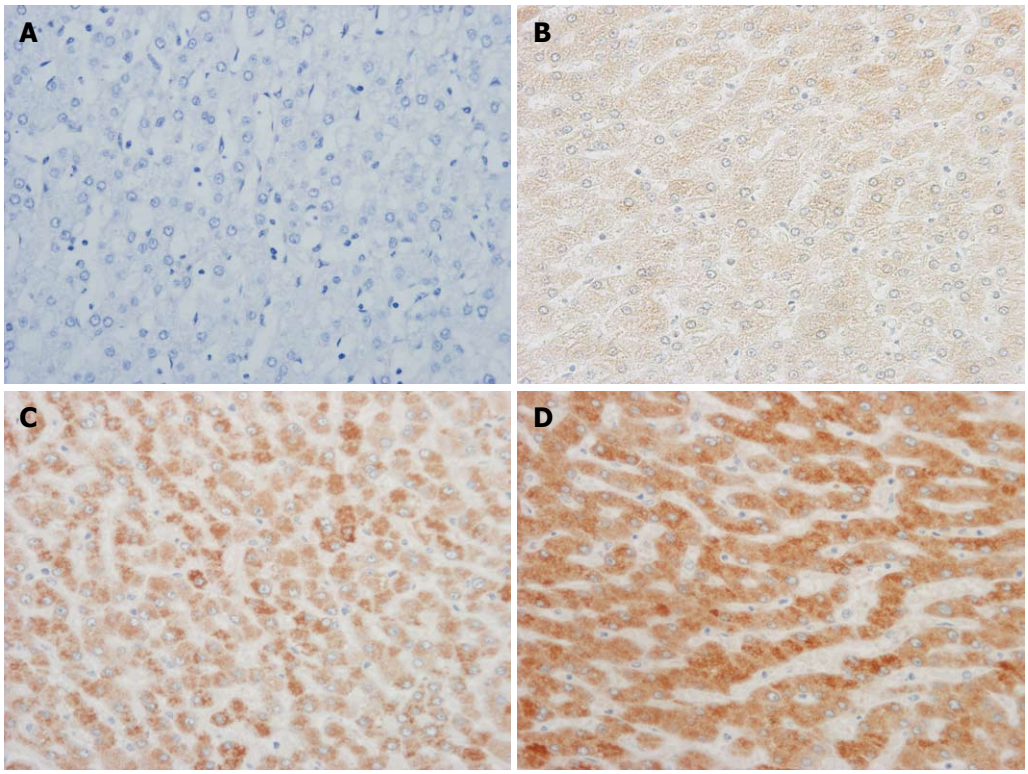

Figure 1 Grading of immunohistochemical staining for microsomal prostaglandin E synthase-1 protein in representative liver tissue (original magnification, × 200). A: No immunoreactivity for microsomal prostaglandin E synthase-1 (mPGES-1) (grade 0); B: Weakly positive for mPGES-1 (grade 1); C: Moderately positive for mPGES-1 (grade 2); D: Strongly positive for mPGES-1 (grade 3).

positive cells ("maximum intensity of staining", Ⅰ) and the intensity level observed in the largest number of positive cells ("most extensive intensity level", Ⅱ). The extent to which positive cells were observed in each specimen ("extent of distribution of positive cells", Ⅲ) was estimated and scored on a scale of 0-4, with 0 = negative, 1 = positive in 1%-25% of cells, 2 = positive in 26%-50% of cells, 3 = positive in 51%-75% of cells, and 4 = positive in 76%-100% of cells. Each section was evaluated for the sum of these three parameters (Ⅰ + Ⅱ + Ⅲ). Immunoreactivity for mPGES-1 protein was compared statistically using the average of the sum in each histological category. The patients in the present study were divided into two groups, including the higher expression (the sum of the categorical score, 6 to 10) and the lower expression groups (the sum of the categorical score; less than 6).

Western blotting analysis

We performed a Western blotting analysis on representative samples of HCC and non-cancerous liver tissues. The tissues were homogenized on ice in RIPA buffer [PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 100 ng/mL phenylmethylsulphonyl fluoride, 4 mg/mL aprotinin, 2 mg/mL leupeptin, 1 mg/mL pepstatin, 10 mg/mL antipain, 10 mg/mL soybean trypsin inhibitor, and 2 mmol/L ethylenediaminetetraacetic acid. The homogenates were clarified by centrifugation. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). After boiling for 5 min in the presence of 2-mercaptoethanol, samples containing 50 mg of tissue lysates were separated on 12.5% SDS-polyacrylamide gels and then transferred onto equilibrated Hybond PVDF membranes (Amersham International, Buckinghamshire, UK). After skim milk blocking, the membranes were then incubated with the mPGES-1 polyclonal antibody (at a dilution of 1:500). Bound antibodies were detected with horseradish peroxidase-labeled rabbit anti-goat IgG (Southern Biotechnology Associates, Birmingham, AL, USA) using an enhanced chemiluminescence detection system (ECL kit; Amersham International, Buckinghamshire, UK).

Analysis of the risk factors for HCC recurrence after curative resection

The following clinicopathological factors were evaluated for their association with HCC recurrence: age, gender, presence of hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus antibody (anti-HCV Ab), platelet count, preoperative blood chemistry (serum levels of total bilirubin, alanine aminotransferase and albumin), presence of liver cirrhosis, and mPGES-1 expression. The evaluated operative factors included the intraoperative blood loss and the hepatectomy method. The tumor factors were the greatest tumor diameter, the number of tumor nodules, the presence of vascular invasion, the presence of capsular formation, the histological grade, and the serum levels of AFP and DCP. The hepatectomy method was classified as anatomical or non-anatomical resection ac-

Table 1 Microsomal prostaglandin E synthase-1 expression in hepatocellular carcinoma and non-cancerous liver tissue *n* (%)

	No. of cases	Patients with higher scores	Patients with lower scores	Scores (mean ± SD)	*P*
Hepatocellular carcinoma tissues					
Well differentiated	18	7/18 (38.9)	11/18 (61.1)	5.1 ± 2.7	-
Moderately differentiated	40	14/40 (35.0)	26/40 (65.0)	5.1 ± 1.7	0.959[a]
Poorly differentiated	6	0	6/6 (100)	3.0 ± 1.8	0.009[a]
Non-cancerous liver tissues					
Normal	2	1/2 (50.0)	1/2 (50.0)	3.3 ± 0.7	-
Chronic hepatitis	31	19/31 (61.3)	12/31 (38.7)	5.4 ± 1.9	0.006[b]
Cirrhosis	31	15/31 (48.4)	16/31 (51.6)	6.4 ± 1.6	0.002[b], 0.039[c]
Normal livers from colorectal cancer	7	0	7/7 (100)	3.5 ± 0.5	-

[a]*vs* well differentiated hepatocellular carcinomas; [b]*vs* normal livers; [c]*vs* chronic hepatitis.

cording to the methods described by Makuuchi *et al*[15] and Takayama *et al*[16]. The anatomic resection consisted of the systematic removal of the hepatic segment which is confined by the tumor-bearing portal tributaries. In the non-anatomic resection, the liver was divided along a line so as to secure a surgical margin of at least 5 mm, if possible.

Statistical analysis

Statistical analyses were performed using either Student's *t*-test or the Mann-Whitney *U* test to compare variables between the groups. A recurrence-free survival curve was plotted using the Kaplan-Meier method. A statistical comparison of the recurrence-free survival was performed using the log-rank test. A multivariate analysis by the Cox proportional hazard model was used to identify the independent risk factors for tumor recurrence. A P value < 0.05 was considered statistically significant. Statistical analyses were performed using the StatView for Windows software program (version 5.0, SAS Institute Inc., Cary, NC, USA).

RESULTS

Characteristics of the patients

There were 42 male (65.6%) and 22 female (34.4%) patients. The mean age was 64 years (range, 38-86 years). Twenty-one patients were positive for HBsAg, 32 were positive for anti-HCV Ab, and 11 were negative for both. Thirty-one patients had a cirrhotic liver, while 33 did not. The maximum tumor size was 12 cm, and 57 patients (89.1%) had a solitary tumor. More than 90% of the patients enrolled in the study had a Child-Pugh classification of A for liver function. Fifty percent of the patients had a tumor size > 3 cm. In the pathological differentiation, HCC was well differentiated in 18 patients, moderately differentiated in 40, and poorly differentiated in 6. The median observation period was 49 mo (range, 3-74 mo).

Immunohistochemical analysis of mPGES-1 protein

The expression of mPGES-1 protein in the HCC and non-cancerous liver tissues was examined immunohistochemically. Various degrees of staining for mPGES-1 protein were observed. The scores for mPGES-1 expression in the HCC and non-cancerous liver tissues are summarized in Table 1. The marked expression of mPGES-1 was demonstrated in well differentiated as well as in moderately differentiated HCC tissues (scores; 5.1 ± 2.7 and 5.1 ± 1.7, respectively). Conversely, mPGES-1 expression was significantly weaker in poorly differentiated HCC tissues (score; 3.0 ± 1.8, $P < 0.05$). Seven of 18 cases (38.9%) with well differentiated HCC and 14 of 40 cases (35.0%) with moderately differentiated HCC had high expression scores, whereas none of the patients with poorly differentiated HCC had high expression scores.

The mPGES-1 levels increased significantly with fibrotic stage of the liver tissues (scores; normal liver 3.3 ± 0.7, chronic hepatitic livers 5.4 ± 1.9, cirrhotic livers 6.4 ± 1.6). High expression scores were observed in 1 of 2 normal livers (50%), 19 of 31 chronic hepatitic livers (61.3%), and 15 of 31 cirrhotic livers (48.4%). There was no significant correlation between tumor differentiation and non-cancerous liver tissue in the expression of mPGES-1 (data not shown). Additionally, mPGES-1 expression in normal livers obtained from 7 patients with liver metastasis was lower than that in damaged livers ($P < 0.05$).

Western blotting analysis of mPGES-1

To confirm the specificity of the mPGES-1 antibody and the presence of mPGES-1 protein in the specimen, Western blotting analysis was performed on representative samples of HCC and non-cancerous liver tissues. Both tissue types yielded a single band with a molecular weight of 16 kDa, indicating the presence of mPGES-1 protein (Figure 2).

Correlation between the levels of mPGES-1 expression and recurrence-free survival time

We evaluated the correlation between the levels of mPGES-1 expression in HCC and non-cancerous liver tissues and recurrence-free survival time. No statistically significant difference was observed in the recurrence-free survival time between the higher and lower expression groups in HCC tissues (Figure 3A). In contrast, a statistically significant difference in the recurrence-free survival time was observed between the higher and lower expression groups in non-cancerous liver tissues ($P = 0.006$, Figure 3B).

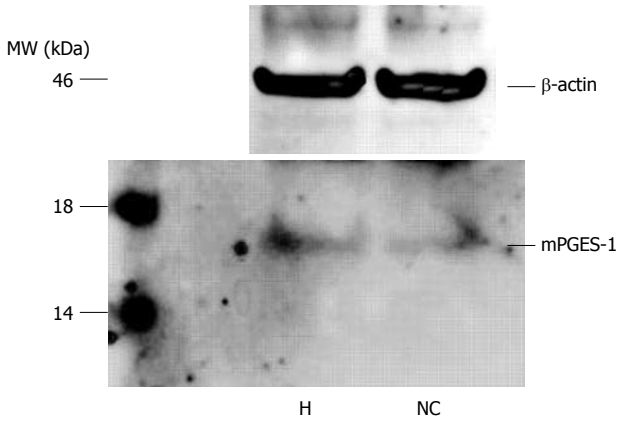

Figure 2 Western blotting analysis for microsomal prostaglandin E synthase-1. A band of 16 kDa in molecular weight, thus indicating the presence of microsomal prostaglandin E synthase-1 (mPGES-1) protein, is identified in both hepatocellular carcinoma tumors (H) and non-cancerous liver (NC) tissues. MW: Molecular weight.

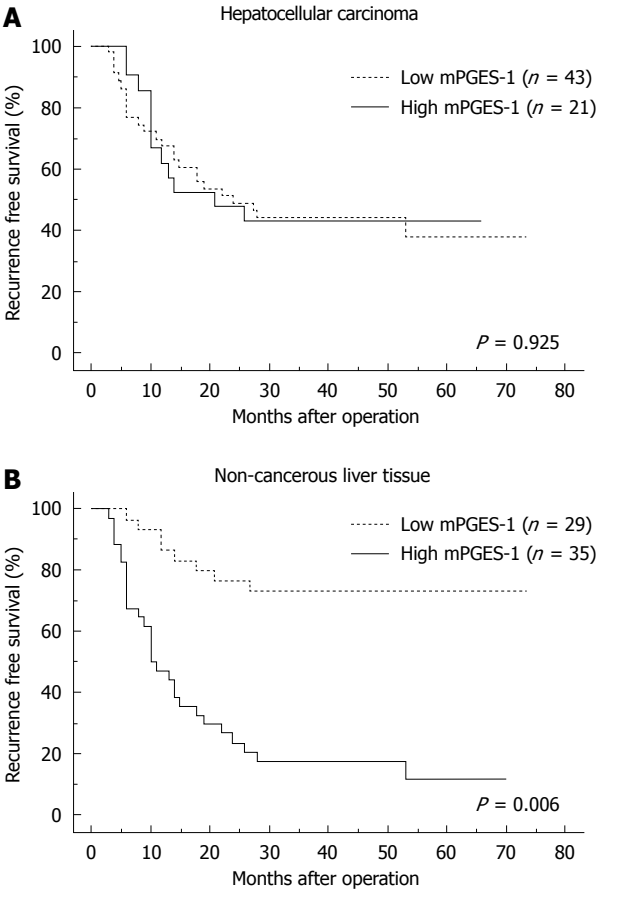

Figure 3 Recurrence-free survival time based on microsomal prostaglandin E synthase-1 expression in hepatocellular carcinoma tissues (A) and non-cancerous liver tissues (B). The recurrence-free survival time is significantly shorter in patients with an increased expression of microsomal prostaglandin E synthase-1 (mPGES-1) in non-cancerous liver tissues.

Correlation between various clinicopathological parameters and recurrence-free survival time

Various clinicopathological parameters were evaluated for their association with HCC recurrence (Table 2). A univariate analysis revealed that recurrence-free survival time was

Table 2 Univariate analysis of clinicopathological features related to postoperative recurrence

	No. of patients	Postoperative recurrence		*P*
		Yes	No	
Age (yr)				
⩾ 60	45	23	22	0.733
< 60	19	9	10	
Gender				
Male	43	20	23	0.481
Female	21	12	9	
Hepatitis B surface antigen				
Positive	21	13	8	0.147
Negative	43	19	24	
Hepatitis C virus antibody				
Positive	32	12	20	0.151
Negative	32	20	12	
Total bilirubin (mg/dL)				
⩾ 1.0	29	7	22	0.987
< 1.0	35	13	22	
Alanine aminotransferase (IU/L)				
⩾ 50	26	11	15	0.635
< 50	38	21	17	
Albumin (g/dL)				
⩾ 3.7	49	24	25	0.022
< 3.7	15	13	2	
Platelets (10^4/μL)				
⩾ 10	46	21	25	0.465
< 10	18	11	7	
Liver cirrhosis				
Present	31	16	15	0.772
Absent	33	16	17	
T mPGES-1				
High	21	12	9	0.925
Low	43	25	18	
NC mPGES-1				
High	35	23	11	0.006
Low	29	9	21	
Hepatectomy				
Anatomic	31	21	10	0.149
Non-anatomic	33	16	17	
Operative blood loss (mL)				
⩾ 500	15	12	3	0.091
< 500	49	25	24	
α-fetoprotein (ng/mL)				
⩾ 100	15	10	5	0.347
< 100	49	27	22	
DCP (mAU/mL)				
⩾ 400	22	15	7	0.081
< 400	42	19	23	
Tumor diameter (cm)				
⩾ 5	16	12	3	0.018
< 5	48	20	29	
Tumor number				
Multiple	7	3	4	0.789
Solitary	57	29	28	
Histological grade				
Well	18	8	10	0.495
Moderate	40	21	19	
Poor	6	3	3	
Capsular formation				
Present	49	25	24	0.320
Absent	15	7	8	
Vascular invasion				
Present	16	11	5	< 0.001
Absent	48	9	39	

DCP: Des-γ-carboxy prothrombin; mPGES-1: Microsomal prostaglandin E synthase-1; T mPGES-1: mPGES-1 expression in hepatocellular carcinoma tumor tissue; NC mPGES-1: mPGES-1 expression in non-cancerous liver tissue.

Table 3 Multivariate analysis of the risk factors for postoperative recurrence

Variables	Hazard ratio	95% CI	*P*
Vascular invasion (present)	4.116	1.813-9.344	< 0.001
NC mPGES-1 expression (high)	4.074	1.760-9.428	0.001
Tumor diameter (≥ 5 cm)	2.060	0.860-4.935	0.105
Albumin (< 3.7 g/dL)	1.745	0.589-3.165	0.315

NC mPGES-1: Microsomal prostaglandin E synthase-1 expression in non-cancerous liver.

shorter in cases with vascular invasion, higher mPGES-1 levels in non-cancerous liver tissue, a larger tumor diameter (≥ 5 cm), and lower levels of serum albumin (< 37 g/L). The operative factors were not significantly correlated with recurrence-free survival time. A multivariate analysis demonstrated that the presence of vascular invasion and higher mPGES-1 levels in the non-cancerous liver tissue were significant independent predictors for the early recurrence of HCC after curative hepatectomy (Table 3).

DISCUSSION

The present study demonstrated that the rate of HCC recurrence was high after curative resection. This finding was consistent with those described in other recent reports[1-6]. Tumor recurrence is caused by metastatic lesions, residual microscopic lesions that remain after curative resection, or multicentric occurrence in the setting of hepatitis or cirrhosis[17,18]. The prevention of tumor recurrence is key to the improvement of prognosis for HCC patients after a hepatectomy[19]. In the present study, a multivariate analysis indicated that the two independent predictors for HCC recurrence after curative resection were the presence of vascular invasion and increased mPGES-1 expression in the non-cancerous liver tissue.

Vascular invasion is a well-known risk factor for a poor prognosis after curative resection. The presence of vascular invasion is considered one of the strongest predictors of intrahepatic metastasis caused by the spread of cancer cells *via* the portal venous system[17-19]. Although several reports have demonstrated that postoperative adjuvant therapy prevented postoperative HCC recurrence[20,21], its efficacy has yet to be determined. Other therapeutic modalities for treating postoperative recurrence are urgently needed.

The most interesting finding in the present study was that increased mPGES-1 expression in the non-cancerous liver tissue was an independent predictor for early HCC recurrence after curative resection. Increased mPGES-1 levels induce PGE_2 synthesis, which may create a suitable environment for occult intrahepatic metastases to survive and spread after hepatectomy. This hypothesis is supported by several studies showing that PGE_2 was implicated in migration, secretion of various types of matrix metallo-proteinases, and cell adhesion in HCC cells[22-24]. Additionally, increased PGE_2 levels in the non-cancerous liver tissue leads to prolonged acceleration of necroinflammation and regeneration in the remnant liver[25]. The inflamed liver may also provide a good environment for occult intrahepatic metastases to grow in response to different growth factors[26]. In the present study, active hepatitic and/or cirrhotic livers had increased mPGES-1 expression compared to normal livers. The repeated cycles of necroinflammation, degeneration, and regeneration increase hepatocyte turnover, which facilitates spontaneous mutation and may hinder DNA repair[26]. The release of reactive oxygen species including superoxide and H_2O_2 in this situation may also contribute to uncontrolled cell growth, apoptosis, and senescence[27]. Another possible mechanism is that mPGES-1 itself may act as a landscaping tumor promoter. mPGES-1 lies downstream of the PGE_2-biosynthetic pathway of COX-2. Recent studies reported that mPGES-1 was expressed in several cancers and was linked to carcinogenesis[12,28]. mPGES-1 derived from the stromal component may promote tumor growth by producing bioactive PGE_2, which acts angiogenetically or immunosuppressively, and affects carcinoma cells in a paracrine fashion[12,28]. Therefore, the increased expression of mPGES-1 in the non-cancerous liver tissue may create conditions suitable for HCC recurrence from metastasis or multicentric occurrence. However, the precise mechanisms remain to be elucidated.

The mPGES-1 expression in HCC tissues did not correlate well with postoperative recurrence. This finding suggested that the mPGES-1 in HCC tissues *per se* did not determine the malignant potential of HCC tissues, although overexpression of mPGES-1 was associated with various types of cancer[12,13].

The data indicate that COX-2 inhibitors are chemopreventive for several kinds of cancers[29], however, there have been no reports on HCC patients. Although the COX-2 inhibitors have a reduced gastrointestinal toxicity in comparison to traditional non-steroidal anti-inflammatory drugs, some adverse effects have been reported[30]. From this standpoint, more selective inhibition of the prostanoid pathway to PGE_2 is thus highly desirable. mPGES-1 is the terminal enzyme for PGE_2 biosynthesis, and thus it is considered the most selective agent for that pathway. Although there have been several reports concerning the selective inhibitors of mPGES-1[31,32], further studies are still needed in clinical settings.

In conclusion, increased mPGES-1 expression in non-cancerous liver tissue is closely associated with the early recurrence of HCC after curative resection. The present study also indicates that an inhibitor of mPGES-1 may be a new therapeutic option to improve the survival rate of HCC patients after curative resection.

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COMMENTS

Background

Microsomal prostaglandin E synthase-1 (mPGES-1) is the terminal enzyme in the formation of prostaglandin E_2 from prostaglandin H_2. Data indicate that increased expression of mPGES-1 is associated with various types of cancers. However, the impact of mPGES-1 expression on the clinical course of hepatocellular carcinoma (HCC) has not yet been elucidated.

Research frontiers

In HCC, tumor recurrence is caused by metastatic lesions, residual microscopic lesions that remain even after curative resection, and multicentric occurrence in the setting of hepatitis or cirrhosis. The research was performed to clarify the risk factors for the recurrence of HCC after curative resection in Nagasaki Medical Center.

Innovations and breakthroughs

The present study demonstrates that various degrees of mPGES-1 expression occur in HCC and non-cancerous liver tissues. This is the first report to demonstrate that increased expression of mPGES-1 in non-cancerous liver tissue is an independent predictor for HCC recurrence after curative resection.

Applications

mPGES-1 expression in non-cancerous liver tissue could be a useful biomarker for screening high risk groups of patients with HCC after curative resection. In the near future, a selective mPGES-1 inhibitor may prevent postoperative recurrence of HCC and improve the prognosis of HCC patients.

Terminology

mPGES-1 is a protein belonging to the membrane-associated proteins involved in eicosanoid and glutathione metabolism super family. mPGES-1 is induced by pro-inflammatory stimuli, down-regulated by anti-inflammatory glucocorticoids, and functionally coupled with cyclooxygenase-2. Thus, mPGES-1 plays a central role in the biosynthesis of prostaglandin E_2.

Peer review

The paper reported that increase in mPGES-1 in non-cancerous liver was an independent prognostic factor in patients received surgical therapy to HCC. Although the results might be of importance, several questions are addressed, and several points to be improved are suggested.

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BRIEF ARTICLE

Suspected uncomplicated cecal diverticulitis diagnosed by imaging: Initial antibiotics *vs* laparoscopic treatment

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Abstract

AIM: To compare the recurrence rate following initial antibiotic management to that following laparoscopic treatment for suspected uncomplicated cecal diverticulitis.

METHODS: We examined the records of 132 patients who were diagnosed with uncomplicated cecal diverticulitis and a first attack during an 8-year period. The diagnosis of uncomplicated diverticulitis was made based on imaging findings, such as inflamed diverticulum or a phlegmon with cecal wall thickening. Concurrent appendiceal dilatation from 8 to 12 mm was observed in 36 patients (27%). One hundred and two patients were treated initially with antibiotics only, whereas 30 underwent laparoscopic treatment, including partial cecectomy (n = 8) or appendectomy with diverticulectomy (n = 9) or appendectomy alone (n = 13). We compared clinical outcomes in both groups over a median follow-up period of 46 mo.

RESULTS: All patients were successfully treated with initial therapy. Of the 102 patients who initially received only antibiotic treatment, 6 (6%) had a recurrence (3 in the cecum and 3 in the ascending colon or transverse colon) during the follow-up period. Five of these patients were managed with repeated antibiotic treatment and 1 underwent ileocolic resection for perforation. Of the 30 patients treated by the laparoscopic approach, 2 (7%) had a recurrence (ascending colon) which was treated with antibiotics.

CONCLUSION: Initial antibiotic management for suspected uncomplicated cecal diverticulitis showed comparable efficacy to laparoscopic treatment in the prevention of recurrence.

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Key words: Antibiotics; Cecal diverticulitis; Laparoscopy; Radiological imaging

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Park HC, Lee BH. Suspected uncomplicated cecal diverticulitis diagnosed by imaging: Initial antibiotics *vs* laparoscopic treatment. *World J Gastroenterol* 2010; 16(38): 4854-4857 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i38/4854.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i38.4854

INTRODUCTION

Right colonic diverticulitis is a common complication of right-sided diverticular disease and has a higher prevalence in Oriental countries than in Western countries[1]. Cecal diverticulitis is uncommon and is rarely detected preoperatively because it is usually misdiagnosed as appendicitis[2].

There has been some controversy regarding the optimal management of cecal diverticulitis. Some surgeons recommend surgical treatment, claiming that cecal diverticulitis

does not usually resolve with medical therapy and has a high rate of recurrence with complications[3-5]. In contrast, other authors favor conservative treatment, stating that it is a safe and effective treatment regimen with a low recurrence rate[6-8]. Differences due to ethnicity or pathophysiological mechanisms including disease state may account for this variation in outcome.

With the increasing use of radiologic evaluation for right lower quadrant (RLQ) pain, the diagnosis of cecal diverticulitis is possible and may be assessed in both uncomplicated and complicated cases[9].

However, the appropriate treatment of suspected uncomplicated cecal diverticulitis diagnosed by radiologic evaluation is not definite and there have been few reports comparing the long-term recurrence rate following antibiotic only management and laparoscopic treatment for suspected uncomplicated cecal diverticulitis in an Asian population.

The aim of this study was to evaluate the treatment outcomes of suspected uncomplicated cecal diverticulitis diagnosed by radiologic imaging. To this end, we reviewed the long-term recurrence in a series of Asian patients who received initial antibiotic management, and compared this to laparoscopic treatment.

MATERIALS AND METHODS

During an 8-year period (2001 to 2008), 8814 patients admitted with RLQ pain were assessed (Table 1). We performed routine computed tomography (CT) in these patients, and in some indeterminate cases, adjuvant specific appendiceal ultrasonography was performed.

Clinical information was reviewed retrospectively using an existing database which revealed 164 patients with suspected cecal diverticulitis following radiologic evaluations. All patients were of Asian descent with a first documented attack. Twenty one patients who underwent surgery for suspected perforation or generalized peritonitis and 11 patients who were lost to follow-up were excluded, thus, 132 patients with suspected uncomplicated cecal diverticulitis were included in the present study. Uncomplicated diverticulitis was diagnosed as inflamed diverticulum or a phlegmon with cecal wall thickening, using radiological imaging (Figure 1). None of the patients had symptoms of peritonitis or the formation of an inflammatory mass. Diverticulitis accompanied by appendiceal dilatation from 8 to 12 mm was observed in 36 patients (27%).

The treatment method was determined at the discretion of the doctor who first examined the patients or patient preference. Therefore, 102 patients who received initial antibiotic management were classified as group 1 and 30 patients who underwent laparoscopic treatment were classified as group 2.

The antibiotic regimen consisted of a second generation cephalosporin and metronidazole which was administered for 4-7 d or until the abdominal pain subsided. Most patients received intravenous antibiotics, however, 8 received oral antibiotics. The diagnosis was confirmed by CT (3-D colon) or colonoscopic examination in all patients at least once during the follow-up period.

Table 1 Clinical and radiological diagnosis in consecutive patients with right lower quadrant pain (n = 8814)

Diagnosis	n (%)
Appendicitis	4718 (53.6)
Cecal diverticulitis	164 (1.8)
Ascending colonic diverticulitis	202 (2.3)
Terminal ileum diverticulitis	10 (0.1)
Mesenteric lymphadenitis	392 (4.4)
Gynecologic disease	501 (5.7)
Urologic disease	93 (1.1)
Uncommon findings (malignancy, IBD, *etc.*)	121 (1.4)
Nonspecific ileocolitis	806 (9.1)
No remarkable findings	1807 (20.5)

IBD: Inflammatory bowel disease.

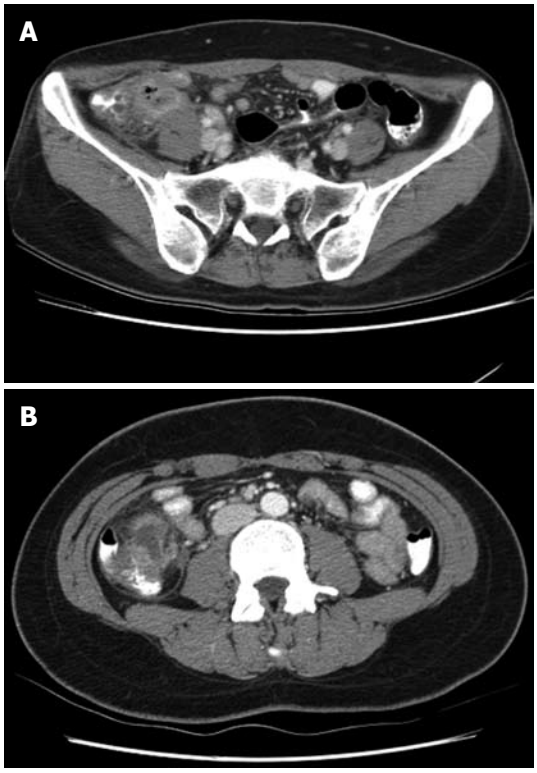

Figure 1 Radiologic evaluation of acute cecal diverticulitis (computed tomography). A: Inflamed diverticulum; B: Diverticulitis with phlegmon.

Laparoscopic treatment consisted of partial cecectomy including appendix and diverticulum in 8 patients, using one or two endoGIA (Covidien, Mansfield, MA, USA). Diverticulectomy was performed in 9 patients where technically feasible, especially in the case of diverticula arising from the anterior aspect of the cecum. Concurrent appendectomy was performed to prevent future diagnostic confusion. The remaining 13 patients underwent appendectomy alone, followed by direct visualization of the inflamed diverticulum located in the lateral or posterior side of the cecum.

Patients were reviewed using their medical records and were interviewed on the telephone to identify any recurring symptoms and surgical interventions. The median

Table 2 Clinical characteristics and outcomes in both groups (mean ± SD) *n* (%)

	Group 1 (*n* = 102)	Group 2 (*n* = 30)	*P*
Age (yr)	37.5 ± 11	39.0 ± 13	0.512
Sex (M/F)	50/52	15/15	0.925
WBC (× 10^3/L)	10.6 ± 3.6	12.3 ± 5.1	0.034
Mean hospital stay (d)	5.8 ± 2	7.3 ± 2	0.003
Mean medical costs ($)	1253 ± 168	1657 ± 157	0.001
Follow-up (mo)	46	45	0.725
Readmission rate	8 (8)	3 (10)	0.710
Recurrence	6 (6)	2 (7)	0.875
Treatment at recurrence	5 antibiotics 1 ileocolic resection	2 antibiotics	-

WBC: White blood cell.

follow-up time was 46 mo (range, 10-112 mo). Using this information, we evaluated the outcomes including readmission and recurrence rate between the two groups using the χ^2 and *t*-tests.

RESULTS

Of the 102 patients in group 1, 7 had undergone a previous appendectomy. Eight patients were suspected of having a pericolic abscess and 31 patients had a visible fecalith on diagnostic imaging. Twenty five patients had concurrent appendiceal dilatation. All patients were successfully treated without complications.

Of the 30 patients in group 2, 2 had a pericolic abscess and 11 had concurrent appendiceal dilatation. The operative findings revealed an inflamed diverticulum and phlegmon with adjacent bowel wall thickening. All patients with partial cecectomy or diverticulectomy were confirmed as having diverticulitis on pathologic examination. Appendix examination revealed that most patients (21/30) had secondary appendiceal serositis and the remaining patients had a normal appendix. The postoperative period was uneventful in most patients with the exception of 2 who developed wound infections. Group 2 patients had a higher white blood cell count at admission, a longer hospital stay, and higher medical costs.

During the follow-up period, 2 patients died of unrelated causes (liver cirrhosis, pancreas cancer). To date, all other patients are alive. Recurrence was defined as the development of the same symptoms and radiological evidence of diverticulitis.

Of the 102 patients in group 1, 6 had recurrence at a median of 15 mo (range, 5-25 mo) after treatment. Three recurrences were in the cecum and 3 were in the ascending colon or proximal transverse colon. Of these 6 patients, 5 were successfully treated with antibiotics and 1 underwent laparoscopic ileocolic resection for perforated diverticulitis. Two patients were readmitted due to RLQ pain, however, these patients were treated medically and did not undergo surgery for appendicitis.

In group 2, 2 patients (7%) had recurrence 21 mo and 24 mo after treatment and required further antibiotic treatment. Of these patients, 1 had undergone partial cecectomy and the other had undergone diverticulectomy. The location of recurrent diverticulitis was the distal ascending colon and hepatic flexure colon in each. One patient readmitted with RLQ pain, was treated medically (Table 2).

DISCUSSION

Although cecal diverticulitis is an uncommon condition and is preoperatively almost indistinguishable from appendicitis, it has a high prevalence in the Oriental population[10,11].

In the same 8-year period, we performed 4871 appendectomies, and the frequency of cecal diverticulitis was high (1 in 30 appendectomies). The high number of appendectomies and a specialized radiologist for RLQ diseases may have contributed to the higher diagnostic rate of cecal diverticulitis in our institution.

Other studies also demonstrated that right colonic diverticulitis can be correctly diagnosed using radiologic evaluation[12-15]. Since diverticula which develop in the right colon are generally of a limited number and are frequently solitary, the evaluation is not difficult. However, in our experience, the differential diagnosis using imaging studies between appendicitis and appendiceal diverticulitis or between perforated appendicitis and perforated diverticulitis is still problematic.

The treatment of suspected uncomplicated cecal diverticulitis diagnosed by radiological imaging has not been uniform. Uncomplicated cecal diverticulitis can usually be treated with antibiotics. However, if the disease is not fully differentiated from acute appendicitis the patient treated with initial antibiotics may be readmitted due to RLQ pain. Moreover, the clinical course of uncomplicated cecal diverticulitis may not be easily determined because most patients are young, and are only followed up for a short period.

Laparoscopic minimal surgery, such as diverticulectomy or partial cecectomy, is a good therapeutic option, however, the procedure is not always easy. Simple diverticulectomy using one or two staplers may be performed in some cases, but concerns regarding conversion or extended dissection of inflammatory tissue have been raised. The location of a diverticulum may be associated with technical difficulties. Diverticulitis that originates from the anterior aspect of the cecum may be more easily managed. However, diverticulitis found in the lateral or posterior aspect of the cecum may result in a difficult laparoscopic procedure. A phlegmon with adjacent inflammation also complicates the situation for surgeons.

To our knowledge, laparoscopic diverticulectomy, particularly in cases of cecal diverticulitis, has been rarely reported[16,17]. A possible explanation for this is that the disease is uncommon and therefore, this procedure may be difficult and risky, if technically infeasible or performed by an inexperienced surgeon.

Treatment with both antibiotics and laparoscopic surgery carry a risk of recurrence. Extensive surgery was often performed during re-operation in order to decrease the

risk of leaving an inflamed diverticulum. However, we presume that the clinical features of patients with cecal diverticulitis might differ between the Asian and non-Asian population. In most Asian patients, a low recurrence rate may be expected and non-operative management could be performed, even in recurrent cases[18-21].

Moreover, diverticulitis does not always recur in the same place. Multiple diverticula are often found in Asian patients with cecal diverticulitis. We also demonstrated right-sided colonic recurrent diverticulitis at a different site.

Many clinicians prefer non-operative management if right-sided uncomplicated diverticulitis is recognized preoperatively and may achieve long-term remission and control of the disease. We also believe that the natural course of cecal diverticulitis has mostly benign features. Many patients were successfully treated with initial antibiotic management at the time of the first attack and had a low readmission and recurrence rate. Moreover, patients with recurrence may be retreated non-operatively. These findings suggest that cecal diverticulitis, if not combined with definite complications, seems to have a benign nature and may be treated non-operatively.

The treatment of complicated cecal diverticulitis or a suspected mass is less controversial due to high morbidity and unexpected pathologies[22]. Surgical treatment is well accepted in these cases.

In conclusion, we suggest that initial antibiotic management is an effective treatment option for suspected uncomplicated cecal diverticulitis diagnosed by radiological evaluation and shows comparable long-term results in the prevention of recurrence, to that of laparoscopic treatment in Asian patients.

COMMENTS

Background

Although the optimal treatment of suspected uncomplicated cecal diverticulitis remains controversial, non-operative management of this disease is increasing. The authors reviewed the long-term recurrence rate following initial antibiotic management for uncomplicated cecal diverticulitis diagnosed by radiological imaging, compared with laparoscopic treatment in Asian patients.

Research frontiers

The efficacy of initial antibiotic management for suspected uncomplicated cecal diverticulitis diagnosed by radiological imaging has not been fully addressed.

Innovations and breakthroughs

Recent studies have reported good outcomes following antibiotic management of uncomplicated diverticulitis. However, there have been few reports on cecal diverticulitis which is common in Asian patients. Cecal diverticulitis is not easily differentiated from appendicitis. Therefore, many clinicians are concerned about the long-term recurrence rate following initial non-operative management, compared with surgical treatment.

Applications

Laparoscopic surgery may be unnecessary in localized or uncomplicated diverticular disease. The surgical treatment options for cecal diverticulitis range from diverticulectomy to right hemicolectomy and are reserved only for definite cases of complicated diverticulitis.

Terminology

Uncomplicated diverticulitis was defined as inflamed diverticulum or a phlegmon with cecal wall thickening, and was not associated with complications, such as perforation, obstruction, or visible abscess.

Peer review

Over all, the study demonstrates success of antibiotic alone therapy in a large number of patients suffering from cecal diverticulitis - a rather uncommon entity.

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BRIEF ARTICLE

Comparative analysis of dideoxy sequencing, the KRAS StripAssay and pyrosequencing for detection of KRAS mutation

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Abstract

AIM: To compare the differences between dideoxy sequencing/KRAS StripAssay/pyrosequencing for detection of KRAS mutation in Chinese colorectal cancer (CRC) patients.

METHODS: Formalin-fixed, paraffin-embedded (FFPE) samples with tumor cells ⩾ 50% were collected from 100 Chinese CRC patients at Beijing Cancer Hospital. After the extraction of genome DNA from FFPE samples, fragments contained codons 12 and 13 of KRAS exon 2 were amplified by polymerase chain reaction and analyzed by dideoxy sequencing, the KRAS StripAssay and pyrosequencing. In addition, the sensitivities of the 3 methods were compared on serial dilutions (contents of mutant DNA: 100%, 50%, 20%, 15%, 10%, 5%, 1%, 0%) of A549 cell line DNA (carrying the codon 12 Gly>Ser mutation) into wild-type DNA (human normal intestinal mucosa). The results of dideoxy sequencing, the KRAS StripAssay and pyrosequencing were analyzed by Chromas Software, Collector for KRAS StripAssay and the pyrosequencing PyroMarkTM Q24 system, respectively.

RESULTS: Among 100 patients, KRAS mutations were identified in 34%, 37% and 37% of patients by dideoxy sequencing, the KRAS StripAssay and pyrosequencing, respectively. The sensitivity was highest with the KRAS StripAssay (1%), followed by pyrosequencing (5%), and dideoxy sequencing was lowest (15%). Six different mutation types were found in this study with 3 main mutations Gly12Asp (GGT>GAT), Gly12Val (GGT>GTT) and Gly13Asp (GGC>GAC). Thirty-three patients were identified to have KRAS mutations by the 3 methods, and a total of 8 patients had conflicting results between 3 methods: 4 mutations not detected by dideoxy sequencing and the KRAS StripAssay were identified by pyrosequencing; 3 mutations not detected by dideoxy sequencing and pyrosequencing were identified by the KRAS StripAssay; and 1 mutation not detected by pyrosequencing was confirmed by dideoxy sequencing and the KRAS StripAssay. Among these discordant results, the results identified by dideoxy sequencing were consistent either with the KRAS StripAssay or with pyrosequencing, which indicated that the accuracy of dideoxy sequencing was high.

CONCLUSION: Taking a worldwide view of reports and our results, dideoxy sequencing remains the most popular method because of its low cost and high accuracy.

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Key words: DNA mutational analysis; KRAS; Mutation; Dideoxy sequencing; KRAS StripAssay; Pyrosequencing

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract, with yearly increasing morbidity and mortality in China. Recently, the development of targeted drugs (for example, cetuximab, panitumumab) has brought advancement in CRC therapy. Cetuximab, which targets the epidermal growth factor receptor (EGFR), shows activity in refractory CRC patients expressing EGFR[1], and in CRC patients with tumors that do not express EGFR immunohistochemically[2]. Thus, there is not an association between EGFR expression and cetuximab efficacy[2,3]. The activating mutations in exon 2 of KRAS play an important role in the progression of CRC, which can induce unlimited proliferation of tumor cells[4,5]. One study reported that KRAS mutations could become an independent prognostic factor in advanced CRC patients treated with cetuximab[6] and there was a significant negative association between KRAS mutations and cetuximab efficacy[7]. Some clinical trials, such as CRYSTAL, OPUS and EVEREST, demonstrated that CRC patients with wild-type KRAS could benefit from the addition of cetuximab to the standard chemotherapy regimen, but patients with mutated KRAS could not[8-10]. Thus, detection of KRAS mutations is strongly recommended before administration of cetuximab.

The mutation rate of KRAS is slightly different among different trials and different areas; for example, the prevalence of KRAS mutation was 35.6% in the CRYSTAL trial[8], but 42.3% in the CO.17 trial[11]; the frequency of KRAS mutation is about 40% in the United States, 34% in the Netherlands, 49% in France and 26.5% in Taiwan, China[12]. The KRAS mutation rate in Chinese CRC patients is about 37% according to our previous study (about 600 patients were studied) using dideoxy sequencing. Most mutations occur in codons 12 and 13 (about 95%), and only a few in codon 61 (about 5%)[13-15]. Up to now, many methods have been used to detect KRAS mutations, including dideoxy sequencing, polymerase chain reaction (PCR)-single-strand conformation polymorphism, PCR-restriction fragment length polymorphism (RFLP), pyrosequencing, denaturing high performance liquid chromatograph, and so on[16]. Along with the advancement of technology, many new kits have been developed, such as the DxS K-RAS Mutation Test Kit and the KRAS StripAssay, which brought new choices for researchers.

As a new method, the KRAS StripAssay has been used in Europe and America, but is not available in China. In order to determine the sensitivity of the KRAS StripAssay, and confirm the feasibility of dideoxy sequencing, we compared the differences between dideoxy sequencing, the KRAS StripAssay and pyrosequencing for mutation detection in codons 12 and 13 of KRAS. Codons 12 and 13 of KRAS were detected by the 3 methods in 100 CRC patients proposed for treatment with cetuximab.

MATERIALS AND METHODS

Patient samples and control samples

A total of 100 patients in Beijing Cancer Hospital with CRC confirmed by histopathology between October 2008 and August 2009 were investigated for KRAS mutations in our laboratory. Formalin-fixed, paraffin-embedded (FFPE) samples with ≥ 50% tumor cells were collected. An A549 cell line was preserved in our laboratory and normal intestinal mucosa was provided by the tissue bank of our hospital.

Genomic DNA extraction

Genomic DNA of FFPE sections was extracted using E.Z.N.A.FFPE DNA Kit (Lot. D3399-01, OMEGA, USA) according to the manufacturer's instructions. Genomic DNAs of A549 and normal intestinal mucosa were extracted using EasyPure Genomic DNA Extraction Kit (Lot. D60916, TransGen Biotech, China) according to the manufacturer's instructions. All genomic DNAs were stored at -20℃ until further research.

Preparation of serial dilutions

The concentrations of DNA from the A549 cell line and normal intestinal mucosa were determined by fluorometry. Serial dilutions were prepared by putting A549 cell DNA into wild-type DNA to produce dilutions with the following contents of mutant DNA: 100%, 50%, 20%, 15%, 10%, 5%, 1%, 0%.

Dideoxy sequencing

A DNA fragment including exon 2 of the KRAS gene was amplified by PCR using primers (KRAS-F: 5'-GGTACTGGTGGAGTATTTGATAG-3', KRAS-R: 5'-TGGTCCTGCACCAGTAATATG-3') with a product size of 248 bp. Each PCR reaction consisted of 10 × LA PCR buffer Ⅱ 2 μL, 10 mmol/L dNTPs 2 μL, LA *Taq* 0.2 μL (DRR200A, TAKARA), genomic DNA 2 μL, 10 μmol/L forward primer 0.5 μL, 10 μmol/L reverse primer 0.5 μL in a final volume of 20 μL. The cycling conditions were 94℃ for 5 min, 45 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ for 20 s, final extension at 72℃ for 10 min, and ended at 4℃. The PCR products were determined by 3% agarose gel electrophoresis and then sequenced using the same forward primer by Invitrogen 3730XL genetic analyzer. The sequencing results were analyzed with Chromas software under the condition of signal/noise > 98%.

KRAS StripAssay

The KRAS StripAssay kit (Lot. 5-590) was kindly pro-

vided by ViennaLab of Austria. All procedures were conducted according to the manufacturer's instructions. Briefly, PCR products were amplified in a tube containing 15 μL amplification mix, 5 μL diluted *Taq* DNA polymerase (1 U), 5 μL DNA template (50 ng genome DNA). The cycling conditions were 94℃ for 2 min, 35 cycles of 94℃ for 60 s, 70℃ for 50 s, 56℃ for 50 s and 60℃ for 60 s, final extension at 6℃ for 3 min. PCR products with fragment lengths 151 bp and 204 bp were determined by 3% agarose gel electrophoresis after PCR amplification. Following hybridization (45℃, shaking waterbath), stringent washing (45℃, shaking waterbath) and color development (room temperature), the results were interpreted using the enclosed Collector sheet.

Pyrosequencing technique

A DNA fragment including codons 12 and 13 of the KRAS gene was amplified by PCR using primers (forward: 5'-biotin-TGACTGAATATAAACTTGTGGTAGTTG-3', reverse: 5'-TCGTCCACAAAATGATTCTGAA-3') with a product size of 91 bp. Each PCR reaction consisted of 10 × PCR buffer 5 μL, 10 mmol/L dNTPs 4 μL, Hotstart *Taq* 0.4 μL [Gene Tech (Shanghai) Company Limited], genomic DNA 4 μL, 10 mmol/L forward primer 0.5 μL, 10 mmol/L reverse primer 0.5 μL in a final volume of 50 μL. The cycling conditions were 95℃ for 3 min, 45 cycles of 95℃ for 10 s, 56℃ for 20 s and 72℃ for 30 s, final extension at 72℃ for 5 min. The PCR products were determined by 3% agarose gel electrophoresis and ssDNA was prepared as described[16]. Mutation detection of KRAS codons 12 and 13 by the Pyrosequencing PyroMark™ Q24 system was done following the manufacturer's instructions (see http://www.pyrosequencing.com/ for more information).

RESULTS

Patient demographics and spectrum of KRAS mutations

The study included 54 males and 46 females with a median age of 59 years (range 22-82 years). The primary locations of tumors were the colon (n = 58) and rectum (n = 42). The mutation rate in females (about 43%) was slightly higher than that in males (about 30%), and the mutation rate in colon and rectal cancers was similar. All patients had a single mutation site. A total of 6 mutation types were detected in this study: GGT>GAT, GGT>GTT, GTT>GCT, GTT>TGT, GTT>AGT, GGC>GAC (wild-type codon 12: GGT; wild-type codon 13: GGC) (Figure 1). Three main mutations Gly12Asp (GGT>GAT), Gly12Val (GGT>GTT) and Gly13Asp (GGC>GAC) accounted for about 80.0% (28/34) of all mutations.

Sensitivity of the 3 methods

Serial dilutions with different contents of mutant DNA were detected by the 3 methods. All 3 methods could correctly identify the Gly12Ser mutation in dilutions containing 15% or more mutant DNA. Dideoxy sequencing failed to detect the mutation in dilutions containing 10% or less mutant DNA. Pyrosequencing failed to detect the mutation in dilutions containing 1% mutant DNA, and only the KRAS StripAssay could unambiguously identify 1% mutant DNA in the dilutions. Thus the sensitivity was highest in the KRAS StripAssay (1%), followed by pyrosequencing (5%), while dideoxy sequencing was lowest (15%) (Table 1).

Table 1 Sensitivity of the 3 methods in mutation detection

Methods	Mutant DNA/total DNA (%)							
	100	50	20	15	10	5	1	0
Dideoxy sequencing	Yes	Yes	Yes	Yes	No	No	No	No
Pyrosequencing	Yes	Yes	Yes	Yes	Yes	Yes	No	No
KRAS StripAssay	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

Dideoxy sequencing could identify mutations in dilutions containing ≥ 15% mutant DNA, pyrosequencing could identify mutation in dilutions containing ≥ 5% mutant DNA, the KRAS StripAssay could identify mutant DNA as low as 1%.

KRAS mutations of 100 CRC patients using the 3 methods

The KRAS mutation was detected in 34/100 (34%) of patients by dideoxy sequencing with 15/54 (27.8%) of males and 19/46 (41.3%) of females, 37/100 (37%) of patients by KRAS StripAssay with 16/54 (29.6%) of males and 21/46 (45.7%) of females, and in 37/100 (37%) of patients by pyrosequencing with 18/54 (33.3%) of males and 19/46 (41.3%) of females. Three main mutations Gly12Asp, Gly12Val and Gly13Asp accounted for 82.4% (28/34), 78.4% (29/37) and 83.8% (31/37) of all mutations by dideoxy sequencing, KRAS StripAssay and pyrosequencing, respectively. The overall results of the 3 methods were similar, with a few discrepancies.

Thirty-three of the 100 patients were identified to have KRAS mutations by all 3 methods, and 8 patients (sample No. 5, 11, 14, 29, 44, 46, 48, 71) showed conflicting results between the 3 methods: 4 mutations (sample No. 5, 44, 46, 48) which were not detected by dideoxy sequencing and the KRAS StripAssay, were identified by pyrosequencing; 3 mutations (sample No. 11, 14, 71) which were not detected by dideoxy sequencing and pyrosequencing were identified by the KRAS StripAssay; one mutation (sample No. 29) not detected by pyrosequencing was idenified by dideoxy sequencing and the KRAS StripAssay (Figure 2). In addition, among these discordant results, the mutations identified by dideoxy sequencing were consistent either with the KRAS StripAssay or with pyrosequencing (Table 2). This indicated that although the sensitivity of dideoxy sequencing was low, its accuracy was high.

DISCUSSION

Along with national development and improvements in standard of living, morbidity and mortality of CRC has increased rapidly in China. The outcomes of the same

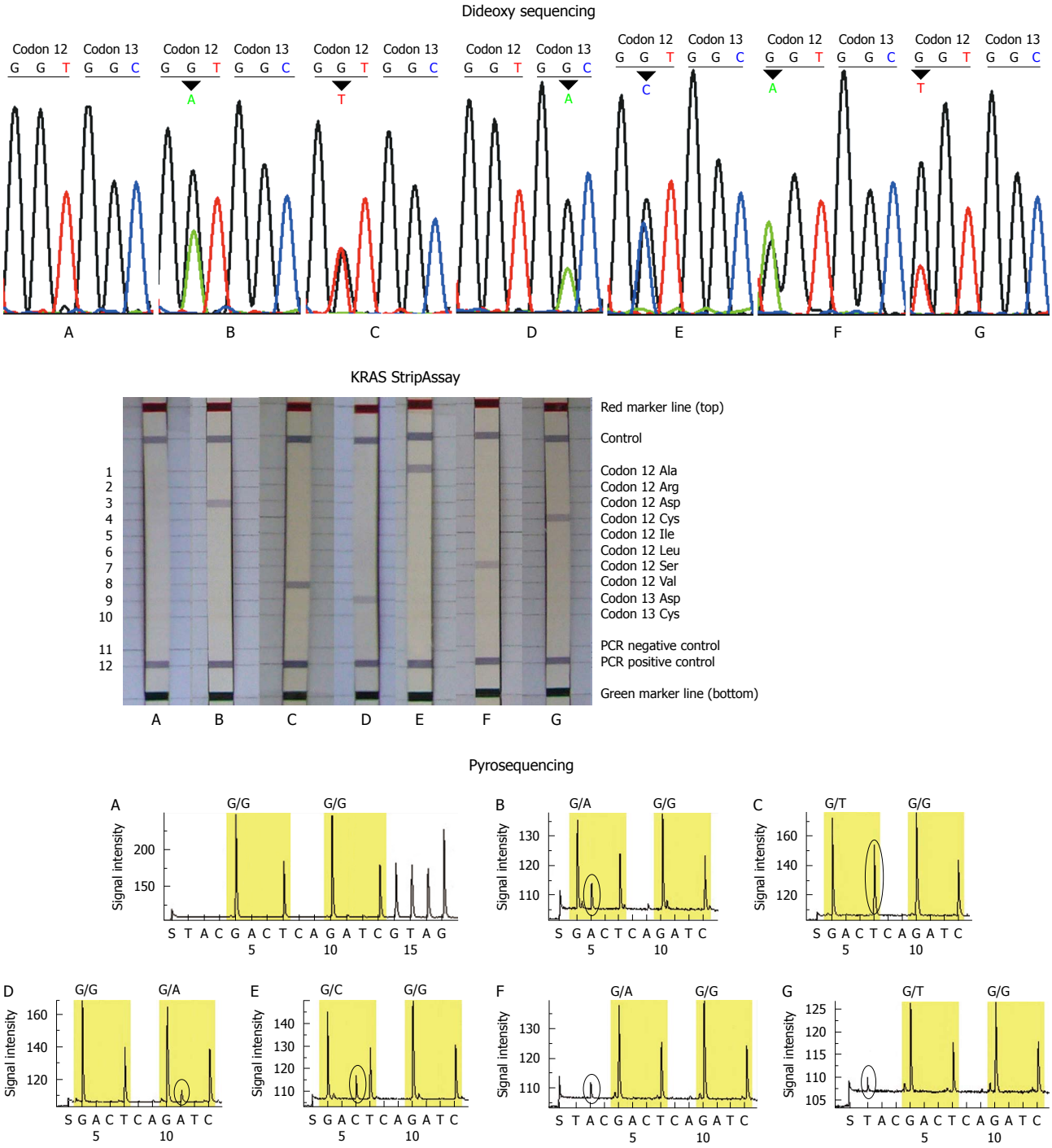

Figure 1 Mutation patterns of KRAS codons 12 and 13 by the 3 methods in this study. A: Wild-type codons 12 and 13 with sequence GGT(12)GGC(13); B: Mutant codon 12 with GGT>GAT; C: Mutant codon 12 with GGT>GTT; D: Mutant codon 13 with GGC>GAC; E: Mutant codon 12 with GGT>GCT; F: Mutant codon 12 with GGT>AGT; G: Mutant codon 12 with GGT>TGT.

treatment regimen in CRC patients were frequently found to differ, and thus it was important to develop individualized treatments. Because the effect of cetuximab was tightly associated with KRAS mutation status, the US Food and Drug Administration recommended that patients who were proposed for cetuximab treatment should undergo KRAS mutation analysis. As a result, besides the conventional methods, more and more techniques have been developed to detect KRAS mutation.

Recent reports have highlighted the advantages of the new methods, and we first compared dideoxy sequencing, the KRAS StripAssay and pyrosequencing for mutation detection in codons 12 and 13 of KRAS in Chinese CRC patients. The mutation rate of KRAS in our study was about 37%, and the mutation rate in females (about 43%) was higher than in males (about 30%) which was different from a report that KRAS mutation in males was higher than in females in Brazil[16].

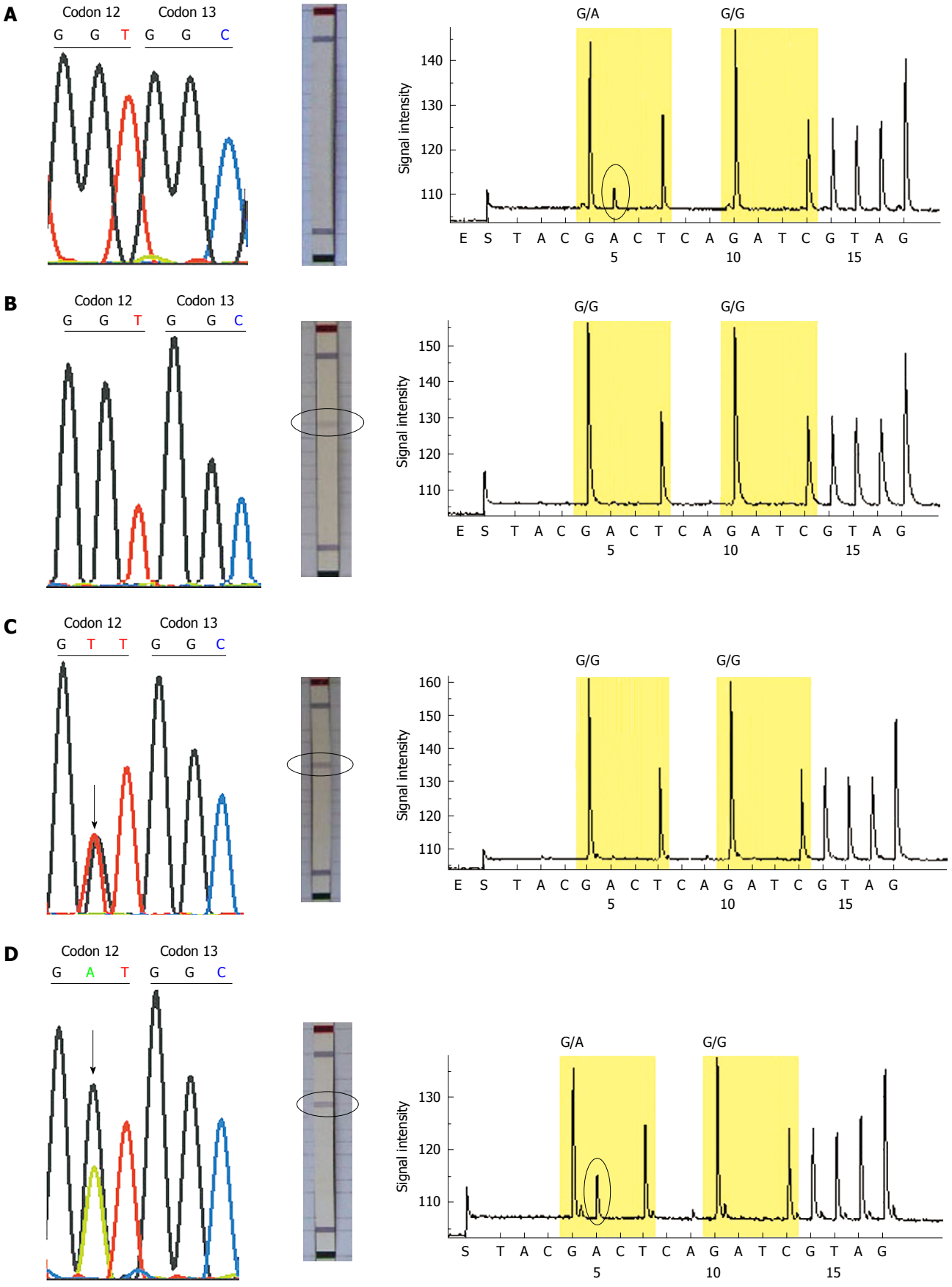

Figure 2 Representative discrepant samples and concordant samples between the 3 methods. A: Mutation of sample 5 was not detected by dideoxy sequencing and the KRAS StripAssay but identified by pyrosequencing; B: Mutation of sample 11 was not detected by dideoxy sequencing and pyrosequencing but identified by the KRAS StripAssay; C: Mutation of sample 29 was not detected by pyrosequencing but identified by dideoxy sequencing and the KRAS StripAssay; D: Mutation of sample 82 was detected by all 3 methods.

Table 2 Discrepant results detected by the 3 methods

Sample No.	Mutation type		
	Dideoxy sequencing	KRAS StripAssay	Pyrosequencing
5	Wild-type	Wild-type	GGT>GAT
11	Wild-type	GGT>TGT	Wild-type
14	Wild-type	GGT>GTT	Wild-type
29	GGT>GTT	GGT>GTT	Wild-type
44	Wild-type	Wild-type	GGT>GAT
46	Wild-type	Wild-type	GGT>GAT
48	Wild-type	Wild-type	GGT>GAT
71	Wild-type	GGT>GCT	Wild-type

These results were repeated at least twice.

The results from the 3 methods were similar, but a total of 8 patients had conflicting results between the 3 methods. We repeated these discrepant samples at least twice, the results being consistent. Because the results by dideoxy sequencing were consistent either with the KRAS StripAssay or with pyrosequencing, the results by dideoxy sequencing were likely to be more accurate. To support our hypothesis, we retrospectively analyzed the patients who were treated with cetuximab. Case 44 with KRAS mutation identified by pyrosequencing was treated with cetuximab and achieved a partial response after 6 weeks' treatment. Because patients with KRAS mutations could not benefit from cetuximab, the result of case 44 by pyrosequencing may be a false positive.

Our results showed that the sensitivities of the KRAS StripAssay and pyrosequencing were higher than that of dideoxy sequencing, but according to our large-scale sampling by dideoxy sequencing, the KRAS mutation rate was stable at 37-39% which was consistent with other reports. From the result of case 44, a false positive could occur in sensitive methods. We can analyze the 3 methods from the aspect of medical economics. At present, the cost is about 100-150 RMB/test for dideoxy sequencing, 1000 RMB/test for the KRAS StripAssay and 200-300 RMB/test for pyrosequencing. Payments in China are limited to the cheapest methods. Up to now, dideoxy sequencing and pyrosequencing have already been widely used to detect KRAS mutations[17,18], but the KRAS StripAssay has not been widely used all over the world.

Studies reported that the KRAS mutation could be used as a prognostic marker in non-small cell lung cancer and as an independent prognostic factor for CRC patients treated with cetuximab[6,19]. Whether there is a relationship between KRAS mutations and prognosis in Chinese CRC patients needs to be studied further.

In conclusion, we compared the differences between 3 methods in the detection of KRAS mutations, and used the KRAS StripAssay to detect KRAS mutations. Although new methods have been developed for detection of KRAS mutation, traditional methods are still in an invincible position and are used widely. In our following large-scale study, dideoxy sequencing will be chosen preferentially because of its ease of use.

ACKNOWLEDGMENTS

The authors thank ViennaLab Diagnostics GmbH for the supply of KRAS StripAssay kits, and Gene Tech (Shanghai) Company Limited for technical assistance in the pyrosequencing technique.

COMMENTS

Background

At present, more and more colorectal cancer (CRC) patients are treated with cetuximab [a monoclonal antibody of epidermal growth factor receptor (EGFR)]. It had been confirmed that KRAS mutated patients could not benefit from cetuximab, so the US Food and Drug Administration recommended that patients proposed for cetuximab treatment should undergo KRAS mutation analysis.

Research frontiers

Besides the traditional methods (e.g. dideoxy sequencing), more and more methods (various kinds of kits) have been developed to detect KRAS mutation. The authors compared methods and demonstrated differences between dideoxy sequencing, the KRAS StripAssay and pyrosequencing, with indications that although dideoxy sequencing is a traditional method, it is still in an invincible position because of its low cost and high accuracy.

Innovations and breakthroughs

Recently, many reports have highlighted the advantages of new methods, and disregarded the traditional methods in the detection of KRAS mutation. This was the first study to compare the differences between dideoxy sequencing, the KRAS StripAssay and pyrosequencing in KRAS detection. The study indicated that although the sensitivity of dideoxy sequencing was lower than the other 2 methods, it was still widely used by many researchers because of its superior accuracy.

Applications

The study could help researchers to choose a suitable method for detection of KRAS mutation according to the sample size, equipment platform and economic status, *etc*. In their opinion, dideoxy sequencing could be easily carried out in any laboratory.

Terminology

In normal physiological conditions, KRAS is regulated by its upstream protein EGFR and plays an important role in the development and progression of tumors. Cetuximab could inhibit the tumors through blocking EGFR and the downstream signal pathway. If KRAS was mutated, KRAS protein was activated without the regulation of EGFR, so cetuximab could not have an effect.

Peer review

The paper deals with the comparison of 3 DNA sequencing methods in order to detect KRAS mutations. The authors postulate that dideoxy sequencing and pyrosequencing techniques have already been widely used to detect KRAS mutations but the KRAS StripAssay has not been widely used all over the world. The paper is well written and well documented.

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BRIEF ARTICLE

Possible key residues that determine left gastric artery blood flow response to PACAP in dogs

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Abstract

AIM: To determine the effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on left gastric artery (LGA) flow and to unveil the structural or functional important sites that may be critical for discrimination of different receptor subtypes.

METHODS: Peptides, including PACAP-27, PACAP-38, amino acid substituted PACAP-27 and C-terminus truncated analogues PACAP (27-38), were synthesized by a simultaneous multiple solid-phase peptide synthesizer. Flow probes of an ultrasound transit-time blood flowmeter were placed around the LGA of beagle dogs. When peptides were infused intravenously, the blood flow was measured.

RESULTS: [Ala4, Val5]-PACAP-27 caused a concentration-dependent vasodepressor action which was similar to that caused by PACAP-27. The LGA blood flow response to [Ala4, Val5]-PACAP-27 was significantly higher than that to PACAP-27, which was similar to that to vasoactive intestinal polypeptide (VIP) at the same dose. [Ala6]-PACAP-27 did not increase the peak LGA flow. [Gly8]-PACAP-27 showed a similar activity to VIP. [Asn24, Ser25, Ile26]-PACAP-27 did not change the activity of peptides at all doses.

CONCLUSION: NH2 terminus is more important to biological activity of peptides and specific receptor recognition than COOH-terminus.

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Key words: Pituitary adenylate cyclase-activating polypeptide; Pituitary adenylate cyclase-activating polypeptide 27; Pituitary adenylate cyclase-activating polypeptide P38; Left gastric artery; Blood flow

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP)

was originally isolated because of its similarity with vasoactive intestinal polypeptide (VIP). PACAP stimulates adenylyl cyclase activity in anterior pituitary cells of rats. So far, two forms of PACAP (PACAP-38 and PACAP-27) have been described. Considering that the 27-residue polypeptide (PACAP-27) corresponds to the N-terminus 27-amino acid sequence of PACAP-38 and shows a 68% identity with VIP[1] (Table 1), PACAP showing a 68% of identity with VIP has been considered a member of VIP-glucagon-growth hormone releasing factor-secretin superfamily.

Both PACAP and VIP exhibit an ability to stimulate adenylate cyclase in pituitary cells and in neural, pancreatic, and liver membrane[2]. However, PACAP is much more potent than VIP in pituitary cells and liver membrane. In the cardiovascular system, PACAP acts as a vasodepressor like VIP. Similarity would demand the validation of effective dose, the duration of response, and the latent period, *etc*.[3].

PACAP and VIP are co-expressed in nerve fibers and neurons in ganglia of guinea pig gallbladder[4]. Our previous study showed that their actions on the gallbladder are opposite, namely VIP relaxes the gallbladder whereas PACAP induces its contraction[5].

It has been shown that PACAP-38 and PACAP-27 are potent VIP-like vasodilators of the femoral arterial bed of dogs, while PACAP-38 differs from PACAP-27 and VIP in its prolonged effects on femoral blood flow[6]. Small arteries and arterioles in the gastrointestinal tract and pancreas are innervated by VIP- or PACAP-positive fibers. Both peptides are very potent vasodilators of gastrointestinal blood vessels in conscious dogs. These findings suggest that PACAP may participate in regulation of the gastrointestinal circulation. However, its effect on gastric blood flow is unknown[7,8].

Three receptor subtypes that mediate PACAP and VIP have been identified[9], including PACAP-specific receptor (PAC1) with a high affinity for PACAP and a much lower affinity for VIP, and PACAP/VIP receptors (VPAC1 and VPAC2) with a similar affinity for PACAP and VIP. All of them belong to the group of 7 transmembrane G protein-coupled receptors. PACAP and VIP act primarily as an inhibitory transmitter on most gastrointestinal and vascular smooth muscle cells, suggesting that PACAP may participate in regulation of the gastrointestinal circulation.

In the present investigation, gastrointestinal blood flow response to PACAP38, PACAP27 and their analogues with amino acid substitutions of corresponding VIP residues as well as substituted analogues at putative functional/structural important sites and C-terminal truncated analogues were studied in conscious beagle dogs to unveil the dose-response and structure-response relationships of these peptides in the left gastric artery (LGA).

MATERIALS AND METHODS

Peptide synthesis

On the basis of sequence homology of PACAP and VIP as well as the structural results by NMR, positions 4, 5, 6, 8, 24, 25, and 26 of PACAP-27 and VIP were selected as substitution sites in the present study. Peptides, including PACAP-27, PACAP-38, amino acid substituted PACAP-27 and C-terminal truncated analogues PACAP 27-38 were synthesized by a simultaneous multiple solid-phase peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan), using the 9-fluorenylmethoxycarbonyl strategy. After cleavage, all peptides were purified with the SynProPep System[10] and characterized by sequencing, amino acid analysis, and fast atom bombardment mass spectrometry to confirm the high homogeneity with the desired structure. VIP was purchased from Peptide Institute (Osaka, Japan).

Table 1 Amino acid sequence of pituitary adenylate cyclase-activating polypeptide-27, pituitary adenylate cyclase-activating polypeptide-38, vasoactive intestinal polypeptide and their analogues

Peptides and their analogues	Amino acid sequence
PACAP-27	HSDG I, FTD S Y, SRYRK, QMAVK, KYLAA, VL-NH2
VIP	HSDAV, FTDNY, TRLRK, QMAVK, KYLN S, ILN-NH2
[Ala4]-PACAP-27	HSDA I, FTDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Val5]-PACAP-27	HSDGV, FTDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Ala4, Val5]-PACAP-27	HSDAV, FTDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Ala6]-PACAP-27	HSDG I, ATDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Gly8]-PACAP-27	HSDG I, FTGSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Asn24, Ser25, IIe26]-PACAP-27	HSDG I, FTDSY, SRYRK, QMAVK, KYLN S, IL-NH2
PACAP 1-33	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQ
PACAP 1-34	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQR
PACAP 1-35	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV
PACAP 1-36	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV, K
PACAP 1-37	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV, KN
PACAP 1-38	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV, KNK-NH2

PACAP: Pituitary adenylate cyclase-activating polypeptide; VIP: Vasoactive intestinal polypeptide.

Methods

The study was approved by the Ethical Committee of the National Institute for Physiological Sciences (Okazaki, Japan) on Animal Use for Experiment. Five beagle dogs of either sex weighing 8-14 kg were used. After fasted for 18 h, the animals were anesthetized with thiamylal (20 mg/kg) and atropine (0.5 mg) and maintained with N_2O-O_2-ether throughout the procedure. Flow probes of an ultrasound transit-time blood flowmeter (Transonic Systems, New York) were placed around the LGA. Connectors of the probes were pulled out of the abdominal cavity through a subcutaneous tunnel and fixed at the chest. After a 4-wk

recovery period, the animals were restrained in Pavlov stands and experiments were conducted in the conscious state. PACAP27, PACAP38, VIP, and PACAP-27 analogues (2.5, 5, 10, 25, 50 and 100 pmol/kg in 1 min) were infused intravenously. The blood flow was measured. Blood flow response to oral ingestion of 300 mL milk served as a control.

Statistical analysis

All data were presented as mean ± SE. Statistical analysis was carried out by one-way analysis of variance using least-significant difference when equal variances or Tamhane's T2 was assumed or when equal variances were not assumed for multiple comparisons. Independent sample *t* test was used for comparison between two independent data. $P < 0.05$ was considered statistically significant with n = the number of animals.

RESULTS

Effects of PACAP-27 and VIP

Different concentrations of PACAP-27, PACAP-38 and VIP were employed. The LGA blood flow responses to PACAP-27 at the doses of 2.5, 5, 10, 25, 50 and 100 pmol/kg were 30.07 ± 8.52, 66.62 ± 16.04, 100, 195.29 ± 35.07, 276.45 ± 47.33, 322.76 ± 60.36, respectively, while those to PACAP-38 at the same doses were 37.35 ± 5.11, 91.69 ± 11.15, 137.60 ± 13.81, 186.91 ± 25.66, 214.12 ± 31.42, 229.73 ± 42.11, respectively. The blood flow responses to VIP at the doses of 10, 25, 50 and 100 pmol/kg were 25.56 ± 8.32, 56.88 ± 9.56, 87.41 ± 1.72, 148.60 ± 17.17, respectively (Figure 1).

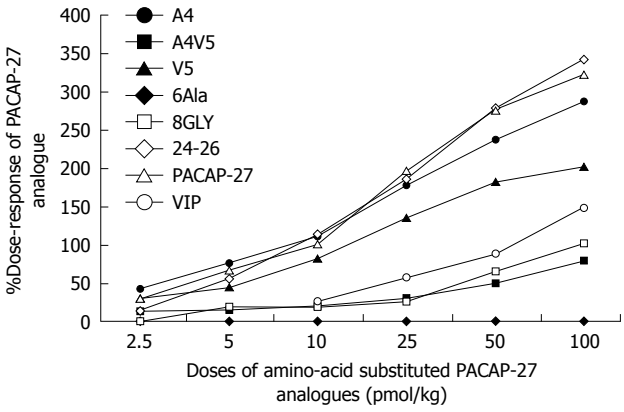

Figure 1 Left artery blood flow response to different doses of amino-acid substituted pituitary adenylate cyclase-activating polypeptide-27 analogues. PACAP: Pituitary adenylate cyclase-activating polypeptide; VIP: Vasoactive intestinal polypeptide.

Effects of N-termini (1-8) substituted PACAP-27 analogues

Effect of N-termini 4 and 5 substituted PACAP-27 analogues with corresponding VIP residues: Intravenous infusion of substituted PACAP27 analogues increased the peak LGA blood flow in a dose-dependent manner. [Ala4, Val5]-PACAP-27 caused a concentration- dependent vasodepressor action similar to that caused by PACAP-27. Both showed a comparable activity to PACAP-27 at the doses of 2.5-100 pmol/kg), demonstrating that a single amino-acid residue substitution at position 4 or 5 of PACAP-27 does not significantly change its biological function. Interestingly, analogues with a substitution at positions 4 and 5, [Ala4, Val5]-PACAP-27 (12.68 ± 4.88, 42.18% of PACAP27) showed a similar activity to PACAP-27 (30.07 ± 8.52) at the dose of 2.5 pmol/kg. However, the responses to [Ala4, Val5]-PACAP-27 (14.58 ± 6.73, 20.63 ± 6.08, 29.99 ± 9.77, 48.53 ± 10.79, 79.20 ± 4.66) at the doses of 5, 10, 25, 50 and 100 pmol/kg were significantly lower than those to PACAP27 (21.88%, 20.63%, 15.36%, 17.55% and 24.54%, $P < 0.05$) and those to PACAP27 at positions 4 and 5 (66.62 ± 16.04, 100, 195.28 ± 35.07, 276.45 ± 47.33, 322.76 ± 60.36), while exhibited a similar activity to VIP (25.56 ± 8.32, 56.88 ± 9.56, 87.41 ± 1.72, 148.60 ± 17.17) at the dose of 10-100 pmol/kg, suggesting that positions 4 and 5 of PACAP-27 are the key NH2-terminal residues of PACAP-27 that discriminate interactions of PACAP with specific receptor subtypes in the LGA (Figure 2).

Effect of N-terminus 6, 8 substituted PACAP-27 analogue: Intravenous infusion of [Ala6]-PACAP-27 did not increase the peak LGA flow, indicating that amino-acid residue replacement at position 6 of PACAP-27 results in loss of its biological function. Phenylalanine, an amino acid residue at position 6 of PACAP -27, was critical for PACAP-27 to exert its action on LGA flow.

The responses to [Gly8]-PACAP-27 were significantly lower at the doses of 5-100 pmol/kg (17.97 ± 4.21, 18.07 ± 4.13, 26.21 ± 7.22, 64.06 ± 15.82, 101.51 ± 17.40) than those to PACAP-27 (26.98%, 18.07%, 13.42%, 23.17%, 31.45%) ($P < 0.05$). [Gly8]-PACAP-27 at the dose of 10-100 pmol/kg showed a similar activity to VIP. Changes in amino-acid residue at position 8 made the biological function of PACAP-27 less potent, suggesting that position 8 of PACAP-27 plays a key role in conformation of PACAP-27 (Figure 2).

Effects of C-termini (24-26) substituted PACAP-27 analogues with corresponding VIP residues

The replacement of C-terminal residues of PACAP-27, [Asn24, Ser25, Ile26]-PACAP-27 (14.75 ± 6.97, 55.43 ± 21.31, 112.66 ± 32.25, 185.23 ± 38.60, 279.30 ± 59.33, 341.83 ± 72.38) did not significantly change the responses at the doses of 2.5-100 pmol/kg, while the responses were 83.21%, 112.66% and 101.03% to PACAP-27 at 5, 10 and 50 pmol/kg, indicating that the three C-terminal residues are not critical for the difference between PACAP-27 and VIP (Figure 2).

Effects of C-terminal deletion in PACAP-38 on peak LGA flow

The effects of C-terminal deletion in PACAP-38 on the peak LGA flow were monitored and compared with the response to PACAP-27, showing that almost all C-terminal deletions in PACAP-38 had no significant effect on the peak LGA flow (Figure 3).

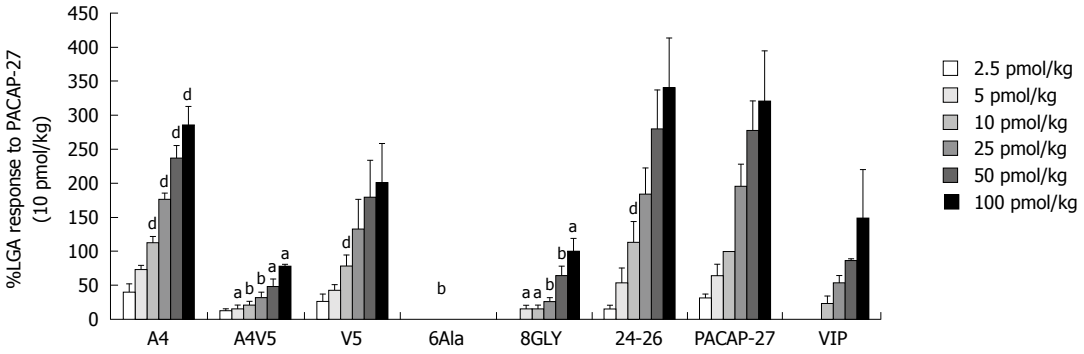

Figure 2 Effects of different doses of amino-acid substituted pituitary adenylate cyclase-activating polypeptide-27 analogues, pituitary adenylate cyclase-activating polypeptide-27 and vasoactive intestinal polypeptide on left artery blood flow. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* pituitary adenylate cyclase-activating polypeptide (PACAP)-27; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* vasoactive intestinal polypeptide (VIP). LGA: Left gastric artery.

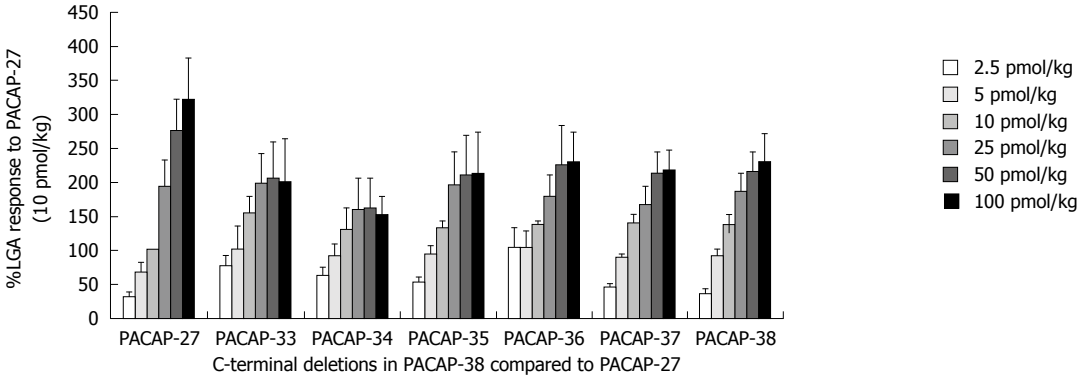

Figure 3 Effects of C-terminal deletions in pituitary adenylate cyclase-activating polypeptide-38 and pituitary adenylate cyclase-activating polypeptide-27 on left artery blood flow (mean ± SE). PACAP: Pituitary adenylate cyclase-activating polypeptide; LGA: Left gastric artery.

DISCUSSION

It has been shown that PACAP has potent gastrointestinal effects[11]. The present study demonstrated that both PACAP and VIP were potent vasodilators of the left gastric arterial bed in dogs, and PACAP was more potent than VIP.

Similarly, it has been reported that although PACAP-27 and N-terminus 27 amino acids in PACAP-38 show a high homology with VIP[12], PACAP is more potent than VIP in stimulating adenylate cyclase in pituitary cells[13].

Autoradiography can clearly identify two PACAP binding sites: one is PACAP preferring, the other has an identical affinity to VIP and PACAP[14].

The physiological actions of these widely distributed peptides, including PACAP, VIP and their analogues, are produced by activating the three common G-protein coupled receptors (VPAC1, VPAC2 and PAC1) which preferentially stimulate adenylate cyclase and increase intracellular cAMP, although stimulation of other intracellular messengers, including calcium[15] and phospholipase D[16] has also been reported.

The three receptor subtypes have been classified into "VPAC receptors" for VIP and PACAP, and "PAC1 receptors" which are the PACAP-preferring subtype[17]. The VPAC receptors can be further divided into VPAC1R and VPAC2R subtypes, based upon helodermin binding/potency (VPAC2R is helodermin preferring).

The actions of PACAP and VIP on gallbladder are opposite, namely VIP relaxes the gallbladder, whereas PACAP induces its contraction both *in vivo* and *in vitro*. The three receptor subtypes that recognize PACAP and VIP as the gallbladder have been found in blood vessels of the gastrointestinal system. Intravenous infusion of PACAP analogues increases the intestinal system blood flow in a dose-dependent manner. The PACAP becomes more potent as the amino chain of PACAP extends.

The aim of this study was to determine the effect of PACAP on LGA flow and to unveil the structural or functional important sites that may be critical for discrimination of different receptor subtypes. PACAP-27 analogues with amino acid substitutions or deletions at selected sites were synthesized.

In the present study, deletion of amino-acid residues from C-terminus of PACAP-38 did not significantly change the effect of PACAP on LGA, which is not consistent with the reported findings in gallbladder[5], demonstrating that the COOH-terminus of PACAP-38 has no key residues for the activity of PACAP-27 in LGA, and that PACAP-27 and PACAP-38 may act on the same receptor subtype.

The C-terminal deletions in PACAP-38 had no signifi-

cant influence on the peak LGA flow in this study, showing that no particular amino acid residue is responsible for the decreased potency of PACAP-27 and PACAP-38, which is consistent with the prolonged response of PACAP-38 to the femoral blood flow in dogs[18].

The change in single amino-acid residue at position 4 or 5 of the amino chain of PACAP-27 did not significantly change the biological function of PACAP-27. However, substituting the amino-acid residues at positions 4 and 5 with corresponding VIP residues significantly changed the biological function of PACAP-27. The responses of LGA flow to [Ala4, Val5]-PACAP-27 and VIP were similar, demonstrating that positions 4 and 5 are the key NH2-terminal residues of PACAP-27 that distinguish interactions with PAC1 receptors from those with VPAC1 and VPAC2 receptors in the LGA.

In our previous study on VIP and PACAP in guinea pig gallbladder[5], VIP induced relaxation while PACAP27 induced contraction of gallbladder. [Ala4, Val5]-PACAP27 were more potent than PACAP27 ($P < 0.01$) in stimulating the gallbladder. It has also been identified in a previous study[5] that [Ala6] PACAP-27 has no significant activity and [Gly8] PACAP-27 is significantly ($P < 0.05$) less potent (25%) than PACAP-27, which are consistent with the findings in the present study, demonstrating that position 4 and 5 are the key residues of PACAP-27 and substitutions at both sites with VIP residues may influence on specific receptor recognition. In this case, positions 4 and 5 substituted PACAP-27 may choose VPAC receptors instead of PAC receptors. Positions 6 and 8 are also important for the effect of PACAP27. It has been shown that a hydrophobic β-coil may form in the N-terminal region and that this structure may be important in receptor-binding affinity[19].

There is evidence that both N- and C-terminal regions are important for the biological activity of peptides and recognition of specific receptors[20,21]. It was reported that replacement of the COOH-terminal of PACAP-27 with VIP has no effect on the relaxation of LGA[22].

In conclusion, NH2 terminus plays an more important role in the recognition of specific receptors than the COOH-terminal. No particular amino acid residue is responsible for the decreased potency of PACAP-27 and PACAP-38. Further study is needed to determine the sites important to the structure and functions of PACAP.

COMMENTS

Background

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated because of its similarity with vasoactive intestinal polypeptide (VIP). PACAP-27 corresponds to the N-terminal 27-amino acid sequence of PACAP-38 and shows a 68% identity with VIP. The effects of PACAP and VIP on the cardiovascular system and gallbladder have extensively studied. The effects of PACAP on excretion and motility of the gastrointestinal tract have also been investigated. The findings suggest that PACAP may participate in regulation of the gastrointestinal circulation. The influence of PACAP on left gastric blood flow was observed in the present study.

Research frontiers

Small arteries and arterioles in the gastrointestinal tract and pancreas are innervated by VIP- or PACAP-positive fibers. Their actions on the gallbladder are opposite: VIP relaxes the gallbladder whereas PACAP induces its contraction. Both peptides were found to be very potent vasodilators of the gastrointestinal blood vessels in conscious dogs, suggesting that PACAP may participate in regulation of the gastrointestinal circulation.

Innovations and breakthroughs

Flow probes of an ultrasound transit-time blood flowmeter were placed around the left gastric artery (LGA). Connectors of the probes were pulled out of the abdominal cavity through a subcutaneous tunnel and fixed at the chest. After a recovery period, the animals were restrained in Pavlov stands and the experiments were conducted in the conscious state. This method can also be used in other experiments on gastrointestinal blood flow.

Applications

The motility and secretion function of gastrointestinal tract are closely related with blood flow. Based on the mechanisms of motility and secretion, the effects of brain-gut peptides on blood flow help understand the physiology of the digestive system and treatment of digestive diseases. The methods can also be used in other experiments on the effects of peptides on gastrointestinal blood.

Peer review

In this study, peptides including PACAP-27, PACAP-38, amino acid substituted PACAP-27 and C-terminal truncated analogues PACAP (27-38) were synthesized and blood flow from the LGA of dogs was measured in response to these peptides infused at various concentrations. The results indicate that amino acid substituted PACAP can cause a concentration dependent vasodepressor action similar to that caused by PACAP-27. The study is interesting, but the data should be further clarified.

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BRIEF ARTICLE

Clinical significance of C-reactive protein values in antibiotic treatment for pyogenic liver abscess

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Abstract

AIM: To investigate the clinical significance of C-reactive protein (CRP) values in determining the endpoint of antibiotic treatment for liver abscess after drainage.

METHODS: The endpoints of antibiotic treatment in 46 patients with pyogenic liver abscess after complete percutaneous drainage were assessed by performing a retrospective study. After complete percutaneous drainage, normal CRP values were considered as the endpoint in 18 patients (experimental group), and normal body temperature for at least 2 wk were considered as the endpoints in the other 28 patients (control group).

RESULTS: The duration of antibiotic treatment after complete percutaneous drainage was 15.83 ± 6.45 d and 24.25 ± 8.18 d for the experimental and the control groups, respectively (P = 0.001), being significantly shorter in the experimental group than in the control group. The recurrence rate was 0% for both groups. However, we could not obtain the follow-up data about 3 patients in the control group.

CONCLUSION: CRP values can be considered as an independent factor to determine the duration of the antibiotic treatment for pyogenic liver abscess after complete percutaneous drainage.

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Key words: Liver abscess; C-reactive protein; Antibiotic treatment; Drainage; Retrospective studies

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INTRODUCTION

Over the past two decades, complete percutaneous drainage combined with antibiotics has been considered as the routine treatment for liver abscess. This combined approach is clinically effective and has significantly reduced the death rate associated with liver abscess[1]. However, the duration and the protocol for the antibiotic treatment after percutaneous drainage is a matter of debate. Conventionally, the duration of antibiotic treatment is determined by the overall health condition after percutaneous abscess drainage[2-4] or by the white blood cell count[5]. The duration of antibiotic treatment was usually prolonged as much as possible[6]. However, assessments performed by considering the overall health condition as the end-point

of antibiotic treatment tend to be subjective. Moreover, standardized criteria for different patients cannot be developed easily. The white blood cell count can be affected by various factors such as physiological situations that may not truly reflect the clinical condition[7]. Prolonged antibiotic treatment has various disadvantages such as pyogenic antibiotic resistance and double infection. To decrease the incidence of antibiotic-resistant bacteria, reduce medical expenses, and increase patient compliance, a more sensitive parameter is required to determine the optimum duration of antibiotic treatment after percutaneous abscess drainage. C-reactive protein (CRP) is an acute-phase protein that is synthesized by liver endothelial cells, which is considered as the most valuable indicator of inflammation, and is a useful marker to determine the usage of antibiotics and assess the efficacy of the antibiotics[8-11]. However, there is no clinical report on the validity of using CRP values to determine the endpoint of liver abscess treatment. We analyzed the CRP values of the patients who were admitted to our hospital between June 2007 and February 2010. The investigation report is as follows.

MATERIALS AND METHODS

Subjects

We performed a retrospective study on 46 patients with liver abscess who were admitted to our hospital between June 2007 and February 2010. The diagnosis was based on typical clinical symptoms such as fever and upper abdominal pain along with the results of liver examination by ultrasonic and computed tomography. There were 32 male and 14 female patients; 8 patients had an abscess on their left liver lobe and 38 had an abscess on their right liver lobe. Ten patients had diabetes and 9 had cholecystitis. Among the 46 cases, 11 had positive culture results, including 4 from blood culture and 6 from abscess culture, and 1 from both; 8 (73%) of 11 patients showed *K. pneumoniae* infection. All the patients received effective antibiotic treatment and underwent percutaneous drainage of the liver abscess when the fluid was identified during ultrasonography.

Study methods

Inclusion criteria: The patients fulfilling the following criteria were included: (1) those undergoing effective antibiotic treatment; and (2) those who had undergone percutaneous abscess drainage when the fluid was identified during ultrasonography.

Exclusion criteria: The patients fulfilling any of the following criteria were excluded: (1) those who had undergone surgery; (2) those who had discontinued hospitalization before the treatment ended or continued medication out of the hospital. The duration of antibiotic treatment could not be determined for these patients; (3) those who had not chosen the right antibiotics and had prolonged hospitalizations; and (4) those who had not undergone percutaneous drainage.

Endpoints of the treatment: (1) Normal CRP values or (2) normal body temperature for at least 2 wk.

Experimental group: Normal CRP values were considered as the endpoint of antibiotic treatment in those patients.

Control group: Normal body temperature for at least 2 wk was considered as the endpoint of antibiotic treatment in those patients.

Mode and duration of follow-up: Visits were continued until March 2010 after hospitalization was ended.

Major endpoints: Duration of antibiotic treatment and recurrence rate.

CRP and white blood cell counts: CRP and white blood cell counts were determined every 3-5 d for the patients with normal CRP values were considered as the endpoint. Antibiotic treatment was stopped when the CRP values returned to normal. CRP and blood tests were not regularly performed for the control group, and the major criteria for discontinuing antibiotic treatment was normalization of body temperature for at least 2 wk.

Statistical analysis

The mean ± SD values were used for quantification. SPSS13.0 software was used for statistical analysis. *T*-test was used to compare the quantitative data. Chi-square analysis was used to compare the measurement data. P values < 0.05 were considered to be statistically significant.

RESULTS

Comparison of the known criteria

Normal CRP values were considered as the endpoint of antibiotic treatment in 18 patients (experimental group), and normal body temperature for at least 2 wk were considered as the endpoint in other 28 patients (control group). The gender, age, size of liver abscess before and after percutaneous drainage, duration of antibiotic treatment before the percutaneous drainage and the time of follow-up were comparable between the two groups (P > 0.05). In both groups, none of the patients showed recurrence of pyogenic liver abscess. We could not obtain follow-up information of 3 patients in the control group, in the intention-to-treat analysis, these patients would be considered as treatment failure.

Comparison of antibiotic treatment

The duration of antibiotic treatment after complete percutaneous drainage for the experimental and the control groups were 15.83 ± 6.45 and 24.25 ± 8.18 d, respectively (P = 0.001). The total duration of antibiotic treatment was 23.06 ± 7.36 d for the experimental group and 31.11 ± 7.30 d for the control group (P = 0.001) (Table 1).

Table 1 Data of liver abscess patients and duration of antibiotic treatment (mean ± SD)

	Experimental group	Control group	*P* value
Age (yr)	57.72 ± 10.39	58.68 ± 11.73	0.779
Women, *n* (%)	5 (28)	9 (32)	0.754
Size of abscess before therapy (diameter)	7.22 ± 1.44	8.01 ± 2.63	0.291
Size of abscess after therapy (diameter)	3.64 ± 0.89	4.02 ± 1.77	0.484
Antibiotic treatment before percutaneous drainage (d)	7.22 ± 5.39	6.82 ± 4.59	0.788
Antibiotic treatment after percutaneous drainage (d)	15.83 ± 6.45	24.25 ± 8.18	0.001[1]
Duration of follow-up (mo)	9.25 ± 5.67	9.24 ± 4.94	0.995
Recurrence rate	0%	11% (follow-up information for 3 individuals was not obtained)	0.151
Total duration of antibiotic treatment (d)	23.06 ± 7.36	31.11 ± 7.30	0.001[1]

[1]Statistical difference between the two groups of data.

Comparison of CRP values and white blood cell counts

The overall average time required for the white blood cell count and the percentage of neutrophils to normalize after the initiation of antibiotic treatment was 17.95 ± 8.00 d. The average time taken for the CRP values to normalize was found to be 21.44 ± 7.06 d for these 46 patients. There was significant difference ($P = 0.045$) between the two durations. The white blood cell count and the percentage of neutrophils had normalized before the CRP values returned to normal. In 5 patients, it was normal even before the percutaneous liver abscess drainage.

DISCUSSION

Liver abscess is a rare disease, with an incidence of 1.0-17.59 cases per 1 000 000 people[12,13]. The death rate for untreated cases is 100%. Currently, the major therapies for liver abscess are antibiotic treatment, percutaneous drainage combined with antibiotics, and surgery. Usually, antibiotics can be used alone for single liver abscess smaller than 3 cm in size. Percutaneous drainage combined with antibiotics is performed for liver abscesses larger than 3 cm. Surgery has to be performed in the cases of multiple abscesses[14]. Due to the effectiveness of these treatments, the death rate of the patients with liver abscess has decreased gradually over the last 20 years; the current death rate is only 6%-14%[1,15].

However, the optimum duration of antibiotic treatment is still a matter of debate. The current treatment protocols are all based on clinical experience, and there is no medical evidence to validate these protocols. Two representative treatment procedures have been suggested: (1) administration of antibiotics alone, for at least 6 wk; after successful percutaneous drainage, antibiotics are continued for another 7 d until all the symptoms disappear[2,3]; and (2) after complete percutaneous drainage, antibiotics are intravenously administered for at least 3 wk, which is followed by oral administration for 1 or 2 mo to prevent recurrence[4]. Furthermore, a study in the United States recommended prolonged antibiotic treatment in the cases of liver abscess[4]. Therefore, antibiotic treatment for liver abscess is considered to be a prolonged procedure. The referred endpoint indicators are non-specific. There are no exact markers to determine the endpoint of antibiotic treatment for liver abscess.

In China, there is no established guideline for the duration of antibiotic treatment after percutaneous drainage. In 45 diabetic patients with pyogenic liver abscess, treatment was not stopped after normalization of body temperature and physical condition; instead, the treatment was continued for 12 wk even after recovery from the abscess[16]. Another study reported that combined use of antibiotic treatment and percutaneous drainage for 4-6 wk was an extremely effective approach[17]. However, the appropriate treatment procedures and the endpoint of antibiotic treatment recommended in these studies required further investigations.

In recent years, Rahimian *et al*[18] reported that antibiotic treatment for liver abscess should not be prolonged. They also reported that short-term antibiotic treatment did not increase the death rate. They had treated 73 patients with liver abscess with intravenous antibiotic administration for 17.5 d, and the associated death rate was 2.5%. However, they did not mention the process of determination of the endpoint of antibiotic treatment. In 2009, the continuing education website of John Hopkins University reported that that after effective percutaneous drainage, the duration of the antibiotic treatment should be determined on the basis of the normalization of the white blood cell count and body temperature, and the treatment should be continued for 14-42 d[5] (unpublished data).

The total white blood cell count and the percentage of neutrophils have been used as standard indicators for the detection of infection, since the measurement methods for these parameters are simple, cheap, and of great clinical utility. These indicators are widely employed in most hospitals, especially in general hospitals. However, the use of the white blood cell count and the percentage of neutrophils as the endpoint of the treatment do not completely reflect the clinical condition. White blood cell count can vary due to various pathological conditions, and they may be influenced by physiological and various other factors such as postprandial intense exercise, cold temperature, pain, and fear. In a retrospective study in China, among 28 patients with thoracic abscess, 4 had normal white blood cell count and significantly elevated

CRP levels[19]. This finding indicates that some patients, especially some elderly patients had lower response to infection. Moreover, among children with lower respiratory system infection, there is no significant difference between the white blood cell counts of patients with pyogenic infection and those with virus infection[20]. Elevated white blood cell count has been traditionally considered as a diagnostic criterion even for patients with appendicitis; however, a prospective study indicated that the sensitivity and specificity of the elevated white blood cell count for diagnosing appendicitis were only 76% and 52%, respectively. The receiver operating characteristic (ROC) curve also indicated that elevated white blood cell count was not clinically relevant for diagnosing appendicitis[7]. ROC curve, is a graphical plot of the sensitivity, or true positives, or false positives, also known as a relative operating characteristic curve, because it is a comparison of two operating characteristics as the criterion changes. So ROC analysis provides tools to select possibly optimal models and to discard suboptimal ones independently. In our data, the white blood cell count and the percentage of neutrophils of some patients were normal even in the initial stage of the disease or before percutaneous drainage. Therefore, white blood cell count did not completely reflect their condition; consequently, it cannot be used as a criterion for medication.

After the onset of inflammation, CRP synthesis increases within 4-6 h, doubling every 8 h. The CRP level reaches the peak value (around 150-350 mg/L) within 36-50 h after infection. The high levels persist through the inflammation period. Therefore, when the infection is controlled, the CRP levels decrease quickly, and the decrease is strongly correlated with the relief from symptoms and with the duration of the treatment. However, the CRP level is not affected by factors such as gender, age, anemia, hyperglobulinemia, and pregnancy[21]. Clinically, the CRP levels have been used to determine whether antibiotic treatment should be started and to judge the effectiveness of the antibiotics. However, very few studies have considered the CRP value as a criterion for determining the endpoint of antibiotic treatment. In 1995, a report suggested that when both CRP and white blood cell counts are normal, antibiotic treatment for the abscess should be discontinued. The accuracy of using CRP values for the assessment of the abscess was as high as 99%, and there were no reports of negative results from the blood culture[9]. However, there have been no further studies on these findings. We realized that CRP value could be considered as an endpoint criterion for liver abscess treatment. In our data, the duration of the shortest treatment was only 11 d, the longest treatment period was not more than 4 wk, and the recurrence rate had not increased.

In summary, the CRP level could be used as an independent factor for determining the duration of the antibiotic treatment in the management of pyogenic liver abscess after complete percutaneous abscess drainage. It can be widely used in clinics. However, for further evaluation of the viability of CRP assessments, studies using more samples and random control trials should be performed.

COMMENTS

Background

Complete percutaneous drainage combined with antibiotics has significantly reduced the death rate associated with pyogenic liver abscess. However, the duration and the protocol for the antibiotic treatment after percutaneous drainage is a matter of debate in those patients. The referred endpoint indicators are non-specific. There are no exact markers to determine the endpoint of antibiotic treatment for liver abscess.

Research frontiers

Antibiotic treatment for liver abscess was considered to be a prolonged procedure. In recent years, it has been reported that antibiotic therapy for treating liver abscess should not be prolonged as short-term antibiotic treatment did not increase the death rate of the patients. However, the process of determination of the endpoint of antibiotic treatment was not mentioned. In 2009, the continuing education website of John Hopkins University reported that that after effective percutaneous drainage, the duration of the antibiotic treatment should be determined on the basis of the normalization of the white blood cell count and body temperature of the patients.

Innovations and breakthroughs

There are no exact markers to determine the endpoint of antibiotic treatment for pyogenic liver abscess at present. C-reactive protein (CRP) is considered as a useful marker to determine the usage of antibiotics and assess the efficacy of the antibiotic. However, there is no clinical report on the validity of using CRP values to determine the endpoint of liver abscess therapy. The authors proposed that the CRP value can be considered as an independent factor to determine the duration of the antibiotic treatment for pyogenic liver abscess after complete percutaneous drainage. In this study, normal CRP values were considered as the endpoint of antibiotic treatment.

Applications

Using CRP value as the endpoint of antibiotic treatment can decrease the duration of antibiotic treatment, thus decreasing the incidence of antibiotic-resistant bacteria, reducing medical expenses, and increasing patient compliance.

Terminology

CRP is an acute-phase protein that is synthesized by liver endothelial cells, which is considered as the most valuable indicator of inflammation. Percutaneous abscess drainage is a procedure performed to remove or drain a contained collection of infected fluid (abscess) from an area of the body such as the chest, abdomen, or pelvis.

Peer review

This manuscript is about a retrospective study to investigate the possibility of using CRP to determine the endpoint of antibiotic treatment along with percutaneous drainage for pyogenic liver abscess patients, and the data indicate that CRP value can be considered as an independent factor to determine the duration of the combination of antibiotic administration and percutaneous drainage. The manuscript is innovative and of putative interest for the readers.

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CASE REPORT

Treatment of portal vein tumor thrombus using ^{125}Iodine seed implantation brachytherapy

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Abstract

We reported two cases of liver metastasis with portal vein tumor thrombus that developed after liver transplantation for hepatocellular carcinoma (HCC). Both the patients were women aged 43 and 55 years, who had liver metastasis and portal vein tumor thrombus formation after liver transplantations for HCC. For the treatment of portal vein tumor thrombus, ^{125}I seeds were implanted into the hepatic tissue under the guidance of preoperative computed tomography (CT) images with a total radiation dose of 130 Gy. Enhanced spiral CT scan was performed for evaluation of the liver at 12 and 16 wk after treatment. Thereafter, upper abdominal CT examination was performed every 2-3 mo. No severe complications associated with the ^{125}I seeds were seen in these two patients. The upper abdominal CT images (obtained after 3 and 4 mo of treatment) showed that the thrombosis reactions were complete reaction and restoration of the patency of the partially obstructed portal vein with partial obstruction. In the case with complete obstruction of the portal vein, the thrombosis was resolved completely, but blood flow could not be restored. After this treatment, one of the patients is still alive, while the other died within 6 mo after the treatment due to lung metastasis complicated with lung infection, leading to respiratory failure.

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Key words: Computed tomography-guided ^{125}Iodine seed implantation brachytherapy; Hepatocellular carcinoma; ^{125}I radioisotopes; Brachytherapy; Portal vein tumor thrombus

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a serious disease which is mainly treated with two approaches: surgical resection and orthotopic liver transplantation. However, these approaches are associated with a high risk of postoperative recurrence and metastasis. Intrahepatic metastasis of HCC after liver transplantation is similar to that after HCC resection in some respects; for instance, both conditions frequently invade the portal vein system. Portal vein tumor thrombosis (PVTT) is very common in liver metastasis after liver transplantation for HCC. To date, no effective treatment for PVTT has been recognized[1].

Three-dimensional (3-D) conformal radiotherapy (CRT) is widely used in treatment of HCC with portal

vein thrombosis. However, this therapy has the following shortcomings: (1) inaccuracy in locating the lesions due to interference by the respiratory movement; (2) a relatively long treatment duration; and (3) damage to the surrounding tissues[2].

^{125}I seed implantation is a type of brachytherapy. Its safety in the treatment of tumors has been recognized over the world. The emergence of computer-aided 3-D treatment planning system (TPS) and computed tomography (CT)-guided precision positioning system has enhanced the accuracy of particle implantation and reduced the extent of damage to the surrounding normal tissues. Therefore, this therapeutic method has been widely used. However, to date, there has been no published report on ^{125}I seed implantation for the treatment of HCC recurrence with PVTT after liver transplantation.

We herein report two cases in which CT-guided ^{125}I seed implantation was used for the treatment of recurrent liver metastasis with PVTT developed after liver transplantation for HCC. Satisfactory results were found during the follow-up in 1 case after 6 mo and in the other up to the present.

CASE REPORT

Case 1

The patient was a 43-year-old Chinese woman who underwent allogenic liver transplantation in the First Affiliated Hospital of China People's Liberation Army Third Military Medical University on February 17, 2006. On July 5, 2007, the abdominal CT examination revealed the left intrahepatic metastasis of HCC. She was treated with ultrasound-guided radiofrequency twice: one in August and the other in October 2007. On December 18, 2007, upper abdominal contrast-enhanced CT scan showed left intrahepatic metastasis, portal vein occlusion of the left extrahepatic segment, and intrahepatic segment-filling defect (2-cm long) in the portal trunk. α-fetoprotein was 43010 μg/L. After informed consent was obtained from the patients for the surgery, CT-guided ^{125}I seed implantation was performed on January 4, 2008. An enhanced abdominal CT scan was performed before seed implantation. The 3-D CT images were transferred to the TPS. On the basis of the obtained CT images of lesions, the spatial distribution of implanted seeds was simulated. A combination of preplanning and real-time technique was adopted, and the number and activity of the implanted seeds were calculated. Spiral CT-guided percutaneous manual implantation of the particles was performed (Figure 1). Since the thrombus originated from the left branch of the portal vein and spread to the trunk, there was no significant extension of the thrombus in the left branch of the portal vein. The ^{125}I seed implantation was done in the portal area around the capsules. We implanted 22 seeds (0.8 mCi/seed) with a total radiation dose of 130 Gy (110-140 Gy) to the thrombus. The liver function tests showed elevated levels of serum transaminase for about 1 wk after the treatment,

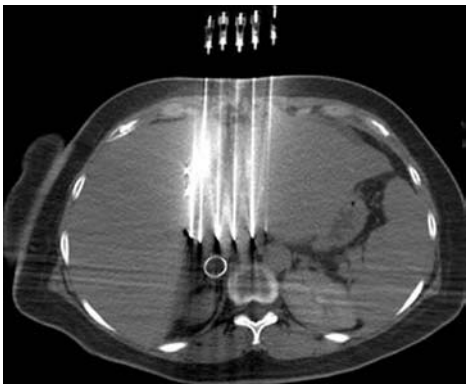

Figure 1 ^{125}I seed implantation process.

which returned to the level of pre-brachytherapy after liver recovery treatment. Abdominal CT after 3 mo showed the disappearance of the portal vein tumor thrombus and the obvious restoration of the portal vein. Thrombosis reaction was complete reaction (Figure 2). This patient died within 6 mo of treatment because of respiratory failure caused by bilateral pulmonary metastasis complicated with pulmonary infection.

Case 2

The patient was a 55-year-old Chinese woman, who underwent allogenic liver transplantation for HCC under general anesthesia on May 18, 2006 in the same hospital. On December 26, 2007, dual-modality positron emission tomography/CT examination showed that the upper posterior part of the right liver had abnormal uptake, thereby suggesting HCC metastasis. On January 12, 2008, ultrasound and CT images showed that the posterior branch of the right portal vein tumor thrombus had invaded the portal vein trunk, the length of the tumor thrombus was about 1.5 cm, and the left main trunk had a small embolus. On January 16, 2008, ^{125}I seed implantation was performed. For the thrombus in the right posterior portal branch, the seeds were implanted directly inside; for the left main trunk, they were implanted in the periportal zone about 1 cm away from the portal area. The treatment protocol was the same as that described in the 1st case, and 27 seeds were implanted at a dose of 0.5-0.8 mCi/seed (Figure 3). No postoperative complications associated with the implantation were observed in this patient. Four months after the treatment, upper abdominal CT showed the following findings: (1) the tumor thrombus in the right branch of the portal vein had resolved with only implanted seeds remaining; (2) the patency of the posterior branch of the right portal vein was not completely restored; and (3) the filling defect in the left branch of the portal vein had disappeared (Figure 4). To date, she is still alive. Transient increase in transaminase occurred in the two patients, which decreased to the preoperative levels after 1 wk. Serious complications, such as acute bleeding during seed implantation, hematoma, infection, abdominal pain, bilirubin, seed migration to the lung, were not observed.

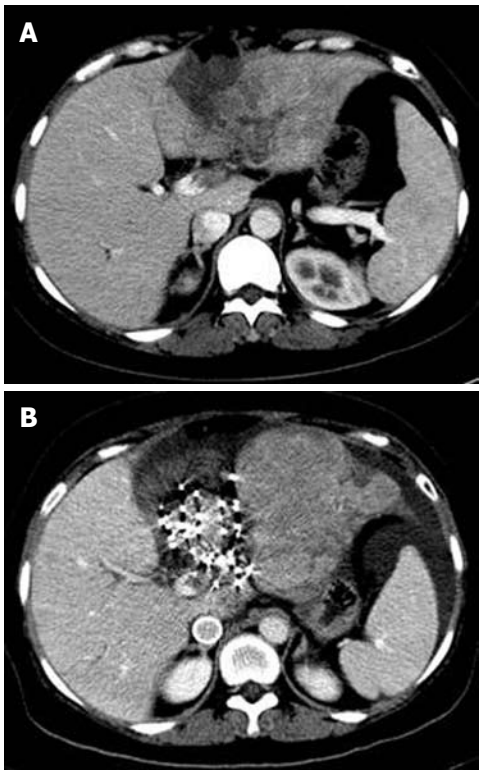

Figure 2 Enhanced computed tomography shows thrombus before ^{125}I seed implantation (A) and absence of tumor thrombi 3 mo after ^{125}I seed implantation (B).

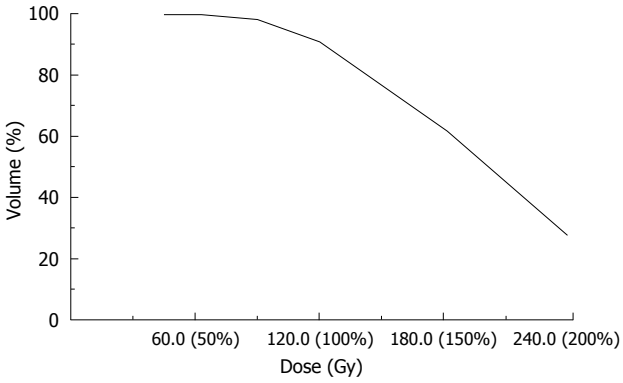

Figure 3 A histogram of therapeutic dose of normal and tumor tissues. target volume = 10.0 cm^3, planning target volume = 13.7 cm^3, target volume ratio = 137.4%, volume fraction for prescription, 100% = 91.2%, volume fraction for prescription, 150% = 63.5%, dose homogeneity index = 30.4%, dose non-uniformity ratio = 69.6%.

DISCUSSION

PVTT is a common complication in HCC patients with liver metastasis after liver transplantation. PVTT not only leads to the development of metastatic tumors in the liver, but also is a sign of liver function deterioration. Conventional radiotherapy can be used in the treatment of PVTT. However, the use of this therapy is limited because the liver has a low radiation tolerance. The tolerance dose for the whole liver is 30-35 Gy[3], but this dose is not sufficient to control the growth of HCC. Lawrence *et al*[4] reported

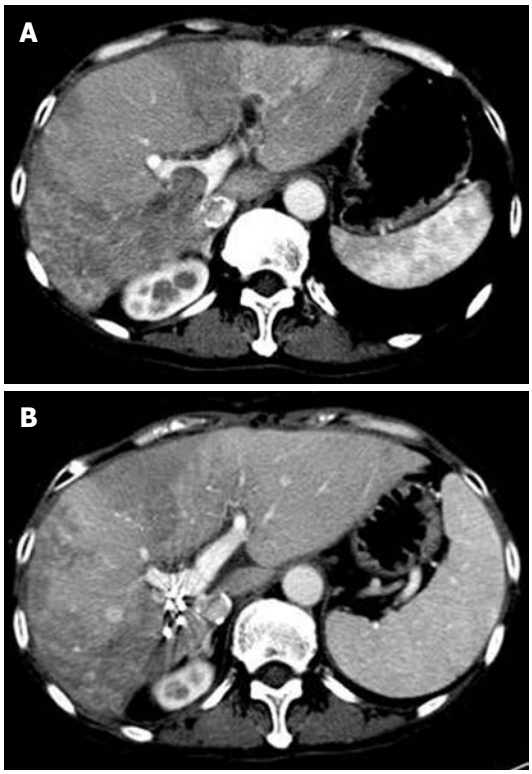

Figure 4 Enhanced computed tomography shows thrombus before ^{125}I seed implantation (A) and absence of tumor thrombi 4 mo after ^{125}I seed implantation (B).

that for lesions in 70%, 50% and 20% of the liver, tolerance doses were 42, 52 and 70 Gy, respectively.

The use of ^{125}I seed implantation in the treatment of PVTT has the following advantages over 3D-CRT: (1) the dose distribution is more conformal to the shape and size of the tumor with the former than with the latter; (2) since irradiation of the lesion is maintained during the decay of the isotopes, the duration of exposure of the tumor is extended; therefore, the tumors receive a higher dose of irradiation, while causing minimal injury to the surrounding normal tissues; and (3) tumor positioning is accurate; the tumor is accurately located without interference from respiratory movements. The application of ^{125}I seed implantation in the treatment of liver cancer has been reported previously. Rafael *et al*[5] performed ^{125}I seed implantations in 56 cases of such intrahepatic metastasis of colorectal cancer where the implantations were either undertaken because the surgeries were contraindicated or performed intraoperatively for residual tumors. In these patients, the actual tumor control rates in the first, third and fifth years were 41%, 23% and 23%, respectively, and the actual survival rates in the first, third and fifth years were 71%, 25%, and 8% (the median survival time was 20 mo), respectively. Subir *et al*[6] reported 64 cases of liver cancers in which intraoperative ^{125}I seed implantations (total dose of 160 Gy) were performed for any of the following reasons: incomplete removal of tumor by surgery, residual primary tumors after surgical resection, and metastasis. In the 64 cases, the actual tumor control rates in the first, third and fifth years were 44%, 22%, and 22%,

respectively, and the actual survival rates in the first, third and fifth years were 73%, 23%, and 5%, respectively.

The complication occurrence of ^{125}I seed implantation in liver tumors has been reported to be low. Nag *et al*[6] reported that in their study of 64 patients treated with ^{125}I seed implantation, only 2 had liver abscesses, but without other complications. Ricke *et al*[7] reported that among 37 patients with liver cancers and some patients with other cancers treated with CT-guided interstitial radiotherapy, 2 patients had severe complications after the treatment (< 5%). A high dose rate (HDR) was only used in temporary implantation because of its high energy of rays and severe damage to the surrounding tissues. There were serious adverse reactions induced by prolongation of seed implantation with HDR, but complications of permanent implantation with low-active seeds were very light. In PVTT treatment, the postoperative liver function abnormalities were transient and treatment-responsive, with only mild liver dysfunction in most cases. There was no seeds migration to the lung. Seeds were put inside the tumor and outside the vessels rather than inside the vessels.

In conclusion, a combination of ^{125}I seed implantation and other interventional means is a new method to control the HCC metastasis. However, analyses of more cases and further researches on brachytherapy are still required to assess the safety and efficacy of this new method.

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Science of weight loss supplements: Compromised by conflicts of interest?

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Abstract

Weight loss supplements often contain powerful pharmacoactive ingredients with the potential to cause harm. Trials used to determine product safety and effectiveness, meanwhile, tend to be small, of short duration, and frequently lack financial conflict of interest disclosures. These factors could conspire to place consumers at risk, especially when published research cited in advertising cloaks products with the suggestion that their safety and effectiveness have been proven by science. Examples of current and former weight loss products backed by potentially conflicted or low quality research include Metabolife-356, Hydroxycut, Xenadrine and LeptiCore. Published research, especially in the field of weight loss supplements, needs better conflict of interest disclosure, and regulators should consider how research findings are used in marketing claims.

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Key words: Weight loss supplements; Cissus quadrangularis; Hydroxycut; Xenadrine; Metabolife; Lepticore; Garcinia cambogia; Conflict of interest

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TO THE EDITOR

Hasani-Ranjbar *et al*[1] recently reviewed evidence behind dietary supplements used for weight loss. While this review provides data suggestive of methodological weaknesses, such as sample sizes and trial duration, and provides a sentence suggesting that safety concerns may not be fully addressed in the reviewed studies, the paper appears to underemphasize inherent flaws in the publications it reviews. Weaknesses that deserve special attention are the small size and short duration of the trials, and links to industry sponsorship, which are frequently not disclosed or inadequately disclosed in the studies themselves. These factors are important for both methodological reasons, and also reasons specific to the weight loss products being reviewed.

Substances tested for weight loss often have potent pharmacological effects[2], may be used by millions of consumers without medical supervision, and can be marketed without meeting the regulatory standards imposed on traditional pharmaceuticals[3]. These factors underscore the importance of assessing product safety as well as efficacy[3,4]. Safety in particular is difficult to establish when studies are of small size and short duration. Of the 19 human studies reviewed by Hasani-Ranjbar *et al*[1], the average number of participants was 64.4 (range 24-153), and the average study duration was 15 wk (range 2-36 wk). Such methodologically weak studies, combined with lax dietary supplement regulation and oversight, have several potential public health implications, including: (1) infrequent or rare side effects may not be detected

Table 1 Selected studies supporting popular weight loss supplements

Xenadrine
Advertisements for this popular and widely advertised weight loss supplement cite a published study supportive of its effectiveness[11]. In addition to being small (n = 47) and of short duration (6 wk), the study lacks financial disclosure, or any mention of a funding source
LeptiCore
Marketing text for this weight loss product cites a published study of 62 participants followed for 6 wk who reportedly experienced significant reductions in weight, body fat, and other metrics associated with chronic disease, such as cholesterol and waist size[12]. The study does not disclose a funding source, beyond the name of the company that provided the tested substance. The paper states that the authors have no competing interests, though one author appears to be a chief scientific officer of a dietary supplement company[1], and appears on US Patent Office[2] filings as the inventor of a weight loss supplement whose patent is held by the same supplement company the author appears to be employed by (The patent was granted in 2010, and originally filed in 2000)
Hydroxycut Advanced
Hydroxycut was the top selling weight loss supplement in the US, then withdrawn from the market after being linked to 23 cases of liver toxicity and one death[3]. Marketing materials for Hydroxycut cited two published studies asserting product effectiveness that were small, of short duration, reported no serious side effects[13,14], and did not disclose relationships between authors and the product manufacturer[15] or that funding was received from the product manufacturer[16]
Hydroxycut has been renamed Hydroxycut Advanced, reformulated and returned to market, distributed by IHS. An active ingredient suspected of causing liver toxicity in the original formulation, Garcinia cambogia[2,3], has been removed and replaced with other ingredients, including CQ. At least 3 recently published studies support the safety and effectiveness of CQ for weight loss but lack financial disclosures or funding sources, beyond mentioning that the CQ being tested was provided by GHA[17-19]. The studies all share an author who is listed as a chief scientific officer for GHA[1] on internet sites, but not in the publications in question, and appears on US Patent Office[2] filings as the inventor of a weight loss supplement whose patent is held by GHA. IHS and GHA have collaborated in the past, though it is unclear whether the CQ currently used in IHS's product is provided by GHA
Concerns
1 Small, short term studies, and those funded by industry[7] may over-state product safety and effectiveness
2 A lack of funding source declaration reduces validity of findings, since readers are unable to assess the potential for this type of conflict of interest
3 Being a patent holder for a weight loss supplement should be considered a financial conflict of interest in these cases, since a patent holder may stand to gain financially from scientific reports of supplement effectiveness
4 The undeclared, potential financial or professional relationships between the patent holder/author and the manufacturer of the substance being studied also appears to be a conflict of interest, since the author would have a personal financial interest in the financial success of the product being studied

[1]http://www.clinicaltrials.gov, and professional networking websites, accessed May 7, 2010; [2]http://www.uspto.gov, accessed May 7, 2010. IHS: Iovate Health Sciences; CQ: Cissus quadrangularis; GHA: Gateway Health Alliances.

by studies of only a few dozen subjects, resulting in a body of evidence that over-states product safety; (2) findings from short-term studies may not reflect actual usage patterns among consumers, who may use products for longer-term weight loss maintenance; (3) sustaining weight loss long-term studies are difficult, so short-term studies may be more likely to garner positive findings, and since publication bias has been reported in favor of positive findings[5], the resulting body of evidence may over-state product effectiveness; (4) to gain market approval under current regulations in the United States, dietary supplement manufacturers only need to submit a relatively low-level of evidence suggestive of product safety[4], (5) products may then go to market backed by findings that over-state their safety and effectiveness; and (6) post market surveillance may only detect adverse events (AEs) once the number of product users blossoms into the tens of thousands, and only after harms have occurred. Unlike in research settings, it is rarely possible to conclusively link harm to product usage in daily life, and in the United States post market surveillance only detects an estimated 1% of such AEs[6].

In pharmaceutical research, industry-funded studies may be more likely to report positive findings[7], and the same is likely for weight loss supplements[8]. Readers can account for this if papers provide clear conflict of interest disclosures. However, the standard financial conflict of interest and funding disclosures regularly seen in rigorous pharmaceutical research may not appear as frequently in dietary supplement research[8]. In some cases, this is further obfuscated when a lack of conflict declaration is used to denote a lack of conflict instead of a declarative statement that there is no conflict. As such it is much more difficult to assess which studies are potentially biased by industry support.

These weaknesses are illustrated by a study, reviewed by Hasani-Ranjbar *et al*[1], of an ephedrine-containing product named Metabolife-356[9]. This manufacturer-funded study reported significant reductions in body-weight and body-fat, with only minor side effects such as dry mouth, insomnia, nervousness, palpitation and headache[9]. However, once the number of users rose into the millions, Metabolife-356 was linked to 92 serious adverse cardiovascular events, including 5 deaths[10], and withdrawn from the US market. These harms were not, and statistically could not be, detected by studies as small (n = 67) as the one in question. The potential for similarly misleading conclusions to be reached about the safety or effectiveness of three currently available products is detailed in Table 1, with the sometimes-opaque relationships between study authors, commercial interests and products delineated in Figure 1. The similarities in potentially flawed conflict-of-interest disclosures suggest a possible trend extending to multiple publications about multiple products in multiple journals.

Industry funding does not necessarily denigrate the

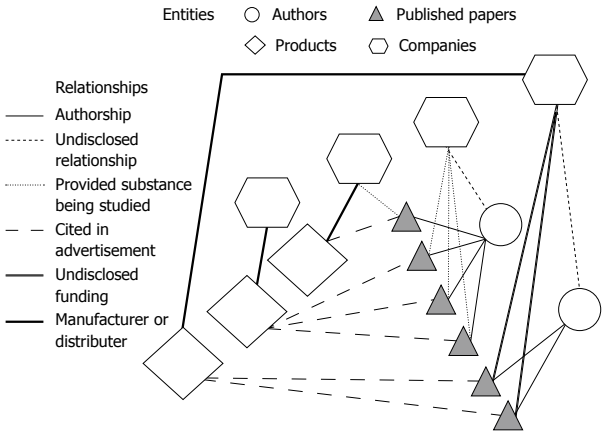

Figure 1 Relationships between select authors, evidence, products and manufacturers (detailed in Table 1).

quality of research, and in many cases is financially necessary to advance scientific understanding. However, undeclared financial conflicts of interest at best reduce face-validity of findings, and at worst represent deception. Financial disclosures that are generally the norm in scientific publications need to be in place in dietary supplement research as well, and should be provided by authors, required by journals, and insisted upon by peer-reviewers. A lack of financial conflicts should be noted by confirmatory statements rather than by omission of conflict declarations. Funding sources should also be noted by reviews of published papers, especially when the reviewed literature contains methodological weaknesses such as small sample size and short duration. When used for marketing, small, short duration pilot studies may have disproportionately large impact, providing false assurance to consumers with low science-literacy that products are "clinically tested" and thus safe and effective. It may also be prudent for regulatory authorities such as the US Federal Trade Commission or Food and Drug Administration to consider how published research is currently used in the marketing of consumer products, especially those with a track record of causing harm.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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The Medical Management of HIV/AIDS

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 39 October 21, 2010

EDITORIAL

4883 Neoadjuvant treatment for resectable pancreatic cancer: Time for phase Ⅲ testing?
Reni M

4888 Role of endoscopic ultrasound during hospitalization for acute pancreatitis
Kotwal V, Talukdar R, Levy M, Vege SS

4892 *Clostridium difficile* infection and inflammatory bowel disease: Understanding the evolving relationship
Navaneethan U, Venkatesh PGK, Shen B

TOPIC HIGHLIGHT

4905 Alcoholic hepatitis 2010: A clinician's guide to diagnosis and therapy
Amini M, Runyon BA

REVIEW

4913 Diagnosis and management of angioedema with abdominal involvement: A gastroenterology perspective
Nzeako UC

ORIGINAL ARTICLE

4922 Anti-inflammatory effects of *Mangifera indica* L. extract in a model of colitis
Márquez L, Pérez-Nievas BG, Gárate I, García-Bueno B, Madrigal JLM, Menchén L, Garrido G, Leza JC

4932 HBx-induced reactive oxygen species activates hepatocellular carcinogenesis *via* dysregulation of PTEN/Akt pathway
Ha HL, Yu DY

BRIEF ARTICLE

4938 Peri-nuclear antibodies correlate with survival in Greek primary biliary cirrhosis patients
Sfakianaki O, Koulentaki M, Tzardi M, Tsangaridou E, Theodoropoulos PA, Castanas E, Kouroumalis EA

4944 Pancreatic function, quality of life and costs at long-term follow-up after acute pancreatitis
Andersson B, Pendse ML, Andersson R

4952 Pulmonary involvement in inflammatory bowel disease
Yılmaz A, Yılmaz Demirci N, Hoşgün D, Üner E, Erdoğan Y, Gökçek A, Çağlar A

4958 Association of p53/p21 expression with cigarette smoking and prognosis in esophageal squamous cell carcinoma patients
Taghavi N, Biramijamal F, Sotoudeh M, Moaven O, Khademi H, Abbaszadegan MR, Malekzadeh R

4968 Etiology and long-term outcome of extrahepatic portal vein obstruction in children
Weiss B, Shteyer E, Vivante A, Berkowitz D, Reif S, Weizman Z, Bujanover Y, Shapiro R

4973 Glycemic index, glycemic load and insulinemic index of Chinese starchy foods
Lin MHA, Wu MC, Lu S, Lin J

4980 CABYR RNAi plasmid construction and NF-κB signal transduction pathway
Shi LX, He YM, Fang L, Meng HB, Zheng LJ

4986 Up-regulation of PIK3CA promotes metastasis in gastric carcinoma
Liu JF, Zhou XK, Chen JH, Yi G, Chen HG, Ba MC, Lin SQ, Qi YC

4992 Five-year long-term outcomes of laparoscopic surgery for colon cancer
Bai HL, Chen B, Zhou Y, Wu XT

4998 Diagnosis of bile duct hepatocellular carcinoma thrombus without obvious intrahepatic mass
Long XY, Li YX, Wu W, Li L, Cao J

CASE REPORT

5005 Laparoscopic wedge resection of synchronous gastric intraepithelial neoplasia and stromal tumor: A case report
Mou YP, Xu XW, Xie K, Zhou W, Zhou YC, Chen K

LETTERS TO THE EDITOR

5009 Haemodynamic and renal effects of tadalafil in patients with cirrhosis
Kalambokis GN, Kosta P, Pappas K, Tsianos EV

World Journal of Gastroenterology
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Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
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EDITORIAL

Neoadjuvant treatment for resectable pancreatic cancer: Time for phase Ⅲ testing?

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Abstract

This paper discusses the rationale for phase Ⅲ testing of neoadjuvant therapy in patients affected by resectable pancreatic adenocarcinoma. The therapeutic management of patients affected by resectable pancreatic cancer is particularly troublesome due to the aggressiveness of the disease and to the limited efficacy and sometimes unfavourable risk-benefit ratio of the available therapeutic tools. Conflicting data on the role of adjuvant chemoradiation have been reported, while adjuvant single-agent chemotherapy significantly improved overall survival (OS) when compared to surgery alone. However, the OS figures for adjuvant chemotherapy remain disappointing. In effect, pancreatic cancer exhibits a prominent tendency to recur after a brief median time interval from surgery and extra-pancreatic dissemination represents the predominant pattern of disease failure. Neoadjuvant treatment has a strong rationale in this disease but limited information on the efficacy of this approach is available from single arm trials with low levels of evidence. Thus, in spite of two decades of investigation there is currently no evidence to support the routine use of pre-surgical therapy in clinical practice. To foster knowledge on the optimal management of this disease, and to produce evidence-based treatment guidelines, there is no alternative to well designed randomized trials. Systemic chemotherapy is a candidate for testing because it is supported by a more robust rationale than chemoradiation. Combination chemotherapy regimens with elevated activity in advanced disease warrant investigation. Caution would suggest the running of an exploratory phase Ⅱ randomized trial before embarking on a large phase Ⅲ study.

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Key words: Pancreatic cancer; Neoadjuvant therapy; Phase Ⅲ trial; Chemotherapy

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INTRODUCTION

Pancreatic cancer represents the fourth most common cause of cancer death, bears the worst prognosis among solid tumors and has seen very limited progress over the last 30 years. Due to intrinsic chemo- and radio-resistance, surgical resection is considered the only therapy that may have an impact on the natural history of the disease and may increase chance for cure. However, 5-year overall survival (OS) rates of less than 20% can be expected even after a curative resection, which is related to a non-negligible risk of mortality and morbidity. Therefore, the therapeutic management of patients affected by resectable pancreatic cancer is particularly troublesome due to the aggressiveness of the disease and to the limited efficacy and, sometimes, unfavourable risk-benefit ratio of the available therapeutic tools.

STANDARD TREATMENT IN RESECTABLE PANCREATIC CANCER

Adjuvant fluorouracil-based chemoradiation followed[1,2] or not[3] by maintenance systemic chemotherapy with 5-fluorouracil has been tested against surgery alone in a few phase Ⅲ trials with conflicting results, ranging from a significant improvement[1] to a detrimental impact on OS[2]. Accordingly, the use of this strategy is a highly controversial topic in the management of patients with resected pancreatic adenocarcinoma.

More recently, randomized trials have suggested that both adjuvant 5-fluorouracil and gemcitabine may obtain an improvement in median survival of 2.6-4.5 mo and in 2-year OS of 6%-10% over pancreatic resection alone[2,4], with no significant difference between the two drugs[5,6].

It is noteworthy that the OS benefit achieved by adjuvant chemotherapy, in addition to being modest, does not apply to the whole population of patients submitted to pancreatic resection. In fact, up to 25% of patients have a complicated course after surgery and are unable to receive the planned treatment in due time[3,7]. Also, patients with evidence of persistent local disease or metastatic disease at the first post-operative radiological assessment are ineligible for prospective trials on adjuvant chemotherapy, whose results are, consequently, not fully generalizable.

PATTERN OF DISEASE RECURRENCE AFTER STANDARD TREATMENT

In the subset of patients receiving postoperative treatment, pancreatic cancer exhibits a prominent tendency to recur locally and to metastasize after a brief median time interval of about 13 mo from surgery[4]. Early relapse after curative surgery may be explained by the presence of micro-metastases or minimal residual disease not detectable at the time of surgery, or by the spread of cancer cells into the portal vein, lymphatic vessels, and the peritoneal cavity due to surgical manipulation of the tumor. In spite of adjuvant systemic chemotherapy, extra-pancreatic dissemination represents the predominant pattern of disease failure, affecting 63%-83% of patients, and occurs earlier than isolated local failure, which can be observed in 17%-37% of cases[2,4-6,8,9].

RATIONAL FOR NEOADJUVANT THERAPY

In this scenario, the administration of neoadjuvant systemic chemotherapy may offer several theoretical advantages. Firstly, micro-metastatic disease may be immediately treated, thus avoiding the harmful delay of at least 2 mo which occurs for patients submitted to upfront surgery. Second, a larger proportion of patients may receive an active systemic treatment compared with the adjuvant setting. Third, the treatment itself may be better tolerated, resulting in a higher rate of treatment compliance and improved dose-intensity. Fourth, neoadjuvant chemotherapy potentially reduces intraoperative tumor spillage. Fifth, the delivery of treatment before surgical manipulation may be favored by better tissue oxygenation, facilitating the distribution of chemotherapy agents into the tumor, and increasing normal tissue tolerance. Moreover, the administration of chemotherapy before surgery allows an *in vivo* assessment of tumor chemo-sensitivity. Finally, neoadjuvant chemotherapy may also lead to more definitive surgical resections by reducing the risk of tumoral infiltration of lymph nodes and of resection margins in the surgical specimen.

On the other hand, the neoadjuvant approach is subject to hypothetical risks such as (1) inaccurate staging and the consequent overtreatment of very early disease; (2) erroneous histology; (3) diagnostic inaccuracy due to difficulties in distinguishing between intra-pancreatic bile duct adenocarcinoma and pancreatic adenocarcinoma; (4) increase in operative morbidity and mortality; and (5) the possibility that the disease might metastasize or become unresectable during the course of induction therapy. The first topic appears to be of little relevance in pancreatic cancer since systemic treatment administration is warranted at virtually any stage of disease, aside from, perhaps, stage I , which is exceedingly rare. Similarly, the risk of yielding an inaccurate pathological diagnosis is limited as the widespread and systematic use of endoscopic ultrasound and fine needle aspiration considerably reduces the possibility of errors. As regards surgical complications, no increase in morbidity or mortality after neoadjuvant therapy has been reported in prior trials[10-12]. Conversely, the topic of disease progression during pre-surgical treatment is of considerable concern because, among patients whose disease was deemed resectable at the time of trial enrolment, only 45%-74% were actually submitted to surgical resection after induction chemoradiation[12-16] and 38%-70% after induction chemotherapy followed[10] or not[11] by chemoradiation. Proponents of neoadjuvant therapy consider these figures another advantage of this strategy, claiming that patients who experience disease progression during induction treatment suffer from an extremely aggressive tumor, which cannot be cured by extensive surgery. In fact, avoiding the risk of surgical mortality and morbidity in this subset of patients may be appealing. However, this is not necessarily true for patients who experience only local progression during neoadjuvant therapy, and in any case, no comparative information from randomized trials on the impact of the different management strategies is available in order to rule out a detrimental impact of delaying surgery. Furthermore, the proper aim for pre-surgical therapy should be that of downstaging disease and of improving both disease control and, ultimately, cure rate, rather than improving patient selection for surgery. Overall, the balance between the theoretical advantages and disadvantages of neoadjuvant therapy in pancreatic cancer appears uncertain.

PRIOR EXPERIENCE WITH NEOADJUVANT THERAPY

There have been no large randomised controlled studies

on the use of neoadjuvant therapy in resectable pancreatic cancer and the sample size of prospective series has usually been limited. In addition to the abovementioned disappointing resection rates, reported median OS and 2-year OS in this single arm selected series ranged from 8 to 23 mo and from 27% to 40%[10-17]. Altogether, these figures do not appear to represent a remarkable improvement when compared to those of patients submitted to surgery alone (median OS 11-17 mo; 2-year OS 15%-31%)[1-3], or to compare favorably with those of adjuvant therapy (median OS 14-25 mo; 2-year OS 29%-55%)[1-4,6,7]. It is noteworthy that prior experiences with adjuvant combination chemotherapy reported more promising results (median OS 27-44 mo; 2-year OS 53%-58%)[9,18,19]. However, inter-trial comparisons, which already have several limitations, are in this case subject to an additional bias due to the different enrolment timing. In fact, the typical population enrolled in a prospective adjuvant trial is better selected than the typical population enrolled in a neoadjuvant trial because it does not include patients with intraoperative or postoperative detection of metastases, patients who die due to surgical complications or those who experience severe morbidity and delayed surgical recovery.

Thus, in spite of two decades of investigation of neoadjuvant therapy in resectable pancreatic cancer, there is currently no evidence to support its routine use in clinical practice, and even a detrimental effect on outcome cannot be ruled out.

TRIAL DESIGN TO ASSESS THE ROLE OF NEOADJUVANT THERAPY

Single arm trials with historical or literature comparison, and divergent study designs and entry criteria have produced modest therapeutic progress and do not allow a proper assessment of the role of neoadjuvant therapy in resectable pancreatic cancer. To foster knowledge regarding the optimal management of this disease and to produce evidence-based treatment guidelines, there is no alternative to well designed randomized trials. Since timing and sequencing of treatments appears to be a crucial and as yet unanswered issue, patients in the ideal trial should be randomly allocated to receive exactly the same treatment for the same period of time before and after surgery. Otherwise, the attribution of any potential outcome improvement to treatment type, timing or duration will be irremediably confounded and trial interpretation inconclusive.

CANDIDATES FOR PROSPECTIVE ASSESSMENT

As mentioned above, pancreatic cancer has an elevated risk of both local and systemic failure after surgery. In this perspective, local therapy represents a poor chance of considerably improving cure rates while the concomitant administration of radiotherapy and systemic chemotherapy may simultaneously address both troubles. The main radiosensitizing antitumor agents available for pancreatic cancer are gemcitabine and 5-fluorouracil. Unfortunately, gemcitabine has to be administered at suboptimal doses which are unlikely to achieve any effect against systemic disease, due to the overlapping toxicity with radiotherapy and both drugs yield scarce activity. In fact, gemcitabine and 5-fluorouracil obtained objective response rates around 10% in advanced disease[20-26]. Any chemotherapy with a low rate of tumor shrinkage is clearly unable to provide any major advantage in terms of either micro-metastatic or local disease control for the majority of patients and may therefore be assumed to have a limited role in the neoadjuvant setting. More active combination chemotherapy regimens appear to be more promising candidates for testing but have feasibility limitations with concomitant irradiation. Furthermore, the value of radiotherapy in this disease is controversial and, at the moment, does not represent the most burning question, while the rational endorsement of the assessment of the role of combination chemotherapy as pre-surgical therapy is more convincing. Among several regimens with conventional or target agents that have been assessed for use against advanced pancreatic cancer, objective response rates over 20% have rarely been reported, while gemcitabine-cisplatin and gemcitabine-oxaliplatin doublets obtained a response rate of 26%[22] and 28%[23], respectively. Unfortunately, these figures were not reproduced in larger trials where partial plus complete response rate was in the range of 10% to 13% with gemcitabine-cisplatin[24,25] and 9% with gemcitabine-oxaliplatin[26]. Response rates with triplets including gemcitabine, a fluoropyrimidin and either a platinating agent (18%-33%)[27-29] or docetaxel (29%)[30], FOLFOXIRI (5-fluorouracil-oxaliplatin-irinotecan; 26%)[31] and G-FLIP (gemcitabine-5-fluorouracil-irinotecan-cisplatin; 26%)[32] regimens have shown promise in single phase Ⅱ series, but no phase Ⅲ or confirmatory trials are available. A PEFG (cisplatin, epirubicin, 5-fluorouracil, gemcitabine) regimen was proven, in a phase Ⅲ trial, to be both clinically and statistically more effective than single agent gemcitabine as upfront treatment in advanced pancreatic cancer[33]. It is noteworthy that this combination chemotherapy had manageable toxic effects and the significant survival improvement was not achieved at the cost of impaired quality of life[34]. Four consecutive trials with the PEFG regimen and its variants reproduced a radiological response rate in the range of 38.5%-51%[33,35-37]. The substitution of infusional 5-fluorouracil by oral capecitabine originated the PEXG regimen that further confirmed the activity figures[38] and rendered the schedule more suitable for clinical use. The reliability of the response rate was also endorsed by the biochemical response rate. In effect, a major biochemical response (i.e. CA19.9 reduction at nadir relative to baseline value reduction $\geqslant$ 90%) was observed in 30% of patients treated with quadruplets *vs* 7% with single agent gemcitabine[39]. The superiority of this four-drug combination over other regimens was also suggested by a recent survey on treatment trends and outcomes of 650 patients with stage Ⅲ pancreatic adenocarcinoma[40]. Based on these data and

considerations, the PEXG regimen appears to be the most deserving candidate for a prospective assessment in the neoadjuvant setting.

CONCLUSION

The topic of treatment sequencing for patients affected by resectable pancreatic adenocarcinoma is of paramount importance and warrants further investigation. Time is mature for the running of a randomized prospective study, which is the only approach capable of providing evidence-based answers. To date, on the basis of activity data from trials on advanced pancreatic cancer, the most robust candidate for testing is the PEXG regimen. However, the lack of a large randomized trial confirming survival improvement over single agent gemcitabine in advanced disease suggests caution before embarking on a phase Ⅲ study in the neoadjuvant setting. An exploratory phase Ⅱ randomized trial seems to embody the optimal approach to avoid the risk of wasting resources and time. Accordingly, a clinical trial involving more than 20 Italian institutions has been designed as a three-arm calibrated study[41] and is currently underway. Patients are randomly allocated to receive either an adjuvant treatment with gemcitabine for 6 mo (calibration arm) or an adjuvant treatment with PEXG for 6 mo or a perioperative treatment (3 mo before and 3 mo after surgery) with PEXG. After completion of recruitment for the phase Ⅱ part of the study, an analysis of the results will be performed to decide whether to continue to the subsequent phase Ⅲ part of the study. It is hoped that this trial will contribute to an expansion of knowledge on the optimal therapeutic management of resectable pancreatic cancer.

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EDITORIAL

Role of endoscopic ultrasound during hospitalization for acute pancreatitis

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Abstract

Endoscopic ultrasound (EUS) is often used to detect the cause of acute pancreatitis (AP) after the acute attack has subsided. The limited data on its role during hospitalization for AP are reviewed here. The ability of EUS to visualize the pancreas and bile duct, the sonographic appearance of the pancreas, correlation of such appearance to clinical outcomes and the impact on AP management are analyzed from studies. The most important indication for EUS appears to be for detection of suspected common bile duct and/or gall bladder stones and microlithiasis. Such an approach might avoid diagnostic endoscopic retrograde cholangio-pancreatography with its known complications. The use of EUS during hospitalization for AP still appears to be infrequent but may become more frequent in future.

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Key words: Acute pancreatitis; Endoscopic ultrasound; Acute biliary pancreatitis; Endoscopic retrograde cholangio-pancreatography; Idiopathic pancreatitis

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INTRODUCTION

In recent years endoscopic ultrasound (EUS) has emerged as a very useful diagnostic modality in the evaluation of patients with acute pancreatitis (AP). Studies have shown EUS to be highly accurate in the diagnosis of gallstone disease (including microlithiasis), chronic pancreatitis, pancreatic tumors and other causes of AP which have negative or inconclusive results as assessed by other imaging methods. However, most of these studies have been carried out after the acute attack has subsided[1-4].

There are very limited data regarding the role of EUS during AP. We have reviewed the literature as to the role of EUS during an episode of acute pancreatitis and attempted to find out its utility in terms of making a diagnosis, finding an etiology and predicting the severity and outcomes in AP.

We could find only 8 studies (7 published and 1 in abstract form) so far which have investigated EUS during an episode of AP. Of these, only one study[5] looked specifically at the diagnostic usefulness of EUS in AP and another[6] looked at its prognostic value. Most of the other studies looked at the role of EUS in evaluation of gallstone pancreatitis and compared it to other diagnostic modalities such as transabdominal US and endoscopic retrograde cholangio-pancreatography (ERCP). We reviewed these studies in an attempt to answer the following questions with regard to AP: (1) how good is EUS in visualizing the pancreas and bile duct? (2) what are the findings

on EUS? (3) whether these findings correlate with the clinical outcomes and (4) what is the role of EUS in suspected biliary pancreatitis?

VISUALIZATION OF THE PANCREAS AND THE BILE DUCT BY EUS DURING ACUTE PANCREATITIS

During acute pancreatitis, edema of the duodenal wall, pancreatic necrosis and inflammation or fluid collection around the pancreas can potentially hinder the visualization of the pancreas, gallbladder or bile duct by EUS. There are only two prospective studies (with 23 and 35 patients, respectively) which have commented about visualization of the pancreas during AP, and the entire pancreas could be visualized in all patients in both of the studies[5,7]. While there are four studies (with a total of 228 patients) which have shown complete visualization of the gallbladder and bile duct in all patients during AP[5,7-9], this was not possible in three studies[10-12]. In one study, the gallbladder could not be visualized in three out of 28 patients (10.7%)[10]. One out of the 3 patients had situs inversus, and in two the gall bladder was located abnormally. In the same study, visualization of the common bile duct (CBD) was complete in 32 patients (88.8%), partial in 3 (8.3%) and unsuccessful in 1 patient (2.7%). Prat *et al*[11] in their study of 123 patients, found that EUS imaging of the CBD was unsatisfactory in three out of 123 patients (2.4%), two with Billroth Ⅱ gastrectomy and one who underwent transcystic drainage. In another study, CBD could not be visualized in one out of 38 patients (2.6%) due to severe pancreatic necrosis[12].

These observations suggest that even though EUS can visualize the entire pancreas, gallbladder and bile duct in acute pancreatitis in most patients, there may be occasional difficulties encountered in patients with severe pancreatic necrosis, unusual location of gallbladder or altered gastroduodenal anatomy.

We would like to stress here that while the entire pancreas can be visualized in AP, changes of chronic pancreatitis and small tumors can be missed and hence EUS for these indications should be avoided during an episode of AP. Another question that needs to be addressed is, how well can the pancreatic duct be imaged by EUS during AP? This has been mentioned in only one study of 23 patients, out of which the main pancreatic duct was seen in 78% of patients[5]. There are no data concerning sensitivity and specificity of EUS in detecting pancreatic duct disruptions and strictures during the course of AP.

EUS FINDINGS OF THE PANCREAS IN ACUTE PANCREATITIS

There are 4 studies[5-7,10] that looked prospectively at the EUS findings in AP early in the disease course. In 3 of the studies[6,7,10], EUS was performed within 72 h of admission and in the other it was done within the 1st week[5]. In two prospective studies by Sugiyama *et al*[5,7], EUS showed a normal or diffusely enlarged pancreas with a normal or diffusely low internal echo pattern in all patients with edematous pancreatitis. The details of these findings were given in only one out of the 2 studies, involving 23 patients[5]. In this study, the pancreas was normal in size in 37.5% of patients with edematous pancreatitis. The echogenicity was normal in 25% and the remaining 75% had diffusely hypoechoic pancreas. However, in another study by Chak *et al*[10], the pancreas appeared normal in size in 63.8% of patients. Only eight out of 36 patients (22.2%) with edematous pancreatitis had a hypoechoic pattern on EUS. Four patients (11.1%) had hyperechoic pattern and the remaining 24 (66.6%) had a mixed pattern. Based on these findings, it appears that in edematous pancreatitis, EUS can show a normal-sized or enlarged pancreas, while hypoechoic pattern may be quite common. In our experience, in some patients the only findings on EUS suggestive of AP can be peripancreatic inflammation/fluid collection (unpublished data). Table 1 shows the salient EUS features of acute edematous pancreatitis.

In the studies carried out by Sugiyama *et al*[5,7], EUS also demonstrated extrapancreatic inflammatory spread as a hypoechoic area. When results of these two studies were combined, EUS correctly identified fluid in the lesser sac in all 18 patients (100%) while fluid in the retroperitoneum was identified in 20 out of 25 patients (80%).

Sugiyama *et al*[5,7] also found that all patients with necrotizing pancreatitis (n = 6) had focal hypoechoic areas with or without interspersed hyperechoic spots. The location and size of focal hypoechoic regions on EUS corresponded to those of avascular pancreatic necrosis on contrast-enhanced CT. Schoefer *et al*[6] in their abstract commented that EUS was able to detect pancreatic necrosis early in 3 patients. However, the number of patients with necrotizing pancreatitis in these studies was very small and therefore these results cannot be extrapolated to all patients with acute necrotizing pancreatitis.

CORRELATION OF EUS FINDINGS WITH CLINICAL OUTCOMES IN AP

Chak *et al*[10], in their study of 36 patients, found that in patients with peripancreatic fluid collection on EUS, there was a trend toward longer duration of hospital stay but it was not statistically significant (9.2 d *vs* 5.7 d, $P < 0.1$). They also found that patients whose pancreas had a coarse echotexture (n = 6) had a significantly shorter hospitalization (2.6 d) as compared to those with fine (n = 19) or grainy (n = 11) echotexture (6.6 and 8.2 d, respectively). However, size of the inflamed gland, parenchymal heterogeneity, parenchymal echogenicity and gastroduodenal wall edema did not correlate with the duration of hospital stay.

Schoefer *et al*[6], in a prospective study of 31 patients, developed an EUS score from 1-30 based on findings such as organ size, aspect of outer margin, echogenicity, location and degree of peripancreatic fluid. The score was correlated with clinical course and CT severity index. EUS score correlated significantly with duration of hospital stay ($P < 0.0001$), number of days with fever ($P < 0.001$),

Table 1 Endoscopic ultrasound features in acute edematous pancreatitis

Study	No. of patients	Normal-sized pancreas (%)	Normal echogenicity (%)	Hypoechoic (%)	Hyperechoic (%)	Mixed (%)
Sugiyama *et al*[5], 1995	16	37.5	25.0	75.0	None	None
Chak *et al*[10], 1999	36	63.8	None	22.2	11.1	66.6

Table 2 Sensitivity, specificity, positive and negative predictive value of endoscopic ultrasound to detect common bile duct stones in acute pancreatitis

Study	No. of patients	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Sugiyama *et al*[7], 1998	35	100	100	-	-
Chak *et al*[10], 1999	36	91	100	100	95
Liu *et al*[8], 2001	100	97	98	-	-
Stabuc *et al*[12], 2008	38	96	85	92	92

days spent in ICU ($P < 0.0001$) and CT severity index ($P = 0.0004$). However, this study was published only in abstract form and therefore requires further validation.

ROLE OF EUS IN SUSPECTED ACUTE BILIARY PANCREATITIS

There are many studies which have looked at the utility of EUS in the diagnosis of CBD stones. However, only 4 studies have so far investigated this during the course of AP[7,8,10,12]. These studies showed that EUS had a sensitivity of 91% to 100% and specificity of 85% to 100% to detect CBD stones (Table 2). It is generally believed that in patients with acute pancreatitis and intermediate probability of CBD stones, EUS can be done safely and may avoid diagnostic ERCP, with its attendant complications. A recent meta-analysis based on 4 randomized controlled trials performed by Petrov *et al*[13] showed that use of EUS for the selection of patients who will need therapeutic ERCP results in significantly lower risk of complications in comparison with the use of ERCP for both diagnosis and treatment of choledocholithiasis. However, it should be noted that only one of these 4 RCTs was carried out in the setting of AP[9]. In this trial, 140 patients with first episode of suspected biliary AP were randomized to EUS or ERCP, within 24 h of admission. In the EUS group, therapeutic ERCP with endoscopic sphincterotomy (ES) and stone extraction was performed if EUS detected choledocholithiasis; otherwise the patients were managed conservatively. None of the patients with negative EUS for CBD stones had recurrent pancreatitis or symptoms suggestive of biliary stones during a median follow up of 26 mo. The only statistically significant difference between the two groups was that EUS could be performed in 100% of patients, while successful cannulation of the CBD in the ERCP group was possible in 86% ($P = 0.001$).

While there were no complications related to EUS, four patients in the ERCP arm had post-ES bleeding. However, there was no significant difference in morbidity or mortality between the 2 groups.

CAN EUS REPLACE CT OR TRANSABDOMINAL ULTRASOUND?

Sugiyama *et al*[5] pointed out that EUS did not have some of the drawbacks of CT such as radiation exposure and contrast load and therefore could be potentially used for both the diagnosis and detection of the cause of AP. However, in patients with edematous pancreatitis, EUS can show normal pancreas. Moreover, the findings on EUS are variable in edematous pancreatitis (hypoechoic, hyperechoic or mixed). Although there is a suggestion that EUS might be able to detect pancreatic necrosis, this is based on a small sample size and therefore larger studies are needed for its validation. Again, EUS cannot detect fluid collection in the retroperitoneal area very well. At the same time, patients with severe pancreatitis may require multiple CT scans to evaluate the dynamics of local complications. Whether EUS, an invasive test, will be feasible and cost effective under these circumstances is a question that needs to be addressed.

Chak *et al*[10] recommended that if the patient would undergo EUS regardless of the transabdominal US findings, it made sense to forgo the expense of transabdominal US. However, in the same study there were 3 patients in whom the gallbladder was not seen on EUS but was seen only on conventional US and all 3 had gallstones.

Therefore, based on current evidence, one cannot propose that EUS can replace CT or transabdominal US. However, in certain situations such as patients with renal failure, contrast allergy or early pregnancy when CT scan cannot be carried out, EUS might have a role to play.

CONCLUSION

The data regarding the role of EUS during AP are limited because its use in this situation is evolving. There is a wide variation in appearance of the pancreas on EUS during AP and it may even be normal in some patients. The finding of focal hypoechoic areas on EUS has been suggested to indicate pancreatic necrosis, but this finding needs to be validated with larger trials. Preliminary data

have shown that some findings on EUS might be able to predict the severity of AP, but further studies are needed for confirmation. One of the important indications for performing EUS in AP is suspected acute biliary pancreatitis when transabdominal US and CT do not show biliary calculi. Although the EUS-guided ERCP approach has been shown to be beneficial in patients with suspected choledocholithiasis, data supporting this in the setting of acute pancreatitis are limited. Identifying other causes such as tumors during the acute stage, while possible, is often done after the resolution of the attack. EUS definitely has a role in the drainage of pancreatic fluid collection and in helping to obtain access to perform necrosectomy. Thus, at the present time, the role of EUS during the acute stage of AP is still limited.

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EDITORIAL

Clostridium difficile infection and inflammatory bowel disease: Understanding the evolving relationship

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Abstract

Clostridium difficile (*C. difficile*) infection (CDI) is the leading identifiable cause of antibiotic-associated diarrhea. While there is an alarming trend of increasing incidence and severity of CDI in the United States and Europe, superimposed CDI in patients with inflammatory bowel disease (IBD) has drawn considerable attention in the gastrointestinal community. The majority of IBD patients appear to contract CDI as outpatients. *C. difficile* affects disease course of IBD in several ways, including triggering disease flares, sustaining activity, and in some cases, acting as an "innocent" bystander. Despite its wide spectrum of presentations, CDI has been reported to be associated with a longer duration of hospitalization and a higher mortality in IBD patients. IBD patients with restorative proctocolectomy or with diverting ileostomy are not immune to CDI of the small bowel or ileal pouch. Whether immunomodulator or corticosteroid therapy for IBD should be continued in patients with superimposed CDI is controversial. It appears that more adverse outcomes was observed among patients treated by a combination of immunomodulators and antibiotics than those treated by antibiotics alone. The use of biologic agents does not appear to increase the risk of acquisition of CDI. For CDI in the setting of underlying IBD, vancomycin appears to be more efficacious than metronidazole. Randomized controlled trials are required to clearly define the appropriate management for CDI in patients with IBD.

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Key words: *Clostridium difficile*; Inflammatory bowel disease; Antibiotics; Colectomy

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INTRODUCTION

Clostridium difficile (*C. difficile*) infection (CDI) is the leading identifiable etiology for antibiotic-associated diarrhea and is associated with substantial morbidity and mortality. Since the initial report of this bacterium as a cause of antibiotic-associated pseudomembranous colitis in 1978[1], the incidence of CDI has increased over the years. Although knowledge in epidemiology, pathogenesis, risk factors, diagnosis and management of CDI has tremendously increased, the frequency and severity of CDI continue to increase at an alarming rate[2-4]. A hypervirulent strain of *C. difficile*, BI/NAP1/027 was reported from North America

and Europe which was associated with a more severe and complicated disease and a higher mortality[3,4]. In addition to its effect on morbidity and mortality, CDI is also associated with increasing duration of hospitalization and costs. The expected health care costs due to CDI alone are estimated as being up to 3.2 billion dollars per year in the US[5]. Clearly the impact of CDI on the health care system continues to grow with emergence of community-acquired CDI[6,7].

Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory conditions. IBD patients frequently require corticosteroids, antibiotics (in CD), immumomodulators, and biological therapy. Some of these agents can increase the risk of acquisition of CDI. In a large population-based cohort study, the use of biologic agents does not appear to increase the risk for CDI[8]. Recently published single-center studies and national inpatient database studies reported rising rates of CDI among IBD patients and their contributions to an increased rate of hospitalizations and mortality[9-12]. The risk of CDI in IBD patients appears to persist even after colectomy. CDI can involve the small bowel[13]. CDI has also been reported in UC patients with restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA)[14,15] .However, the exact pathogenic role of *C. difficile* in these clinical settings are unclear. *C. difficile* may cause an isolated infectious colitis superimposed on IBD, or in some patients, may precipitate an IBD flare leading to two separate but simultaneous inflammatory processes. The other possibility is that *C. difficile* may be just a colonizer and that IBD flare probably occurs independently. When patients with IBD develop worsening symptoms and *C. difficile* is isolated in their stool, there are no clear guidelines to suggest whether to withhold or continue IBD-related medications, including antibiotics, corticosteroids, immunosuppressants, or biologics, while instituting appropriate therapy for CDI. In a retrospective study, patients treated by combination therapy of antibiotics and immunomodulators had a trend towards increased mortality when compared with those treated by antibiotics alone[16]. Lastly, there is no consensus on which antibiotic regimen should be considered as the first-line agent for the treatment of CDI complicating IBD.

Given the uncertainty in the pathogenesis and controversy on management of patients with concurrent CDI and IBD, we embarked on this project to clarify some issues on evolving CDI in IBD. The main goals of this article are to provide information on the pathogenesis and impact of CDI on disease course of IBD, to discuss diagnosis and treatment modalities of CDI in IBD, and to compare the clinical, laboratory, macroscopic and microscopic features between isolated CDI and superimposed CDI on IBD.

REVIEW CRITERIA

In February 2010, we searched MEDLINE from 1970 to the present using the Medical Subject Headings terms "Clostridium difficile, inflammatory bowel disease, Ulcerative colitis, Crohn's disease, Clostridium difficile and inflammatory bowel disease, Clostridium difficile and diagnosis, Clostridium difficile and treatment". Full papers and abstracts without language restrictions were considered. Important developments in research, reports from centers of excellence, and our own research developments form the basis of this article.

EPIDEMIOLOGY

CDI occurs predominantly in hospitalized patients and the incidence is increasing across the US with 3 million new cases of CDI occurring each year and as many as 10% of patients being affected within 2 d of hospitalization[17]. The prevalence of carrier state of the bacterium ranges from 0% to 3% in healthy individuals to 20% in hospitalized patients[17]. Interestingly, only one third of all infected patients developed diarrhea, while the remaining two thirds were asymptomatic carriers. Antibiotic exposure is the major risk factor for CDI.

The Center for Disease Control (CDC) reports of community acquired *C. difficile* colitis in the US has made the picture more concerning[6,7]. The traditional risk factors, such as recent hospitalization, being elderly, or having an underlying health condition were often absent. Close to 25% of patients who developed community acquired *C. difficile* colitis were young, healthy patients with no recent hospitalization in the past year.

Recent papers have highlighted a hypervirulent form of *C. difficile* strain, BI/NAP1/027 that was shown to be associated with a more severe and complicated disease course and a higher mortality. This strain appears to spread across the US. In a recent CDC report with regard to the BI/NAP1/027 strain of *C. difficile*, 38 states were reported to have the hypervirulent strain of the bacterium in their population[6,7]. This particular strain of *C. difficile*, toxinotype Ⅲ, North American PFGE type 1, and PCR ribotype 027 (NAP1/027) carries the binary toxin gene cdtB (cytolethal distending toxin B gene) and an 18-base pair deletion in tcdC; it produces 16-23 times more toxin A and B than the routine strain[3,5]. In addition, this hypervirulent strain was reported to be associated with increased disease severity[18] and possibly transmissibility and to cause outbreaks in Europe and the US[3,4]. The increasing use of fluoroquinolones may be one of the reasons for selecting the hypervirulent BI/NAP1/027 *C. difficile* strain since it is resistant to this class of antibiotics and possibly less responsive to other antibiotics.

PATHOGENESIS OF *C. DIFFICILE*-INDUCED DIARRHEA

Pathogenic strains of *C. difficile* produce two potent toxins, toxin A, an enterotoxin, and toxin B, a cytotoxin. The genes encoding toxin A and B are encoded in the *C. difficile* pathogenicity locus (*tcdA* and *tcdB*) which also encode two additional regulatory genes (*tcdC* and *tcdD*)[19]. The *tcdD* gene product up-regulates toxin transcription, while *tcdC* prob-

ably encodes a toxin gene repressor[19]. The fifth gene of the pathogenicity locus, *tcdE* is postulated to release both toxins A and B into the colonic lumen by lysing the cell walls[20]. Both toxins A and B have a 49% amino acid homology and possess a N-terminal domain that possesses cytotoxic activity, a transmembrane domain that facilitates toxin entry into the cytoplasm and a C-terminal domain that favors toxin binding to the epithelial cells[19]. Both toxins A and B are UDP-glucose hydrolases and glucosyltransferases and contribute to infectious and inflammatory diarrhea; however toxin B may be the major inflammatory toxin[21]. The toxins initially attach to non-proteinaceous disaccharide Gal beta 1-4GlcNac residues in the colon. Both toxins play a role in the initial binding to the colonic epithelial cells. After adhesion, the toxin enters the cell through receptor-mediated endocytosis and catalyzes the transfer of a glucose residue from UDP-glucose to guanosine triphosphate-binding rho proteins[19], the intracellular signaling molecules regulating cytoskeletal organization and gene expression. Glucosylation of rho proteins in turn leads to disruption of protein synthesis, and cell death. This leads to the inflammatory diarrhea seen in patients with CDI[22].

INFLAMMATORY BOWEL DISEASE AND CDI

Almost three decades before, LaMont *et al*[23] postulated that *C. difficile* toxin complicates chronic IBD and contribute to relapse in some patients. Since then, isolated case series of CDI contributing to symptomatic relapse in patients with IBD have been reported[24-27].

Incidence and prevalence

Paralleling the rising burden of CDI in the general population, recent years have witnessed a dramatic increase in CDI in patients with IBD. Recently, two single-center studies and two national inpatient database studies have reported a rising rate of CDI among IBD patients and their contributions of increased rates of hospitalization and mortality[9-12]. In a retrospective study of all confirmed CDI patients from a tertiary care center over a 7 years period, there was a doubling in the CDI rate in CD patients (9.5 to 22.3/1000 admissions) and tripling in UC patients (18.4 to 57.6/1000 admissions)[9]. A similar increase in the rate of CDI in IBD patients from 1.8% in 2004 to 4.6% in 2005 was observed in a subsequent study from a different tertiary-care center[10]. Furthermore, both studies identified that IBD patients, in particular those with UC, were at a disproportionately higher risk for acquiring CDI than non-IBD patients. In a large study utilizing the Healthcare Cost and Utilization Project Nationwide Inpatient Sample inpatient care database in the US, hospitalized patients with concurrent CDI and IBD had a 4 times greater mortality than those admitted to hospital for IBD or CDI alone[11]. In a subsequent study utilizing the same National Inpatient Sample database to study the temporal pattern of CDI, the prevalence of CDI among UC patients (37.3 per 1000) was higher than that among CD patients (10.9 per 1000), non-IBD gastrointestinal (GI) patients (4.8 per 1000), and general medicine patients (4.5 per 1000). In addition the incidence of CDI among UC patients almost doubled (26.6 per 1000 to 51.2 per 1000) over the 7-year period. CDI was independently associated with a greater mortality among patients with UC, but not CD[12].

Superimposed infections of pathogenic bacteria or viruses may contribute to exacerbation of IBD. Concurrent CDI is one of them. In a Scandinavian study in 1983, only 5% of patients admitted for a flare had CDI which would make routine screening not cost-effective[28]. However recent studies reported that approximately 5%-19% of newly-admitted patients for relapsing IBD tested positive for *C. difficile* toxins[29,30]. Similar to the adult population, pediatric IBD patients also seem to be susceptible to CDI as a recent Italian study identified *C. difficile* toxins in 24.7% of patients with diarrhea or abdominal pain[31]. *C. difficile* carriage status was studied with stool culture and molecular microbiological methods in IBD patients in clinical remission with no recent hospitalization or antibiotic exposure[32]. Toxigenic *C. difficile* was demonstrated more frequently in IBD patients (8.2%) than in healthy volunteers (1.0%). However, none of these patients developed CDI after a 6 mo follow-up and all the ribotypes identified were community-acquired, highlighting the acquisition of *C. difficile* in IBD patients even in remission from a wide variety of community sources[32]. The clinical relevance and significance of this community-acquired *C. difficile* carriage is interesting and its effect on the outcome of IBD has not been studied.

Risk factors

Environmental exposure continues to be the most common route of acquisition of CDI. Recent hospitalization increases the risk for nosocomial acquisition of CDI, the most common setting for the infection. Antibiotic-resistant *C. difficile* spores survive in hospital environment and can be isolated on toilets, bedrails, floors, telephones, call buttons, stethoscopes, and the hands of healthcare workers[19-21]. Sharing a room with an infected patient also increases the risk of infection[19].

Interestingly in a majority of IBD patients, CDI seems to often be contracted outside of the hospital. In a recent study, the median time to development of CDI in non-IBD patients was 4 d in contrast to less than a day with CDI in IBD patients[9]. In another study, 76%-79% of patients acquired CDI from the community[10]. Toxigenic *C. difficile* was demonstrated more frequently in IBD patients in complete remission with no recent hospitalization or antibiotic exposure (8.2%) than in healthy volunteers (1.0%) and the ribotypes identified were community-acquired, highlighting the acquisition of *C. difficile* in IBD patients[32]. In a case-control study from our institution, we also observed that 47.2% of patients had acquired the infection outside the hospital[33].

Almost any antibiotic has been associated with the development of CDI. The risk of CDI varies depending on the type of antibiotic, frequency, duration, route of antibiotic use, and the use of concurrent medications[34-37]. How-

ever, even a short-term use of prophylactic antibiotics can cause CDI[38]. The most common implicated antibiotics associated with CDI till recently were ampicillin, amoxicillin, cephalosporins, and clindamycin[34-37]. However, with widespread use, fluoroquinolones have become one of the common predisposing factors for CDI[38-41]. The exact mechanism of antibiotic-associated CDI in IBD is unclear. In addition, frequency of antibiotic exposure in relation to CDI risk in IBD has been highly variable. Antibiotic use prior to 3 mo before the development of CDI was seen in only 40% of IBD patients compared with 69% in the non-IBD population in a study[42]. A separate study showed that 61% of IBD patients with antibiotic exposure developed *C. difficile*[10]. In a cohort study, 57.2% of patients acquiring a CDI received antibiotics in the previous 6 mo[8]. In our recent study, antibiotic exposure within 30 d prior to *C. difficile* testing was found to be associated with an increased risk for CDI with odds ratio of 12.0 [95% confidence interval (95% CI): 1.2-124.2][33].

Immunosuppression is also proposed as another risk factor for the development of *C. difficile* infection. Cancer chemotherapy, particularly methotrexate[43] or patients with organ transplantation on immunosuppression appear to be at risk. The role of immunomodulators in the development of CDI is controversial. Previously studies have reported the association of immunomodulators with CDI in IBD[9,10]. However, in the studies from our institution and others suggest that immunosuppressive treatment was not associated with the risk of CDI[33,44,45].

The relationship of the use of corticosteroids to the risk of CDI has been studied in IBD patients. In a large cohort study of IBD patients, corticosteroid use with or without simultaneous use of other immunomodulating drugs was associated with a 3-fold increase in the risk of CDI (relative risk = 3.38, 95% CI: 1.88-6.10)[8]. Even in the absence of other immunomodulating drugs, the risk increased 2.5 fold in patients using corticosteroids[8]. Corticosteroids have also been shown to increase the risk of CDI relapse in solid organ transplant patients[44]; however corticosteroids and their risk of association to CDI have not been studied in IBD patients.

The use of biologics, specifically infliximab to the risk of CDI was studied in IBD patients and there appears to be no association of the use of biologics on the risk of development of CDI[8].

The normal bacterial flora in the bowel is an important natural defense and inhibits the growth of *C. difficile*[46]. In addition, the gastric acid barrier is a host mechanism to protect against ingested microorganisms[47]. The use of proton pump inhibitors and the risk for CDI is a subject of controversy, as published results of studies have been conflicting. Some initial studies demonstrated a higher risk of development of CDI with proton pump inhibitor therapy but this finding has not been consistently demonstrated[42,48].

Similar to the non-IBD populations, increasing age has been proposed as a risk factor for CDI in the IBD population[49-51]. IBD patients with CDI, however, were younger than the corresponding non-IBD population who developed CDI. In addition to immunosuppressive medication, host immunity, particularly the humoral arm, may play a role in determining susceptibility to CDI[52]. Thus serum and intestinal secretory antitoxin antibodies may afford protection and may be associated with mild colitis or carriage, while patients with deficient response develop severe or recurrent CDI[52]. Recurrent CDI is also suggested to be because of alterations in the fecal microbiota with markedly decreased diversity as demonstrated by phylogenetic analysis of 16S rRNA-encoding gene sequences[53].

IBD itself has been shown to be a specific risk factor for the development of CDI, particularly in those with colonic involvement[9,11,54]. Patients with UC appear to be at a higher risk for the development of CDI than CD and the presence of colonic disease conferred 3-fold greater risk (odds ratio = 3.12, 95% CI: 1.28-5.12) for CDI[11]. Similarly CD patients with colonic involvement seem to be at a greater risk for CDI than those with isolated small bowel disease[11]. The risk of CDI in relation to disease activity is unclear. A recent study suggested that patients with a greater disease activity may be at a higher risk for CDI[31]. However, in the population based study by Nguyen *et al*[12], the inverse association between CDI and colectomy rate led to the suggestion that IBD patients with CDI have lesser disease activity, although there was no information on the disease activity. Similarly, in our recent study we did not find any difference in the endoscopic disease activity in UC patients with and without CDI[33].

OUTCOME AFTER TREATMENT AND NATURAL HISTORY OF CDI

Short-term outcome

Patients with CDI are at risk for complications including toxic megacolon, colonic perforation, and peritonitis with sepsis. Patients with IBD are similarly at risk for these complications. Single-center and nationwide studies have studied the outcome of CDI in patients with IBD. The results have been highly variable with some studies reporting shorter stay in patients with CDI in IBD than those in non-IBD patients[42], and some studies showing similar lengths of stay[55], while other studies highlighting increased hospitalization duration and costs[10].

The colectomy rate in CDI is an important measurement of short-term outcome in CDI. Colectomy has been shown to be independently associated with a greater than 2-fold increase in inpatient mortality (incidence rate ratio = 2.4, 95% CI: 1.8-3.2)[56]. However studies have reported varying rates of colectomy for CDI in the setting of IBD. In a large study utilizing the Health Care inpatient care database, the development of CDI was inversely related to the risk of colectomy[11]. Similarly a subsequent study reported low rates for colectomy after CDI (1 of 15 patients)[42]. In our recent study with colectomy at 3 mo following CDI infection being the end point, we did not find CDI as a risk factor[33]. In a single-center case-control study, the rate of emergent colectomy in their CDI-UC population was 23% with the indication being toxic complications (4 of 11) or

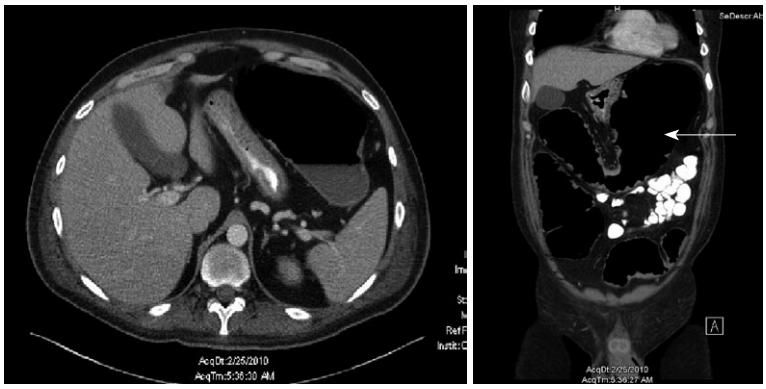

Figure 1 Toxic megacolon in a 27-year-old patient with *Clostridium difficile* infection who had underlying ulcerative colitis, resulting in emergent subtotal colectomy. Arrow indicate dilated colon.

medically refractory disease (7 of 11) compared to 13.4% in the *C. difficile*-negative IBD population[55]. However, it is interesting that 7/11 patients had medically refractory disease and may be *C. difficile* was a colonizer. Also there was no statistically significant difference in the short-term risk of colectomy at 1 mo[55]. In another study, underlying IBD was associated with 6 fold greater risk of bowel surgery compared with patients with CDI without underlying IBD[10]. We believe that the lower risk of colectomy with UC-CDI in most studies may be due to the fact that patients with UC exacerbation resulting from CDI are much more likely to improve with proper pathogen-directed medical therapy. Therefore, treating CDI in UC patients may actually prevent the need for colectomy in the short term (Figure 1).

Long-term outcome

There are limited studies available investigating the long-term outcome of CDI in patients with IBD. In a recent retrospective case control study, UC-CDI patients had worse clinical outcome than UC patients without CDI, with a follow-up of up to a year after CDI[55]. On the other hand, the study did not discriminate between recurrent CDI *vs* worse IBD disease activity because of the retrospective nature and study design. However, *C. difficile*-positive patients had significantly more UC-related hospitalizations (58 hospitalizations *vs* 27 hospitalizations) and emergency room visits in the year following initial admission (8 visits *vs* 1 visit). Also, up to a year following the index admission, patients with CDI had significantly higher rates of colectomy compared to *C. difficile*-negative patients (44.6% *vs* 25%). In a case-control study comparing the disease course for 1 year before and 1 year after the initial infection in 87 patients with IBD with *C. difficile,* colectomy occurred in only 10.3% of patients (9/87) following CDI[57]. While 8% had fewer hospitalizations in the year following infection, 41.3% of patients (36/87) followed for a year after CDI had no difference in the number of hospitalizations. However, 46% of patients (40/87) had more hospitalizations in the year following CDI (range 1-9 hospitalizations)[57]. Also 53% (46/87) of IBD patients with CDI required an escalation in their IBD medical therapy including initiation of biologic therapy (26%; 23/87), dose escalation of current biologic (8%; 7/87), escalation or initiation of azathioprine/6-MP (11.5%; 10/87) or methotrexate (7%; 6/87)[57]. Both these studies are limited by their retrospective nature and it is unclear whether underlying IBD severity was responsible for this outcome or whether *C. difficile* produces certain immunological changes that leads to a worse long term clinical outcome. Table 1 summarized both the short and long term outcome of CDI in IBD.

Table 1 Short and long-term outcomes with *Clostridium difficile* infection and inflammatory bowel disease

Short-term outcomes
Toxic megacolon
Colonic perforation
Peritonitis with sepsis
? Increased hospitalization duration and costs
Colectomy rates highly variable
Long-term outcomes
Increased UC related hospitalization and emergency room visits
? Escalation of medical treatment
Increased rate of colectomy

UC: Ulcerative colitis.

CLINICAL, RADIOGRAPHIC, ENDOSOPIC, AND HISTOLOGIC FEATURES

Patients with CDI can present with a wide variety of clinical manifestations ranging from an asymptomatic carrier state to fulminant colitis with megacolon. The most common clinical presentation of CDI is diarrhea and abdominal pain. The diarrhea is usually watery in patients with CDI; however in patients with underlying IBD, it may be bloody or mucous[49,58]. There are associated systemic symptoms and low-grade fever with a polymorphonuclear leukocytosis.

Although 0% and 3% of healthy adults may carry *C. difficile*, the frequency of asymptomatic carriage of *C. difficile* in patients with IBD is not exactly known. *C. difficile* carriage status in 122 IBD patients in clinical remission in the outpatient setting with no recent hospitalization or antibiotic exposure was studied with stool culture and molecular DNA-based microbiological methods. The strains were characterized by toxin typing, ribotyping, and pulsed-field gel electrophoresis. Toxigenic *C. difficile* was demonstrated more frequently in IBD patients (8.2%) than in healthy volunteers (1.0%). However, none of these patients developed CDI after a 6 mo follow-up and all the ribotypes identified were community acquired highlighting

the acquisition of *C. difficile* in IBD patients even in remission from a wide variety of community sources[32].

Some patients may present with severe disease causing paralytic ileus, which may evolve into toxic megacolon characterized by a dilated colon (> 7 cm in its greatest diameter), and signs and symptoms of severe toxicity (fever, chills, dehydration, high white count). There is associated dilatation of the small intestine in patients with megacolon mimicking an intestinal obstruction. Bowel perforation may also occur[59-61]. Diarrhea may be absent because of paralytic ileus, particularly in postoperative patients who receive narcotics for pain control. Patients may also have anasarca due to severe hypoalbuminemia[62]. Patients may present without diarrhea but only with abdominal pain, fever and leukocytosis (a leukemoid reaction with a white blood cell count up to 100 000 cells/cu.mm.)[62]. A high degree of suspicion is required to diagnose CDI in these settings.

Abdominal imaging

Plain radiography is usually normal in patients with CDI, unless they have complications like ileus or toxic megacolon or perforation. CT imaging is useful in the diagnosis of severe or fulminant CDI and the characteristic features include colonic-wall thickening, pericolonic stranding, the "accordion sign", and the "double-halo sign"[63]. The accordion sign is seen with oral contrast and shows the high attenuation in the colonic lumen alternating with a low attenuation inflamed mucosa, while the double-halo sign is seen with intravenous contrast[63]. The presence of these signs in the right clinical setting may suggest a diagnosis of CDI.

Endoscopy

Lower endoscopic visualization forms an important part in the evaluation of patients with CDI in IBD. Isolated CDI produces the classic endoscopic appearance of pseudomembrane formation which is described in 50% of patients[64,65]. However in patients with underlying IBD, classic endoscopic or histologic features of pseudomembranes are conspicuously absent, making it hard to diagnose CDI in patients with worsening diarrhea[10,42]. In fact, recently published studies from Milwaukee and Belgium did not identify pseudomembranes in any of the IBD patients with CDI who underwent endoscopic evaluation[10,42]. However endoscopy may be useful to assess disease activity of IBD and also to rule out other secondary causes of diarrhea including concurrent cytomegalovirus infection[66].

Histology

The classic histologic picture in CDI is the presence of pseudomembranes. Pseudomembrane formation is caused by sloughing and necrosis of the mucosa with ulceration secondary to inflammation. Pseudomembranes are actually characteristic "volcano" lesions with focal ulceration with inflammation composed of polymorphonuclear leukocytes, fibrin, chronic inflammatory cells, and epithelial debris[66]. In patients with IBD, pseudomembranes are not commonly present, CDI tends to produce a nonspecific mucopus; erythema and friability are commonly encountered endoscopic findings[10].

LABORATORY DIAGNOSIS

Although a variety of laboratory tests are used for the diagnosis of CDI, enzyme linked immunoassay (ELISA) is the most commonly used test to detect the toxin.

Enzyme linked immunoassay

These assays are based on the detection of toxins A and/or B using either a monoclonal antibody or a polyclonal antiserum that recognizes the specific toxin. The ELISA test is inexpensive and the results are available within 2-6 h. The most widely used ELISAs for detection of both toxins A and B in stool are somewhat less sensitive (70%-90%) than the cell cytotoxicity assay (see below). Up to 30% of tests may be falsely negative in comparison to the cell cytotoxicity assay or culture[67,68]. They do, however, demonstrate excellent specificity (99%)[68,69]. The lower sensitivity of these tests can be improved by performing ELISAs on 2 or 3 specimens rather than on 1 specimen, which increases the diagnostic yield by 5%-10%[70]. In IBD patients, the diagnostic yield of ELISA testing may be much lower. Four sequential stool samples were shown to increase the diagnostic yield to 92%[42].

Latex agglutination assay

Latex agglutination assay is based on the glutamate dehydrogenase (GDH) enzyme produced by *C. difficile*. The sensitivity of these tests approached almost 96%-100% in a recent study[71]. However, certain other organisms can also produce GDH and also the positivity indicates only the presence of the organism, rather than *in vivo* production of *C. difficile* toxins. It is not recommended for routine clinical use.

Cell cytotoxicity assay

Cell cytotoxicity assay is the gold standard test for diagnosis of CDI. It detects as little as 10 picograms of toxin and it is the most sensitive available test for detection of toxin B[72-75]. It is based on the principle that the toxins in the stool exert a cytopathic effect characterized by cell rounding which can be demonstrated in tissue culture. The high sensitivity (94%-100%) and specificity (99%) of the cytotoxicity assay is its major advantage. Disadvantages are its relatively high technical expertise and the 24-48 h needed to complete the assay[76].

C. difficile culture

Stool culture is seldom used for routine diagnosis because of labor intensiveness, long turnaround time (24-48 h) and a low specificity. The *in vivo* production of toxins can be seen in hospitalized patients who are asymptomatic carriers. It fails to differentiate toxin-producing from non-toxigenic strains. However, because culture permits molecular typing of the organisms, it is essential for monitoring molecular epidemiology and antibiotic susceptibility[72].

We do not recommend its routine use in the diagnosis of CDI in clinical practice.

Polymerase chain reaction for toxin gene detection

Polymerase chain reaction (PCR) based primers for the detection of genes for toxins A and is highly sensitive and specific for the diagnosis of CDI[77,78]. Culture of the organisms may be required for PCR, which makes the process more technically demanding and challenging. A study based on the nested PCR assay reported a 99% concordance with the cytotoxicity assay and a sensitivity of 96.3% and a specificity of 100%[78].

TREATMENT OF CDI

The Society for Healthcare Epidemiology of America recommends initiating empiric therapy for CDI immediately after stool procurement for patients with severe symptoms consistent with CDI[34]. Empiric treatment is warranted if the clinical suspicion is high without waiting for the results as early initiation of treatment is critical in improving the outcome. Agents that decrease intestinal motility, such as narcotics and loperamide, should be avoided because of the risk of decreasing toxin clearance and the risk for ileus and/or megacolon[79].

Specific antibiotic therapy should be initiated as soon as possible. Oral metronidazole in a dose of 250-500 mg four times a day for 10-14 d or oral vancomycin at 125-500 mg four times a day for 10-14 d is the treatment of choice in patients with CDI. Metronidazole can be administered intravenously (in doses of 500 mg four times daily) in patients who are unable to take oral agents[66]. Bacitracin, teicoplanin and fusidic acid have been used in the treatment of CDI, but their efficacy has not been proved superior to vancomycin/metronidazole in large systematic meta-analysis[80,81]. A large meta-analysis of 1157 patients from 12 randomized trials assessed the efficacy of eight antibiotics for the treatment of CDI. None of the antibiotics are superior to others for symptomatic cure and/or reduction in complications[82]. Thus metronidazole is the initial drug of choice because of similar efficacy, lower cost and lesser risk of selecting vancomycin resistant *enterococci* in mild to moderate disease. However in patients with severe disease, multiple studies have shown a failure rate of 22%-38% with metronidazole[83]. Studies have shown similar cure rates in patients with mild disease with either use of metronidazole or vancomycin, while in severe disease the eradication rate with metronidazole is 76%, as compared with vancomycin, which gives a cure rate of 97%[84]. These data support the use of vancomycin as the first line treatment for severe CDI, also in patients with mild to moderate CDI who do not improve within 72 h of initiation of treatment with metronidazole should be switched to vancomycin. Severe CDI requires aggressive treatment and doses up to 2 g/d of vancomycin may be required in patients with severe disease. A recent phase 3 trial compared the efficacy and safety of OPT-80, fidaxomicin that is bactericidal *via* inhibition of RNA polymerase and oral vancomycin in treating CDI. The clinical cure rates after OPT-80 (fidaxomicin) or vancomycin treatment were comparable[85]. However, OPT-80 was associated with a highly significant lower recurrence rate than vancomycin[85]. Further evidence of its efficacy needs to be studied. Anion-binding resins, such as cholestyramine and colestipol have also been used along with antibiotics[86]. These are proposed to bind to the *C. difficile* toxins and may have adjunctive benefit. However these agents have not been studied in IBD patients.

The efficacy of metronidazole or vancomycin specifically in the IBD population with CDI is unknown, but one study reported that just less than one quarter of the IBD patients with CDI required to be initiated on oral vancomycin because of lack of sufficient response with metronidazole[42]. Neither vancomycin nor rifaximin have been studied in randomized controlled trials for CDI in IBD patients.

There are no guidelines or evidence to suggest that one particular antibiotic regimen is better than the other in IBD patients who develop CDI. However colectomy rates in hospitalized patients with IBD was reportedly less from 45.5% in 2004, to 3.5% in 2006 in a single center study where vancomycin was adopted as the first line therapy in IBD patients with CDI after 2005[87,88].

Patients with fulminant colitis require initiation of treatment with oral vancomycin at a high dose of 500 mg every 6 h which may be administered with a nasogastric tube because of paralytic ileus. We also tend to use intravenous metronidazole along with vancomycin in these cases in our clinical practice. Emergent surgery is required for patients who do not respond to the above medical management and in patients with impending perforation and toxic megacolon. Patients usually undergo a subtotal colectomy and a temporary ileostomy and are associated with a high perioperative mortality rate approaching close to 40%[89].

There is no consensus on whether IBD-related medications, particularly immunomodulators and corticosteroids should be discontinued during the anti-CDI therapy. In a retrospective study of 155 patients from Europe with CDI complicating IBD, 104 (67%) were cotreated with antibiotics and immunomodulators (defined as the use of prednisone, azathioprine/6-mercaptopurine, methotrexate, biologics, cyclosporine, tacrolimus) for their *C. difficile*-associated IBD exacerbation, while the remaining 51 (33%) were treated with antibiotics alone[32]. The primary outcome of the study was colon perforation or toxic megacolon, shock, colectomy, and mortality. Patients treated by combination therapy had a trend towards a worse outcome when compared to those treated by antibiotics alone (likelihood ratio = 11.9; 95% CI: 0.9-157)[32]. Thus in most patients with CDI, it may be inappropriate to escalate immunosuppressive therapy during the acute CDI episode. However, the question of whether to add immunomodulator therapy in patients who are not on it before the CDI episode remains unanswered. In a recent survey of 169 North American gastroenterologists, there was significant disagreement on whether combination antibiotics and immunomodulators or antibiotics alone should be given

Table 2 Differentiating *Clostridium difficile* infection and inflammatory bowel disease

Features	Isolated CDI	CDI and IBD
Setting	Often hospital acquired	Often community-acquired
Risk factors	Antibiotic exposure prior to infection common Immunomodulator and corticosteroid use Increasing age	Many patients lacking of history of antibiotic exposure Immunomodulator and corticosteroid use playing even a greater role Increasing age Risk greater with ulcerative colitis than Crohn's disease, more with colonic involvement than small bowel disease
Clinical features	Usually watery diarrhea	May be bloody or mucous diarrhea
Outcome	Short term complications including toxic megacolon, colonic perforation, and peritonitis with sepsis	Short term complications including toxic megacolon, colonic perforation, and peritonitis with sepsis similar to patients without IBD Hospitalization costs and length of stay variable in studies Increased mortality in some studies Risk of colectomy unclear Long term outcome unclear, increased hospitalizations and escalation in medication use and colectomy rates reported with retrospective data
Diagnosis	ELISA testing for toxins	ELISA testing may be less sensitive
Endoscopy and histology	Pseudomembranes common	Pseudomembranes rare
Treatment	Metronidazole for mild to moderate severity Vancomycin for severe disease	? Vancomycin for any hospitalized IBD patient
Recurrence	20% after the first episode of CDI	Rates highly variable 10%-58%, may be higher
Extra-colonic gastrointestinal manifestations	Small bowel can be affected	Most cases of small bowel involvement in IBD patients Pouchitis can also be seen

IBD: Inflammatory bowel disease; ELISA: Enzyme linked immunoassay; CDI: *Clostridium difficile* infection.

to flaring IBD patients with CDI. Overall, 77/169 (46%) of the respondents elected to add on corticosteroids as a combined treatment with antibiotics, whereas 82/169 (54%) treated the flare with antibiotics alone. When maintenance azathioprine was regularly taken, only 11% of respondents withdrew it upon the diagnosis of CDI[90].

RECURRENT CDI

Recurrence of CDI is common, affecting approximately 20% of patients. Recurrence typically occurs 1 to 2 wk after stopping metronidazole or vancomycin, but it can be delayed for up to 12 wk[49,90]. Risk factors for recurrent CDI include a prior history of recurrence, increasing age, use of additional antimicrobials, and an inadequate protective immune response to *C. difficile* toxins[49,91].

There are limited data available on the risk of CDI recurrence in IBD patients. In a study published in abstract form from Milwaukee in 2005, recurrent CDI was reported in 27/46 (58%) of patients[92]. In a subsequent study from the same center, recurrent *C. difficile* occurred in (10/87) 11.5% of patients[57]. Thus the risk appears to be highly variable and prospective studies need to be undertaken to clearly clarify the risk of CDI recurrence in IBD patients.

Management of a first recurrence of CDI is identical to a primary episode. Long tapering courses of vancomycin or pulsed treatment reduce recurrence and are suggested for treating second episode of recurrence[93,94]. Because of the risk of often-irreversible neuropathy with long-term use of metronidazole, it is not used for treatment of second relapse. Recently, several small series reported the efficacy of rifaximin in treating recurrent CDI[95,96]. Similarly reconstitution of the fecal flora by administration of stool is effective in small series[97,98] as previous studies have shown loss of diversity of fecal flora[53]. Other treatments including the use of active and passive immunization by administration of immunoglobulins or oral administration of antibodies from colostrum of cows immunized against toxins are under investigation for future use[91].

The treatment of recurrent disease in IBD patients is unclear in the absence of evidence based studies. In a study from Milwaukee of 14 IBD patients, rifaximin at a dose of 200 mg three times a day for 2 wk, followed by 200 mg once daily for 2 wk and 200 mg every other day for the final 2 wk of the taper resulted in resolution of infection in all the patients[92]. In the absence of data, we recommend treating patients in a similar way to the non-IBD population as far as recurrence is concerned (Table 2).

CDI IN SPECIAL SITUATIONS

C. difficile enteritis

Small intestinal *C. difficile* has increasingly been reported. The spectrum of CDI has definitely expanded with small bowel involvement (Figure 2)[99]. They are more frequently reported in patients with IBD who have undergone total colectomy or some form of gastrointestinal surgery[13]. The most common presentation is increased ileostomy output with associated dehydration. In patients with small bowel CDI, the risk factors seem to be slightly different. Antibiotic use and IBD predispose to small bowel CDI similar to CDI of the colon. Prior surgeries of the colon/colectomy, and host factors including advanced age, immunocompromised state are proposed as additional risk factors for small bowel CDI[100]. More than 90% of patients reported in the literature had gastrointestinal surgery of the colon.

The reason for the predisposition of patients who undergo colonic surgery to small bowel CDI is not clear al-

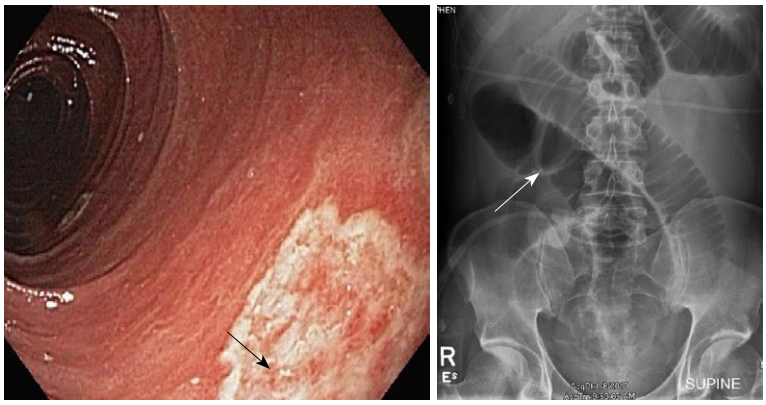

Figure 2 Recurrent *Clostridium difficile* enteritis in neoterminal ileum in a 36-year-old patient with diverting ileostomy for ileal pouch who had a preoperative diagnosis ulcerative colitis. Arrows: Enteritis due to *Clostridium difficile* infection and dilated loops of small bowel.

though multiple hypotheses are proposed. Firstly, changes occur in the small-bowel bacterial flora resembling colonic flora and after colectomy this may make it susceptible to overgrowth with *C. difficile,* particularly with concomitant antibiotic use[101]. This is based on the fact that the neoterminal ileum is colonized by colonic-type bacterial flora after ileocolonic resection[102]. Prolonged exposures to fecal stream may make the small bowel mucosa undergo metaplastic changes, as seen in patients with IPAA[103]. This notion was further supported by the fact that similar changes may occur in patients with end ileostomy and the long latent period between the surgery and the infection supports this. Changes occur in the ileostomy flora resembling the fecal flora[104]. In patients who develop infection in the immediate post operative period, a majority of patients had CDI of the colon prior to surgery which leads to the hypothesis that in those patients CDI of the small bowel may be secondary to migration of *C. difficile* into the small bowel after surgery. CDI is a toxin-mediated disease process. Although receptors for *C. difficile* toxins are typically on colonic epithelium, the receptors for toxin B is ubiquitous and may be present on small bowel epithelium which could mediate diarrhea in the immediate post operative period in the absence of colonic phenotype changes[105]. Secondly, colonization of the small bowel occurs because the protective mechanisms are compromised by colonic resection surgeries. The mechanical action of the ileocecal valve may be lost because of surgery[106]. In addition, continued peristalsis in the small bowel also inhibits colonization of the small bowel with *C. difficile*[106]. Therefore, surgeries involving only the left side of the colon with preservation of the ileocecal valve do not seem to increase the risk of CDI of the small bowel, highlighting the importance of the ileocecal valve in preventing colonization.

Initial studies highlighted that infection of the small bowel with *C. difficile* was associated with an increased mortality[13]. The increased permeability of the small intestinal mucosa was hypothesized to be due to result in profound sepsis[107]. However, recent studies showed a favorable prognosis. In fact, two large recent case series reported no mortality[100,108]. This may be probably secondary to increased awareness of the problem and early intervention.

The treatment of small bowel CDI is controversial and stratification of the disease severity as CDI of the colon could be used to initiate appropriate management plan. In a series of 11 patients, more than 50% responded to metronidazole alone[108]. In another series, all six patients were treated with a combination of metronidazole and vancomycin[100]. Thus similar to colonic CDI, oral vancomycin may be a first-line agent for severe CDI, while in mild to moderate disease, metronidazole may be used. However in patients who do not improve within 72 h of initiation of treatment with metronidazole, vancomycin needs to substituted instead of metronidazole.

C. difficile pouchitis

CDI has been reported in patients with IPAA[14,15,109,110]. CDI in IPAA can either present with asymptomatic colonization or with chronic antibiotic-refractory pouchitis or occasionally with fatal outcome. As the majority of patients have a history of short- or long- term exposure to antibiotics, CDI should be excluded in pouch patients with persistent symptoms with or without endoscopic findings of pouchitis or other pouch disorders.

In patients with IPAA, the epithelium of pelvic pouches undergoes morphologic changes facilitating fecal flora establishment[109]. These histologic adaptive changes include villus atrophy, Paneth cell hyperplasia, and a partial transition to colonic mucin phenotype without complete metaplasia[103]. In a recent study of 115 patients with IPAA, 21 (18.3%) were tested positive for *C. difficile* toxin A or B[14]. Three of those patients had chronic antibiotic-refractory pouchitis and all 3 patients had clinical remission and disappearance of *C. difficile* toxin from the stool with anti-*C. difficile* treatment with rifaximin or tinidazole. Three additional patients with other pouch-associated disorders also symptomatically improved with treatment of CDI. We also recently reported a patient who developed CDI of the pouch and neoterminal ileum immediately after ileostomy closure with a fatal outcome[111]. Fulminant outcomes of CDI of the pouch have also been described recently in a case report[112]. Similar to IBD patients with CDI who do not have the classic endoscopic or histologic features of pseudomembranes[10,42], superimposed CDI in pouch patients hardly have endoscopic or histologic features of pseudomembranes which makes the diagnosis challenging.

The treatment of CDI in IPAA is empiric at this point. There are no published prospective trials. The traditional

drugs used in the management of CDI are metronidazole and vancomycin. Previous studies suggest that metronidazole may be not completely protective against CDI of the pouch, as the bacterial infection can develop while the patients had been still on metronidazole[109,110]. Therefore, in patients with *C. difficile*-associated pouchitis, metronidazole may not be considered as the first-line agent. Based on our own experience and limited published literature, rifaximin, tinidazole, or vancomycin have been used with satisfactory results[14,110].

C. difficile infection in diverted bowel

Diversion colitis is common in segments of the colorectum after surgical diversion of the fecal stream, which may persist indefinitely unless the excluded segment is reanastomosed[113]. Patients with diverted bowel appear not immune to the development of CDI in the excluded downstream bowel segment. There has been a case report in which, following subtotal colectomy and end-ileostomy for medically refractory disease, a UC patient subsequently developed severe CDI in the rectal remnant (Hartmann pouch) and the patient responded to metronidazole suppositories[110].

RECOMMENDATIONS

In patients with IBD who present with worsening symptoms, CDI needs to be thought off and ruled out. In patients with a suspected diagnosis of CDI in IBD, stool studies for CDI are sent and empiric treatment is started. ELISA is the most commonly used method of diagnosis of CDI. We do not usually wait for the stool studies to return back to start treatment. We start all our IBD patients with suspected CDI on vancomycin 125 mg orally every 6 h and continue their previous immunosuppressive therapy. We do not add any new immunomodulators or escalate immunosuppressive medications in patients with suspected CDI in IBD unless CDI is ruled out with serial stool studies (at least 3-4). The duration of antibiotic use is 14 d. Routine endoscopy is not performed in these patients as the yield of pseudomembranes is very low unless an alternative diagnosis such as cytomegalovirus infection is being entertained. We also follow these patients serially to study the impact of CDI on the short term and long term outcome of IBD.

FUTURE DIRECTIONS

The pathogenesis and natural history of CDI in IBD patients is not entirely clear. The role of CDI in IBD exacerbation needs to be further investigated. It is unclear how to distinguish whether CDI is precipitating an IBD flare or whether it is an innocent bystander, as medical treatment targeted CDI does not necessarily induce IBD into remission. There is need for research to study the role of asymptomatic carriage of *C. difficile* and its impact on the longer-term outcomes of CDI in IBD. Although some retrospective studies have suggested worse long-term outcome of CDI in IBD patients, it needs to be prospectively studied. Management of these patients can be challenging. Future studies to ascertain the appropriate management of CDI in IBD is required in particular, as there is little consensus on whether antibiotics and immunomodulators or antibiotics alone should be administered to these patients. Randomized controlled trials comparing metronidazole and vancomycin are also required to clearly understand the best management of *C. difficile* flares in IBD patients. A multidisciplinary approach involving gastroenterologists and colorectal surgeons, together with a team of GI pathologists and GI radiologists is necessary to successfully manage and treat patients with these disorders. Development of animal models with concurrent CDI and IBD would help us to understand the pathogenesis and manage these patients better.

CONCLUSION

CDI has continuously evolved over the years rising from a relative "benign" disease entity due to antibiotic exposure to a significant public health problem. CDI poses substantial challenge to epidemiologists, infection control practitioners, infectious disease specialists, gastroenterologists, gastrointestinal surgeons and hospital administration. The rising incidence, with increasing hospitalization rate, length of hospital stay, morbidity and mortality is of great concern. There has been a tremendous increase in the burden of CDI over the past few years with higher rates of surgery and mortality in the IBD population compared with the non-IBD cohort. The increase in the risk of community-acquired CDI in IBD population highlights that a high index of suspicion should be maintained even in the absence of conventional risk factors, such as antibiotic use or health care exposure. Patients with IBD even after colectomy are not immune to CDI. Pseudomembranes on endoscopy and histology appear to be uncommon in CDI superimposed on IBD. Randomized controlled trials are required to define the appropriate strategy for risk stratification and management for CDI in patients with IBD. In addition, preventive measures are the key and require concerted effort from all quarters from epidemiologists to hospital administration and clinicians.

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TOPIC HIGHLIGHT

Anastasios Koulaouzidis, MD, MRCP (UK), *Series Editor*

Alcoholic hepatitis 2010: A clinician's guide to diagnosis and therapy

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Abstract

Alcoholic hepatitis (AH) remains a common and life threatening cause of liver failure, especially when it is severe. Although the adjective "acute" is frequently used to describe this form of liver injury, it is usually subacute and has been developing for weeks to months before it becomes clinically apparent. Patients with this form of alcoholic liver disease usually have a history of drinking heavily for many years. While certain aspects of therapy, mainly nutritional support and abstinence are well established, significant debate has surrounded the pharmacologic treatment of AH, and many institutions practice widely varying treatment protocols. In recent years a significant amount of literature has helped focus on the details of treatment, and more data have accumulated regarding risks and benefits of pharmacologic treatment. In particular, the efficacy of pentoxifylline has become increasingly apparent, and when compared with the risks associated with prednisolone, has brought this drug to the forefront of therapy for severe AH. This review will focus on the clinical and laboratory diagnosis and pharmacologic therapies that should be applied during hospitalization and continued into outpatient management. We conclude that the routine use of glucocorticoids for severe AH poses significant risk with equivocal benefit, and that pentoxifylline is a better, safer and cheaper alternative. While the full details of nutritional support lie beyond the scope of this article, nutrition is a cornerstone of therapy and must be addressed in every patient diagnosed with AH. Finally, while traditional psychosocial techniques play a major role in post-hospitalization care of alcoholics, we hope to make the medical clinician realize his or her role in reducing recidivism rates with early and frequent outpatient visits and with the use of baclofen to reduce alcohol craving.

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Key words: Alcoholic hepatitis; Alcoholic liver disease; Pentoxifylline; Baclofen

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INTRODUCTION

The term alcoholic hepatitis (AH) was first used by Beckett *et al*[1] in 1961, but reports of clinical jaundice after excessive ethanol consumption were not unusual in the early medical literature and they likely represented instances of AH[1,2]. Despite this longstanding observational relationship between alcohol consumption and liver disease, significant work has been required to determine how much alcohol must be consumed to cause liver disease, and which cohort of patients are at highest risk of developing significant liver injury.

VOLUME OF ALCOHOL REQUIRED

Observational studies have shown an increased risk of cirrhosis with ingestion of greater than 10-20 g of alcohol per day in women and more than 20-40 g/d in men[3]. In addition to total duration of alcohol intake, a variety of genetic, environmental and gender-related factors appear to independently influence the development of alcoholic liver disease. Age, female gender, and excess body weight [body mass index (BMI) > 27 kg/m^2 in men, BMI > 25 kg/m^2 in women] have been identified as independent risk factors for development of liver disease including AH[3-6]. In addition to a smaller volume of distribution, women are at higher risk due to a relative deficiency of gastric alcohol dehydrogenase compared to men[7]. An oral dose of alcohol in a woman is more like an intravenous dose. We have recently seen several women who developed AH after gastric bypass, in which the amount of gastric mucosa available to metabolize alcohol was reduced. More severe forms of AH are associated with consumption of large amounts of alcohol or binge drinking, and concomitant malnutrition[8]. Not surprisingly, the presence of coexisting hepatitis C has also been linked to a poorer prognosis[9].

DIAGNOSIS

Diagnosing AH can be challenging as the disease has widely varying presentations and in severe cases can mimic a bacterial infection and/or biliary obstruction. A detailed and thorough history remains the cornerstone of diagnosis[10]. Obtaining such a history can be rather difficult if patients feel ashamed about their drinking habits. Often, lengthy discussions are required to reveal the full extent of alcohol intake.

Medical history

Questions that are relevant to ask are detailed in Table 1. Patients regularly tell us that they "quit drinking" when in reality they simply reduced the amount or switched from hard liquor or fortified wine to beer. It is important to obtain the exact time sequence and volume and type of alcohol consumption. Many patients have the mistaken impression that beer is not alcohol. It is actually difficult to drink enough beer on a daily basis to develop AH-perhaps 48 beers per day. To develop AH, patients usually have to supplement beer with wine, fortified wine, and/or hard liquor.

Alcoholism and alcohol-related health problems are common in patients seen in county or university healthcare systems. Detailed histories regarding alcohol are therefore common in these settings. In contrast, patients admitted or seen in private systems may not be questioned at all about alcohol or may be asked only a few superficial questions that the patient finds easy to respond negatively to.

In general, patients with AH have been drinking heavily for years and then report a dramatic increase in the amount of alcohol intake, usually relating to a major life stressor, such as death of a parent, loss of a job, divorce, *etc*. Also, patients have often stopped drinking alcohol days to weeks prior to presentation due to malaise, poor appetite, and/or the realization that their drinking finally "caught up" with them. Most commonly patients present with nonspecific complaints such as anorexia, nausea and vomiting, abdominal pain, and weight loss (Table 2)[10,11].

Table 1 Questions to ask patients with suspected alcoholic hepatitis

When did you first start to drink alcohol?
How many days per week do you usually drink?
How many years have you been drinking on a regular or daily basis?
How many times have you been arrested for driving under the influence of alcohol?
How many times have you been arrested for public intoxication?
What type of alcohol do you usually drink? Beer? Wine? Hard liquor?
How many drinks of each type of alcohol do you drink on an average day?
Do you usually drink at home? Bars?
Have you been through an alcohol rehabilitation program? What type-inpatient or outpatient? How many times?
Have there been prolonged times when you drank no alcohol?
When was your last drink?

Table 2 Symptoms and signs of alcoholic hepatitis

	%
Common Presenting Symptoms of Alcoholic Hepatitis[10-13]	
Anorexia	27-77
Nausea and vomiting	34-55
Abdominal pain	27-46
Weight loss	29-43
Physical Examination Findings	
Hepatomegaly	71-81
Ascites	35
Encephalopathy (from asterixis to coma)	18-23
Gastrointestinal bleeding requiring transfusion	23
Jaundice	37-100
Malnutrition	56-90
Hepatic bruit	59

Physical examination

In addition to the patient's history, noteworthy physical findings that may help the clinician focus on AH as the diagnosis include hepatomegaly, ascites, encephalopathy (ranging from asterixis only to coma) and gastrointestinal bleeding requiring transfusion, especially if the cause is esophageal varices. Nonspecific findings of jaundice and malnutrition are also commonly seen[10-12]. With severe AH, jaundice is present in essentially 100% of patients. Notably, fever ranging from 100.4º to 104º, due to AH and not attributable to infection can be seen in over half of patients diagnosed with severe AH[11]. Fever is a common cause of confusion among physicians and can lead to extensive and relatively useless evaluations for fever of unknown origin, when AH should be the obvious explanation. The presence of a hepatic bruit is also very helpful in providing strong evidence for AH, if there is no malignant mass that could also cause a bruit. In one large series, 59% of patients with severe AH had a bruit[13].

Laboratory data

Laboratory findings in AH are often nonspecific, but can on occasion provide clues to the diagnosis. These include mild to moderately elevated transaminases, usually with an aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio above 1.5 with AST greater than 45 U/L but usually < 300 U/L[10,14]. However, an unusual variant of AH, known as alcoholic foamy degeneration, can lead to an AST as high as 730 U/L[15]. A serum bilirubin > 2 mg/dL is often required to make a diagnosis, but clinical jaundice is usually present and the bilirubin is regularly greater than 10 mg/dL. Other nonspecific but established markers of alcohol intake include gamma-glutamyltransferase activity (GGT) and erythrocyte mean corpuscular volume[14]. As the severity of alcohol-related liver injury increases, the bilirubin can increase with a concomitant decrease in GGT[16]. Also, it has been our experience that total blood cholesterol levels < 100 mg/dL can predict poor outcome; the lower the cholesterol, the worse the prognosis. Finally, a mild to very elevated leukocytosis (up to 40 000/mm^3) is very characteristic of AH. While less common, reports of severe leukemoid reactions are readily found with documented peripheral white blood cell counts > 130 000/mm^3[17-20]. In general, a severe leukemoid reaction in AH portents a very poor prognosis[17,19].

Clinicians can be led astray by the presence of both fever and prominent leukocytosis, leading to concern for sepsis and overshadowing the possibility of AH, particularly if the patient's history is unclear or unattainable due to altered mental status. As these patients may be profoundly nutrient deficient and commonly have comorbidities such as cirrhosis, they are generally at increased risk of infection and therefore an evaluation for bacterial infection should be performed[11]. This evaluation should, at a minimum, include a chest X-ray, blood cultures, abdominal paracentesis (ascites is frequently present), and urinalysis with urine culture. More specific testing should be pursued for localizing clinical signs of infection such as excessive sputum production or diarrhea. However, the clinician must also remember that profound leukocytosis can be seen without a concomitant infection, and extensive evaluation and prolonged use of broad spectrum antibiotics can lead to loss of time and resources, but more importantly, can carry specific risks (superinfection with resistant bacteria or fungi) and delay appropriate therapy[17-19].

Liver scanning

In rare instances in which diagnosis is still unclear despite a thorough history, physical examination and laboratory evaluation, a technetium sulfur colloid liver spleen scan can confirm the diagnosis of AH noninvasively[21]. This is perhaps the last remaining indication for this very old imaging modality. However the single photon updated version, otherwise known as the perfused hepatic mass, may have utility in assessing prognosis in parenchymal liver disease.

The dramatic "colloid shift" to the bone marrow and spleen seen in the older version of this scan is characteristic of severe AH and is unusual in other diagnoses. The liver may be nearly invisible on a liver spleen scan and the bone marrow may be so visible that one can count ribs easily.

Liver biopsy

Liver biopsy is rarely needed outside of research protocols or perhaps when the patient and family fabricate a story of total alcohol abstinence when AH is clinically obvious. This conspiracy is common when the patient is pursuing liver transplantation. A histologic diagnosis of AH rules out the possibility of liver transplantation, at least in the Unites States. Because of the high risk of recidivism, organs can not be allocated to patients with a diagnosis of AH. Only patients who survive AH and continue to have liver failure after 6 mo of observed and documented abstinence can be listed for transplant. Patients usually improve so dramatically with several months of abstinence that a transplant is not needed.

The rare biopsy is usually performed transjugularly because of ascites and/or coagulopathy. The most common characteristic finding on pathology is macrovesicular steatosis which can also be seen in nonalcoholic fatty liver disease[22]. Intrahepatic cholestasis can also be seen and requires the clinician to rule out mechanical obstruction of the bile ducts, and evaluate the patient for other causes of cholestasis such as drug toxicity or viral hepatitis. Mallory bodies can be seen in up to 65% of patients with AH but can also be found in other causes of hepatocyte injury and has been described as indicative but not pathognomonic of AH[2]. Giant mitochondria, and in particular Type I megamitochondria, can also provide a diagnostic clue for AH and correlate with the presence and amount of daily alcohol consumption[23,24]. Ultimately however, biopsy must be correlated with the patient's history and will rarely provide all the diagnostic information needed in the absence of other details.

Some inexperienced clinicians may assume that such a biopsy could represent nonalcoholic steatohepatitis (NASH). However, patients with NASH do not present with deep jaundice, ascites, coagulopathy, *etc*. NASH is a much less inflammatory condition. In fact, patients with NASH do not develop jaundice until their advanced cirrhosis is near terminal.

CLINICAL ASSESSMENT AND TREATMENT

Once a diagnosis is made, treatment should be initiated that addresses all aspects of the disease, including alcohol cessation, correction of nutritional deficiencies and initiation of pharmacologic therapy when needed. In fact, a 3-pronged approach can help clinicians formulate a plan to guide therapy from the time of presentation through hospitalization and following into the outpatient setting to help reduce recidivism and prevent recurrence.

Nutrition

The first consideration for the hospitalized patient, after

evaluation and treatment for any signs of alcohol withdrawal, should be nutrition and electrolyte repletion, because AH induces a profound catabolic state. In part because of malnutrition, AH carries a considerably high mortality rate, therefore nutrition remains a key aspect of therapy. Nutrition should be provided orally if the patient is able to eat or via nasojejunal feeding if nausea, vomiting or poor appetite prevent adequate intake of calories. Calorie counting is essential to assure adequate intake as patients require a higher than average caloric intake (approximately 1.2-1.4 times the normal resting intake)[25]. Furthermore, nighttime supplementation of nutrition (approximately 700 kcal/d) may prevent muscle wasting and improve lean muscle mass and should be considered in hospital and beyond if the patient has any evidence of cirrhosis[26]. Furthermore, patients with longstanding alcohol abuse usually require liberal multivitamin, folic acid and thiamine supplementation. Many of these patients are profoundly depleted of potassium due to high aldosterone levels and lack of intake of solid food for weeks to months. Many have been living on a total liquid alcohol diet. Serum potassium levels do not accurately reflect intracellular levels. It may take many days of potassium repletion to finally achieve normokalemia. As these nutritional considerations are being addressed, the next step for the clinician is deciding upon whether pharmacologic therapy and anticipating potential complications of AH.

Ascites

Patients with pure severe AH in the absence of cirrhosis have relatively little problem with ascites. They eat so little that they do not take in enough sodium to retain much fluid. Maintenance intravenous fluids should be avoided to minimize fluid retention. When cirrhosis is also present, they may have more problematic fluid retention. In this case, if blood urea nitrogen and creatinine are normal, spironolactone can be given. This drug will increase urinary excretion of sodium and water, increase serum potassium, and decrease the need for potassium supplementation. Once serum potassium is normal without supplementation, oral furosemide can be added, if needed. If azotemia occurs, diuretics should be stopped and the patient should be evaluated for hepatorenal syndrome. The first step is to give 1 g of 25% albumin/kg body weight (100 g maximum) intravenously daily for 2 d and to monitor creatinine. If creatinine improves with albumin, the azotemia is probably diuretic-induced. If creatinine continues to rise, hepatorenal syndrome is probably present, as this commonly occurs in severe AH (see below).

Variceal hemorrhage

Similar to the situation with ascites, the authors have observed that patients with pure severe AH in the absence of cirrhosis have relatively little problem with upper gut hemorrhage. The relatively short duration of AH usually does not lead to formation of varices that are large enough to bleed. However, patients with underlying cirrhosis can bleed from esophageal varices. Urgent endoscopy with banding of varices is warranted when this occurs. Patients with severe AH are very intolerant of hypotension and seldom survive shock superimposed on AH.

Pharmacologic therapy

Deciding upon appropriate pharmacologic management of patients with AH relies heavily upon assessment of the severity of disease. Mild forms of AH may improve with abstinence and conservative management, while more severe disease is associated with significant mortality and should be treated more aggressively[27]. The Maddrey discriminant function (DF) [4.6 × (prothrombin time (PT) in seconds - control PT) + serum bilirubin (mg/dL)] was first introduced in 1978 in order to aid in the assessment of disease and guide therapy, which at that time relied mostly upon corticosteroid use[28]. A cutoff value of 32 was used to identify patients with a mortality rate above 50% without pharmacologic therapy. The shortcomings of the DF were outlined by Dunn *et al*[10], the most notable of which are the lack of standardized PT measurement techniques and values across different laboratories, and more importantly a relatively high risk of mortality (up to 17%) with DF values under 32. The model for end-stage liver disease (MELD) has been evaluated and compared with DF in predicting mortality and has helped guide initiation of pharmacologic therapy[10,29,30]. A MELD score of 11 has equal sensitivity but higher specificity then DF in predicting 30-d mortality[29]. A MELD score of 20 or higher at the time of admission has the highest sensitivity and specificity for predicting in-hospital mortality and outperformed both DF and Child-Pugh-Turcotte (CPT)[30]. We propose using a MELD score ⩾ 20 or a DF of 32 or higher to prompt initiation of pharmacologic therapy (Figure 1).

There may be a component of malabsorption of vitamin K due to jaundice in addition to poor synthesis of coagulation components by the diseased liver. The International Normalized Ratio regularly decreases after 3 daily doses of 10 mg of vitamin K intravenously or subcutaneously. Oral dosing of vitamin K is not appropriate because of poor absorption in the setting of deep jaundice.

After diagnosing and assessing the severity of AH, the decision of which pharmacologic therapy to initiate has become a major point of debate among experts, but recent publications may help narrow the choices down considerably. A few small trials had suggested that glucocorticoids can improve short-term survival in patients with the severe AH (DF ⩾ 32)[31]. Since then, however, significant work has challenged the efficacy of steroids and raised concerns regarding side effects. The first of these was a meta-analysis by Christensen *et al*[32] in 1995 which did not support the routine use of glucocorticosteroids in these patients. In addition, a 2008 Cochrane review of 15 randomized controlled trials with a total of 721 patients concluded that glucocorticosteroids did not statistically reduce mortality compared with placebo or no intervention. Only when a subset of patients with DF ⩾ 32 or with encephalopathy were evaluated was a mortality benefit seen[33]. In addition to the limited efficacy found by these analyses,

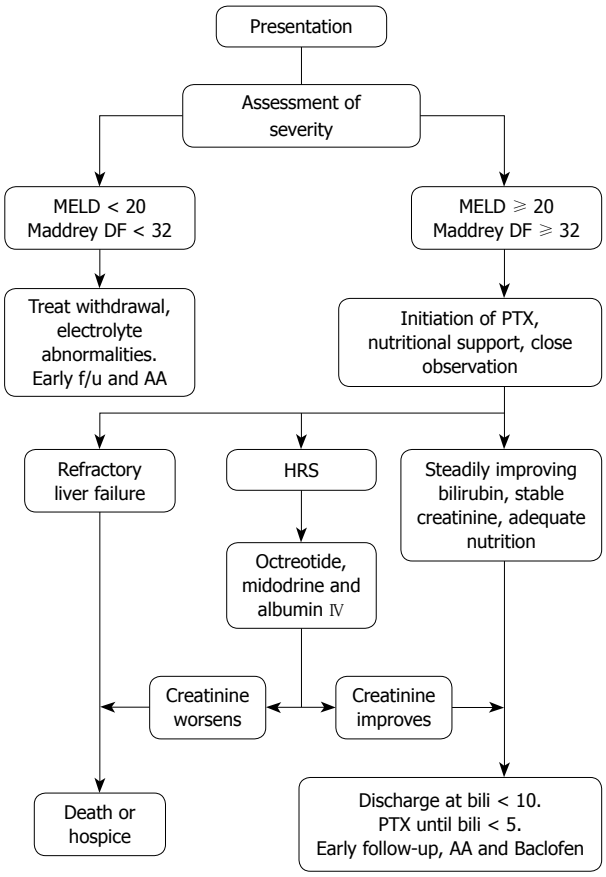

Figure 1 Treatment algorithm for hospitalized patients with alcoholic hepatitis. MELD: Model for end stage liver disease; DF: Discriminant function [4.6 × prothrombin time (PT) in seconds - control PT]; PTX: Pentoxifylline; AA: Alcoholics anonymous; HRS: Hepatorenal syndrome; Bili: Bilirubin (in mg/dL).

there is also a propensity for side effects with even relatively short-term use of steroids. The clinician must take these side effects into consideration. Approximately 16% of patients experience adverse effects, primarily in the form hyperglycemia or Cushing's syndrome, but also with increased risk of infection as compared with only a 4% adverse event rate in control patients [relative risk (RR), 3.63; 95% confidence interval (CI): 1.95-6.76][33].

As there is a lack of strong evidence of a survival benefit and a propensity for adverse events, routine use of glucocorticosteroids is discouraged. In the ongoing obesity epidemic, many patients with severe AH now have frank diabetes or insulin resistance. Corticosteroids can make diabetes overt or convert non-ketosis-prone patients to a ketosis-prone state.

Another serious practical issue regarding corticosteroid treatment is discontinuation of this highly problematic drug. In some of the trials, the drug was stopped abruptly. In others the dose was tapered. Many physicians are reluctant to stop steroid treatment abruptly and the patients may remain on it too long. Many patients with severe AH are in county hospitals or other settings in which they see a different physician in the clinic than in the hospital. The patient may be homeless and not return to the clinic at all.

In the USA these patients are frequently taken to or transferred to an academic hospital but then discharged to a county hospital because of a lack of insurance that is required to return to the academic system. The new clinic physician may not have access to the patient's medical information and may not even know why the patient is on steroids. It has been the authors' experience that too often the drug is continued for more than 30 d because the clinic physician does not know what to do with the steroid dose. Too often it is just continued at the high dose, putting the patient into what the authors call "steroid auto-pilot". The drug may be continued for months in this setting with the dose being adjusted up or down, for no apparent reason, unless someone finds out that it is supposed to be stopped. Then the issue of tapering the dose comes up.

Yet another issue is prednisone *vs* prednisolone for treatment. When steroids are avoided, such a debate is unlikely.

Unless a physician makes a commitment to see the same patient in the clinic and stop the drug when appropriate, it is the authors' opinion that the physician should not start steroid treatment for this condition. The hospitalist movement in the USA has led to different physicians caring for the patient in the hospital *vs* in the clinic and has made continuity and follow-through on a plan of care very difficult.

More recently, trials and reviews of pentoxifylline (PTX) have shown a better risk benefit profile than that of steroids, and point to PTX as a better first-line agent in treatment of severe AH than glucocorticosteroids. The efficacy of PTX in severe AH was demonstrated by Akriviadis *et al*[13] in 2000 with a randomized, placebo-controlled trial showing significant benefit in both short-term survival and in preventing development of hepatorenal syndrome (HRS), a key cause of mortality in AH.

Since then, there has been debate regarding the magnitude of the effect PTX has on mortality, and recent publications have examined the results of the Akriviadis trial along with other available data to clarify this issue. A Cochrane analysis of PTX, published in 2009, performed a detailed analysis of all the combined randomized, controlled trials available at that time. Routine meta-analysis showed reduced mortality (RR, 0.64; 95% CI: 0.46-0.89) and reduced hepatic-related mortality due to HRS (RR, 0.40; 95% CI: 0.22-0.71), and trial sequential analysis found strong support for PTX in lowering serum creatinine, a surrogate marker of HRS. However, the group concluded that there was not a significant effect on mortality based primarily upon trial sequential analysis which included, among other data, an abstract by Lebrec *et al*[34] which was published fully in 2010. This abstract did not demonstrate the same mortality benefit as that seen in the Akriviadis trial and therefore led to doubt regarding overall efficacy. Since that time, however, the full manuscript by Lebrec has been published and reveals that a likely reason for the discrepancy is based upon the significantly different populations used in each study[13,34]. The recent article included only CPT class C patients with cirrhosis while the older study excluded such patients completely. This may explain the lack of a clear cut mortality benefit, as any advanced cirrhosis patient diagnosed with AH

stands to have a predictably poorer outcome than his counterpart without cirrhosis. More important than the differences between the 2 trials, however, is the common finding that PTX therapy correlated significantly with a lower rate of liver-related complications (including HRS and hepatic encephalopathy).

A recent randomized trial comparing PTX to steroids in the setting of severe AH (DF ⩾ 32) also showed significantly lower mortality with PTX (35.29% *vs* 14.71%, $P = 0.04$) and no incidence of HRS[35]. In terms of adverse events with the use of PTX, gastrointestinal upset including diarrhea, vomiting and or epigastric pain, are the primary complaints. Publications vary widely on the reported rate of adverse events, with gastrointestinal complaints ranging from 9.9% to 26.5%, and overall events including headache, skin rash, spontaneous bacterial peritonitis and urinary tract infection reported as 67.3% for PTX *vs* 28.2% in the control group. In our experience of treating many hundreds of patients with PTX, side effects rarely lead to discontinuation of this life-saving drug. These patients have so many digestive symptoms routinely that they do not notice the upset stomach that healthy patients may experience with PTX. No life threatening or severe reactions were reported with PTX and therefore a trial should be attempted. We have treated hundreds of patients with PTX with results similar to the Akriviadis trial.

We therefore recommend PTX as the routine first line treatment of severe AH at a dose of 400 mg orally 3 times daily for a period of at least 4 wk. We usually continue this safe, inexpensive drug until the bilirubin is < 5 mg/dL. Patients are usually discharged from hospital when the bilirubin is approximate 10 mg/dL (see below). The drug is usually stopped in the outpatient setting. This strategy may require up to several months of treatment with the latter component taking place in the clinic.

Another advantage of PTX over steroids is that it can be safely given for months, whereas steroids can not.

Hepatorenal syndrome

Due to the prevalence of HRS in AH patients, the clinician must also be prepared to diagnose and treat this disorder. If the serum creatinine is abnormal or the patient has only minimal fluid overload on admission, it is prudent to withhold diuretics. If diuretics are initiated, it can be confusing as to whether subsequent azotemia is diuretic-induced or HRS. A retrospective review of patients with advanced liver disease and renal failure found that misdiagnosis of HRS occurred in approximately 40% of cases[36]; therefore care must be taken to make an appropriate diagnosis before initiating therapy, and a table paraphrasing the diagnostic criteria set by the International Ascites Club is provided[37] (Table 3). Diagnosis should begin with a careful review of medications, cessation of diuretics and any potentially nephrotoxic drugs, urinalysis and urine electrolytes to give a rapid assessment of underlying nephropathy and acute tubular necrosis. This should be followed by a 24 h urine collection to rule out proteinuria, and Doppler ultrasound of the kidneys to rule out parenchymal disease and obstructive uropathy.

Table 3 International ascites club criteria for hepatorenal syndrome[37]

Cirrhosis with ascites
Serum creatinine > 1.5 mg/dL (> 133 μmol/L)
No improvement in serum creatinine (< 1.5 mg/dL) after at least 2 d with diuretic withdrawal, and volume expansion with intravenous albumin. The recommended dose is 1 g/kg of body weight per day up to a maximum of 100 g/d
Absence of shock
No current or recent treatment with nephrotoxic drugs
Absence of parenchymal kidney disease as indicated by proteinuria > 500 mg/d, microhematuria (> 50 red blood cells per high power field) and/or abnormal renal ultrasonography

After the appropriate evaluation is performed and HRS is diagnosed one should initiate therapy with intravenous albumin infusion of 1 g/kg per day (100 g maximum) for a total of 2 d and pharmacotherapy. A study of octreotide and midodrine used in combination showed significant reduction in mortality (43% *vs* 71%, $P < 0.05$) and sustained reduction in serum creatinine (40% *vs* 10%, $P < 0.05$)[38]. We recommend initiation of octreotide 50 μg/h continuous infusion and midodrine 5 mg orally given every 8 h followed by gradual increases in the dose of midodrine by 2.5 mg increments with each dosing. There is no reason to wait 24 h between dose increases. Time is of the essence in treating this life-threatening complication of severe AH. The goal is to achieve an increase in mean arterial pressure of 15 mmHg or until a systolic blood pressure of 140 mmHg is reached.

Finally, consideration of the use of an anabolic steroid, in particular oxandrolone, can be made. In a study comparing oxandrolone to prednisolone and placebo, oxandrolone was found to improve conditional mortality rates beyond 30 d. This effect was especially pronounced in patients with moderate severity AH[39]. A recent review of oxandrolone use in AH, as well as other catabolic diseases associated with muscle wasting, showed evidence of clinical efficacy and few side effects[40]. Improvements in body composition, muscle strength and function, recovery from acute catabolic injury and nutritional status have been shown in various trials[40].

It has been the authors' policy to add oxandrolone at a dose of 40 mg orally daily for 30 d maximum in the following circumstances: (1) Maddrey score ⩾ 80 on admission, or (2) lack of improvement in Maddrey score or MELD after 10-14 d of PTX. Androgenic steroids could theoretically increase the risk of hepatocellular or prostate carcinoma. Because of this potential risk, physicians who have used oxandrolone extensively for severe AH do not prescribe it for more than 30 d. Oxandrolone seems to improve survival in patients with ultra-severe or refractory AH (Table 4).

ABSTINENCE AND POST-HOSPITALIZATION CARE

The final consideration, for those patients who survive

Table 4 Use of oxandrolone for alcoholic hepatitis

Dose	Oxandrolone 40 mg orally daily
Duration of therapy	30 d maximum
Circumstances for use	Maddrey score ≥ 80 on admission No improvement in Maddrey score or MELD after 10-14 d of pentoxifylline

MELD: Model for end-stage liver disease.

the initial bout of AH, involves establishing a plan for increasing the likelihood of abstinence from alcohol. This usually involves a multidisciplinary approach involving self-help or 12-step programs, some level of psychiatric or behavioral therapy and also pharmacotherapy. The efficacy of self-help programs is well established and usually accessible if not always funded, and we recommend routine referral and strong encouragement of attendance[41].

The role of the clinician in this recovery program has been relatively undefined, primarily as most pharmacologic therapies have been less than efficacious. Furthermore, clinicians may feel that closely monitoring a patient's abstinence may be difficult to confirm without measurable markers.

With regard to pharmacotherapy, baclofen has recently been evaluated in terms of safety and efficacy in the setting of alcoholic cirrhosis. Baclofen significantly reduced alcohol cravings and significantly lengthened time to relapse with no significant adverse effects noted after 12 wk of continuous use in a well run randomized, controlled trial[42]. Notably, patients with CPT class C cirrhosis had the most significant effect from treatment, and may reflect the importance of the patient's commitment to the treatment program. Discharge is considered as the bilirubin level approaches 10 mg/dL, and the clinician can use this to help plan initiation of baclofen.

Many of the early randomized trials of treatment for AH were conducted on a dedicated approximate 100 bed Liver Unit at the University of Southern California. This unit was mostly populated by patients with AH. The founders of this unit, Drs Reynolds and Redeker, determined through 5 decades of experience in treating these patients, that a bilirubin approximate 10 mg/dL was a good marker of stability for discharge. When patients were sent out with higher bilirubin levels, they were regularly rapidly readmitted with further deterioration.

Currently, we are initiating baclofen in the final days of hospitalization, starting at 5 mg orally 3 times daily then increasing to 10 mg on day 3. The authors have had great success in eliminating alcohol craving and eliminating alcohol consumption with baclofen. No side effects have been recognized in this patient population. We are continuing it indefinitely. Some patients who have stopped it have then redeveloped an alcohol craving and requested that it be continued.

With respect to monitoring abstinence, close follow-up with interview may not be adequate and usually requires a committed family member or friend to corroborate abstinence and honestly report recidivism. In addition to, or in place of such an informant, the clinician may find the use of serologic markers useful for monitoring or diagnosing alcoholic recidivism. Carbohydrate-deficient transferrin (CDT) has been approved by the US Food and Drug Administration for identification of heavy alcohol use and is found in high prevalence in alcoholics. Furthermore, CDT is not detectable after approximately 2 wk of abstinence and may help confirm patient reports of abstinence[14]. A second helpful test is measurement of ethyl glucuronide (EtG), a non-volatile, water-soluble, direct metabolite of ethanol which tests positive shortly after consumption and remains positive for up to 80 h after complete alcohol excretion[43]. EtG may play a role in monitoring for recidivism or in drug and alcohol treatment programs that monitor patients more closely.

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REVIEW

Diagnosis and management of angioedema with abdominal involvement: A gastroenterology perspective

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Abstract

Abdominal involvement in angioedema is often a challenge to diagnose. Acute onset abdominal pain is its most common presenting symptom, and misdiagnosis may lead to unnecessary surgical intervention. Familiarity with the types and presentations of angioedema can be invaluable to clinicians as they consider the differential diagnoses of a patient presenting with abdominal pain. Detailed personal and family histories, careful physical examination of the patient, combined with knowledge of angioedema types, can help clinicians perform their diagnostic evaluation. An accurate diagnosis is essential in order to provide appropriate treatment to patients with angioedema. Depending upon the diagnosis, treatment may be the avoidance of provoking factors (such as allergens or medications), inhibiting histamine-provoked reactions, or treating C1 esterase inhibitor deficiency.

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Key words: Acquired angioedema; Angiotensin-converting enzyme-induced angioedema; Gastrointestinal; Hereditary angioedema; C1 esterase inhibitor deficiency

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INTRODUCTION

About 10% to 20% of people worldwide will develop an episode of angioedema or urticaria at some point in their lifetime, women being more prone than men[1]. Angioedema is characterized by localized temporary swelling, which can affect all layers of the skin or of the walls of hollow viscera, such as the oropharynx, respiratory system, or the gastrointestinal (GI) tract. Peripheral non-pitting edema is typical of cutaneous manifestations. However, the effects of visceral angioedema are variable, ranging from life-threatening episodes when the respiratory system (larynx) is involved, to pain of varied severity associated with nausea or vomiting, when abdominal viscera such as the intestines are involved[2].

Abdominal pain associated with angioedema may manifest as severe acute onset abdominal pain, or as chronic recurrent abdominal pain of moderate severity. The abdominal pain is described as cramping or colicky and is rated as severe to excruciating in 87% of patients[3]. Vomiting and diarrhea occur in 78% and 65%, respectively, of patients with abdominal symptoms[3].

Patients with these symptoms typically present to one of four medical specialists, namely emergency room physicians, primary care physicians (internists, pediatricians, family medicine), general surgeons, and gastroenterologists. Physicians in each of these specialties should be familiar with the signs and symptoms of cutaneous and visceral angioedema, and be able to order appropriate tests to investigate this group of differential diagnoses. This brief review provides a perspective of angioedema diagnoses and management, including new therapeutic options, for practicing gastroenterologists.

Table 1 Molecular mechanisms of angioedema[2,4,5]

Type of AE	Mediator	Mechanism
Allergic AE	Histamine (mast cells)	Allergens react with IgE antibodies on the surface of mast cells, causing degranulation and release of histamine
ACE-I-induced	Bradykinin	ACE-Is prevent the conversion of bradykinin to inactive metabolites, leading to bradykinin accumulation
NSAID-induced AE	Leukotrienes (mast cells)	Inhibition of COX-1 leads to overproduction of vasoactive substances by shunting arachidonic acid metabolism through the lipoxygenase pathway, creating leukotrienes. Vasoactive leukotrienes act on cell-surface receptors to increase vascular permeability and promote inflammation
HAE type 1	Bradykinin	Genetic mutations in the *C1 INH* gene result in low levels of C1 INH. Major roles of C1 INH include inactivating coagulation factors XIIa, XIIf and XIa; blocking C1 complement autoactivation; and inhibiting activated kallikrein. Removal of these inhibitory actions results in complement activation and elevated bradykinin levels
HAE type 2	Bradykinin	Genetic mutations in the *C1 INH* gene result in normal levels of C1 INH, but the C1 INH is dysfunctional. Plasma cascades are unregulated in the presence of dysfunctional C1 INH, leading to bradykinin accumulation as in HAE type 1
Inherited AE with normal C1 INH	Bradykinin	Missense mutation in factor XII gene confers a significant increase in the protease activity of each activated factor XII molecule, which increases bradykinin generation. Decreased activity of enzymes such as ACE and aminopeptidase P have also been noted
Acquired AE	Bradykinin	Type 1: Immune complex formation associated with rheumatologic, lymphoproliferative, and neoplastic disorders continuously activate C1, causing C1 INH depletion and bradykinin accumulation Type 2: Autoantibodies inactivate C1 INH, leading to bradykinin accumulation
Idiopathic recurrent AE	Unknown	Unknown

AE: Adverse events; ACE-I: Angiotensin converting enzyme inhibitor; HAE: Hereditary angioedema; COX-1: Cyclooxygenase-1; NSAID: Non-steroidal anti-inflammatory drug; C1 INH: C1 esterase inhibitor.

TYPES OF ANGIOEDEMA AND THEIR CHARACTERISTICS

Classification of angioedema types is based on their etiology or pathophysiology. Broadly, these include allergic angioedema, angiotensin converting enzyme inhibitor (ACE-I)-mediated angioedema, non-steroidal anti-inflammatory drug (NSAID)-mediated angioedema, hereditary angioedema (HAE), inherited angioedema with normal C1 esterase inhibitor (formerly called HAE type 3), and acquired C1 esterase inhibitor deficiency angioedema [acquired angioedema (AA)]. Angioedema can result from mast cell degranulation with massive histamine release or from increased accumulation of bradykinin either *via* increased production or decreased inactivation (Table 1).

Allergic angioedema is caused by reaction to foods (such as shellfish, nuts, some fruits), medications, insect bites, latex, or other environmental allergens, and results from IgE-mediated mast cell degranulation, with resultant histamine release that causes local tissue swelling[2]. Sensitization, through prior exposure to the allergen, is usual. Swelling in allergic angioedema can occur throughout the body and is typically associated with urticaria and pruritus. Ingested allergens may cause angioedema symptoms that include abdominal pain and vomiting. Most episodes of allergic angioedema resolve 1 to 3 d after ceasing contact with the allergen[2].

A variety of medications can induce a non-IgE-mediated form of angioedema, including ACE-Is, NSAIDs, and rarely, angiotensin-2-receptor blockers (ARBs).

ACE-I-induced angioedema occurs in 0.1% to 2.2% of patients receiving these drugs[6,7]. It manifests within the first month of treatment in one quarter of patients taking ACE-Is, but delay of onset as long as 10 years has been reported[8]. Bradykinin is converted by ACE into inactive metabolites. Thus, ACE-Is inhibit the degradation of bradykinin, causing it to accumulate[9]. This accumulation causes angioedema *via* bradykinin-induced vasodilation, increased capillary permeability, and plasma extravasation[8,10]. ACE-I-induced angioedema primarily affects the head and neck (especially the lips and tongue), and is more common in women and people of African descent[8]. However, there have been case reports of abdominal visceral involvement with ACE-I-induced angioedema presenting with abdominal pain as the only symptom[11]. ACE-Is should always be considered in the differential diagnosis of unexplained abdominal pain. Although switching patients to ARBs is safe in most patients with ACE-I-induced angioedema, continued bouts of angioedema have been reported in some patients after switching to ARBs[12]. Observational data suggest that the combined use of ACE-I and ARBs may be more likely to result in angioedema[13].

NSAID-induced angioedema is present in 0.1% to 0.3% of patients receiving NSAIDs[14,15]. This is a class-specific reaction mediated by inhibition of cyclooxygenase (COX)-1, which results in the over-production of a variety of vasoactive substances, including cysteinyl leukotrienes. It is often characterized by periorbital swelling and occurs in combination with respiratory symptoms in one third of patients[14]. Observational data suggest that the combined use of ACE-Is and NSAIDs may also be more likely to result in angioedema adverse effects[13].

HAE occurs in 1:10 000 to 1:50 000 people and results from mutations in the C1 esterase inhibitor (*C1 INH)* gene[16]. Type 1 HAE is caused by a deficiency in the amount of functional C1 INH produced, while type 2 HAE is characterized by dysfunctional C1 INH. Although primarily inherited in an autosomal dominant manner, HAE appears *de novo* in one quarter of patients

due to new mutations. C1 INH plays an important role in complement, contact, and fibrinolytic pathways, which have been described in other literature[17]. The end result of quantitative or functional C1 INH deficiency is massive bradykinin release, which is thought to mediate many symptoms of HAE and AA[17]. Bradykinin causes edema, ascites, and swelling *via* increasing vascular permeability; congestion, hypotension, and erythema due to vasodilation; and cramps, spasms, and pain due to contraction of nonvascular smooth muscle[4].

HAE can manifest anywhere in the body, including the head and neck, extremities, GI tract, genitals, trunk, and larynx, and shows wide variability in presentation within patients and families[18,19]. Up to 80% of patients with HAE have recurrent abdominal pain, while half will have a potentially life-threatening laryngeal attack[19-22]. Such abdominal pain symptoms may occur for many years without concomitant cutaneous or respiratory symptoms[23]. HAE-mediated abdominal pain can be mistaken for other causes of abdominal pain, such as acute appendicitis[16]. Attacks are frequently accompanied by a prodromal phase. In the case of GI manifestations, nonspecific complaints of irritability, aggressiveness, fatigue, or hunger may precede an attack[3]. Intestinal wall swelling (thickening on imaging studies), ascites, and rarely, hypovolemic shock, occur due to massive fluid accumulation in the intestinal wall and lumen, and in the peritoneal cavity[23]. Attacks typically resolve over 2 to 5 d, and may be triggered by trauma, stress, medical procedures (e.g. instrumentation or surgery), estrogens, and certain medications (e.g. ACE-Is)[4].

Inherited angioedema with normal C1 INH is a rare disorder caused by a mutation in the coagulation factor Ⅻ gene, which in turn leads to increased gene expression, and consequently increased levels of bradykinin[24]. It is clinically indistinguishable from HAE, is thought to be autosomal dominant, and although occurring predominantly in women, has also been reported in men[25]. Similar to HAE, estrogen-containing birth control pills, estrogen-replacement therapy, and pregnancy may precipitate or worsen symptoms[25,26].

AA is a rare syndrome that occurs as a result of increased catabolism of C1 INH and overactivation of the classical complement pathway[27]. Although the clinical picture is identical to HAE, the underlying immunologic disturbance is not hereditary in nature, and precise mechanisms remain unclear. AA has been associated with lymphoproliferative/neoplastic disorders (type 1 AA) or autoimmunity (type 2 AA). Symptoms often localize to the abdomen and upper respiratory tract, as well as skin. Abdominal symptoms of angioedema have been described previously. Symptoms typically resolve over 2 d.

Idiopathic recurrent angioedema refers to swelling episodes that occur at least three times within a 6 to 12 mo period, have no identifiable cause, and are typically recalcitrant to treatment[1]. The mechanism is unknown, but autoimmune processes have been implicated. In most cases, swelling is accompanied by urticaria. When urticaria is present, it is accompanied by pruritus. Swelling can last from hours to days. Abdominal symptoms include pain, nausea, and vomiting.

MOLECULAR MECHANISMS AND PATHOPHYSIOLOGY OF ANGIOEDEMA TYPES

Different cells, and different cell components, play roles in the prevention or causation of various types of angioedema.

Mast cells, with cell surface IgE receptors, mediate allergic angioedema when an allergen interacts with the Fab fragment of the IgE molecule (Figure 1). Such allergen-Fab interaction results in intracellular signaling, which causes degranulation of the mast cells with release of histamine and leukotrienes, thereby resulting in angioedema symptoms.

HAE and AA occur due to the loss of inhibitory control of the contact/fibrinolytic pathway and the classical complement pathway caused by low levels or subnormal activity of C1 INH (Figure 2). Unlike allergic angioedema, these events occur in the extracellular milieu. C1 esterase inhibitor is synthesized mainly by hepatocytes and, to a lesser extent, by circulating blood monocytes. In AA, C1 INH is catabolized at a rate which is faster than the rate of synthesis, thus resulting in attacks of angioedema. HAE is caused by genetic mutations which result either in significantly low production of normal C1 INH (type 1), or production of normal or elevated quantities of a dysfunctional C1 INH which is unable to bind the usual substrates and thus is unable to inhibit activation of the contact/fibrinolytic and classical pathways (type 2)[4].

Angiotensin converting enzyme (ACE) is also known as kininase Ⅱ, because one of its main roles is to metabolize bradykinin into inactive metabolites. When ACE activity is inhibited, another enzyme - aminopeptidase P - metabolizes bradykinin, thus preventing excess accumulation. ACE-I-induced angioedema results from accumulation of bradykinin after inhibition of ACE by ACE-I drugs, and appears to occur more often among individuals who have subnormal activity of aminopeptidase P. Such subnormal aminopeptidase P activity may be genetic (caused by a mutation), or acquired (caused by another drug the patient is taking)[28].

Inherited angioedema with normal C1 INH results from missense mutations in the factor Ⅻ gene, which confer a significant increase in the protease activity of transcribed factor Ⅻ, resulting in increased bradykinin production[29]. Some of these patients also appear to have concomitant mutations in the ACE gene, which result in production of ACE with reduced ability to degrade bradykinin[30]. Angioedema attacks are therefore more common in patients with these mutations due to increased production, and decreased metabolism, of bradykinin.

NSAID-induced angioedema results from the inhibition of COX-1, which results in the channeling of larger quantities of arachidonic acid into the lipoxygenase pathway, resulting in the increased production of vasoactive leukotrienes (Figure 3).

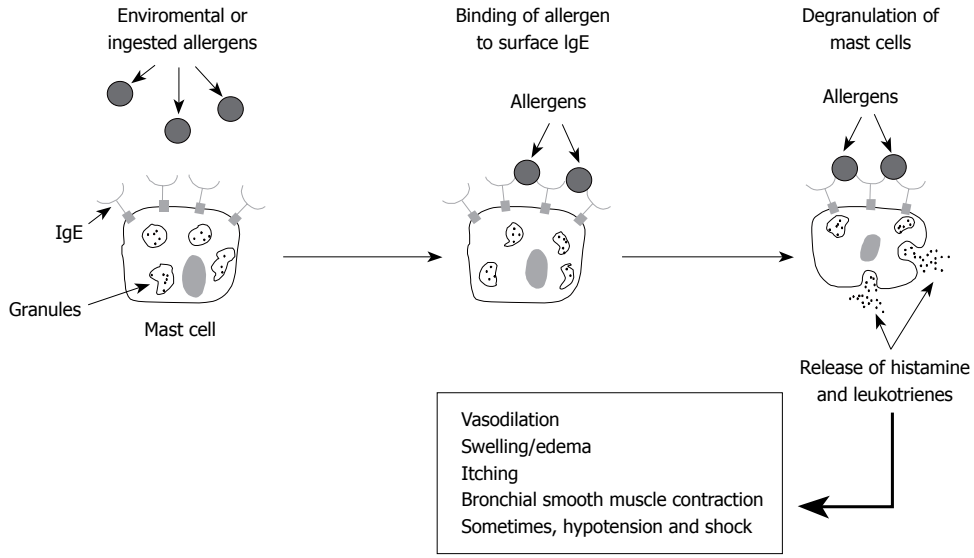

Figure 1 Allergic angioedema pathway.

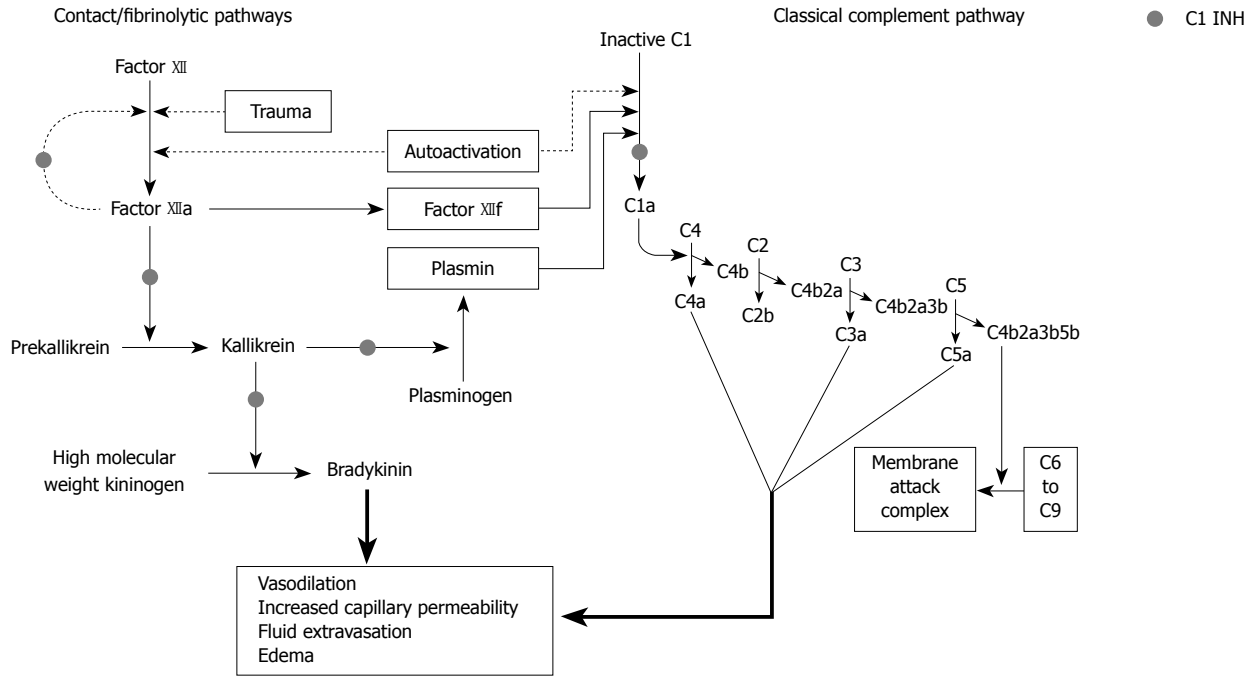

Figure 2 Pathways Involved in hereditary angioedema and acquired angioedema. C1 INH: C1 esterase inhibitor.

DIAGNOSIS

For the practicing gastroenterologist, several patients with abdominal involvement by angioedema will present as referrals for evaluation at a time when the acute episode has subsided, or during a subacute episode of abdominal pain. In such cases, identification of angioedema triggers (e.g. medications, allergens, trauma, or disease) and careful consideration of patient and family history may provide clues. The absence of a family or prior personal history of angioedema should not preclude consideration of this diagnosis.

A thorough history of patient illness, family history, and review of current and recent medications will raise suspicion of the diagnosis in appropriate circumstances. Physical examination, cross-sectional imaging studies of the abdomen, and relevant laboratory tests will often confirm the diagnosis during the acute episode. The physical examination may reveal characteristics of cutaneous angioedema (urticaria, pruritis, cutaneous swelling). For episodes with abdominal involvement, palpation may reveal diffuse abdominal tenderness, with or without rebound[23]. Bowel sounds may be hypoactive or hyperactive[20]. Shifting dullness may be present.

Contrast-enhanced abdominal computed tomography (CT) scan may show intestinal wall and mucosal thickening consistent with edema, fluid accumulation in dilated small or large bowel loops, and ascites[23]. Plain

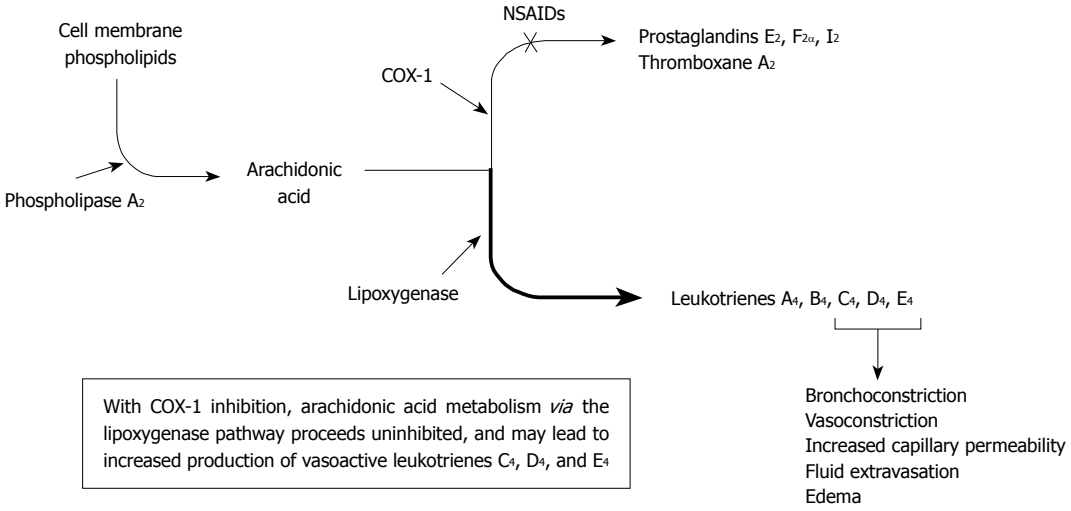

Figure 3 Non-steroidal anti-inflammatory drug-induced angioedema pathway. NSAIDs: Non-steroidal anti-inflammatory drugs; COX-1: Cyclooxygenase-1.

Table 2 Distinguishing features of angioedemas[8,27,28,33]

	HAE	Inherited	AA	Idiopathic	ACE-I	NSAID	Allergic
Location	Anywhere	Anywhere	Anywhere	Especially lips and face	Especially lips, tongue, intestines	Especially face	Anywhere
Urticaria	No	No	No	Usually	Rare	Usually	Usually
Family history	Yes	Yes	No	No	No	No	No
Age of onset	6-20 yr	6-20 yr	> 40 yr	Any age	Any age	Any age	Any age
Trauma as trigger	Yes	Yes	Yes	No	No	No	No
C1q	Normal	Normal	Low (type 1) Low/normal (type 2)	Normal	Normal	Normal	Normal
C1 INH levels	Low (type 1) Normal (type 2)	Normal	Low (type 1) Low/normal (type 2)	Normal	Normal	Normal	Normal
C1 INH function	Low (type 1) Low (type 2)	Normal	Low	Normal	Normal	Normal	Normal
C4	Low	Normal	Low	Normal	Normal	Normal	Normal
C3	Normal	Normal	Low/normal	Normal	Normal	Normal	Normal

HAE: Hereditary angioedema; AA: acquired angioedema; ACE-I: Angiotensin converting enzyme inhibitor; NSAID: Non-steroidal anti-inflammatory drug; C1 INH: C1 esterase inhibitor.

abdominal X-ray may show various degrees of obstruction with or without air-fluid levels, thumb printing, and dilated intestinal loops. Abdominal ultrasonography may detect ascites and edematous viscera. These findings are visible only during acute angioedema attacks and are fully reversible.

Endoscopy of the GI tract or oropharynx is not recommended for patients with suspected HAE due to the risk of inducing a potentially life-threatening laryngeal attack. In cases where endoscopy must be used for additional clinical reasons, prophylactic measures to protect against the possibility of laryngeal swelling should be initiated. Diffuse mucosal edema and erythema, and bulging masses of gastric mucosa (resembling a submucosal tumor), have been reported upon upper GI endoscopy in patients with HAE and abdominal attacks[31].

Laboratory testing should be conducted for patients suspected of angioedema to aid in differential diagnosis. Elevated serum tryptase and urine histamine levels can detect IgE-mediated angioedema, and allergy testing can be used to help identify the source of the offending allergen[32]. Assessment of complement markers provides useful information for helping delineate between acquired and hereditary forms of angioedema. Patient characteristics and test results can help differentiate between types of angioedema, as shown in Table 2.

MANAGEMENT

The various types of angioedema have symptoms that overlap with several other acute and subacute conditions, and as noted above, can occasionally present atypically. As such, the diagnosis may be obscured. Isolated abdominal pain from angioedema, without associated skin or respiratory system symptoms, may lead to wrong diagnoses or unnecessary interventions. In one study, misdiagnosis of abdominal symptoms led to unnecessary appendectomy, laparotomy, or both, in 35% of patients[20]. Such inappropriate treatment may be attributed to the multiple interpretations that can be made of GI findings. For example,

Table 3 Clinical studies of agents used in the treatment of hereditary angioedema

	Trial design	Primary efficacy outcome result	AE	Other safety notes
Routine prophylaxis				
CINRYZE[34] C1 inhibitor (human) (1000 units every 3-4 d for 12 wk) Ⅳ	Randomized, double-blind, placebo-controlled, cross-over study (n = 24)	Decreased the number of attacks (mean 12.7 for placebo *vs* 6.1, P < 0.0001)	Sinusitis, rash, headache, upper respiratory tract infection	Precautions: hypersensitivity reactions, thrombotic events, and risk of transmission of infectious agents
DANOCRINE[35,36] Danazol (range, 40-1000 mg, mean: 171.2 mg/d) oral route	Retrospective (n = 118)	Decreased the number of attacks (33.3 per year when untreated *vs* 9.7 per year when treated)	Clinical: weight gain, menstrual irregularities, virilization in women, headache Laboratory: elevated liver enzymes, elevated cholesterol	8 patients with long-term therapy had serious adverse events (i.e. myocardial infarction, stroke, deep vein thrombosis, acute pancreatitis, hepatocellular adenoma) Warnings: use in pregnancy is contraindicated. Thrombotic events, peliosis hepatis, benign hepatic adenoma, and intracranial hypertension have been reported
Acute attacks				
BERINERT[37] C1 esterase inhibitor (human) (10 or 20 units/kg body weight) Ⅳ	Randomized, double-blind, placebo-controlled study (n = 124)	20 mg/kg dose: reduction in time to onset of symptom relief (> 4 h for placebo *vs* 50 min, P = 0.0016)	Headache, nausea, abdominal pain, dysgeusia, vomiting	Treatment-emergent AEs: laryngeal edema, facial attack with laryngeal edema, swelling (shoulder and chest) exacerbation of HAE, and laryngospasm Precautions: hypersensitivity reactions, thrombotic events, and risk of transmission of infectious agents
FIRAZYR[38,39] Icatibant (30 mg) SQ	Randomized, double-blind, comparator-group study [n = 56 (placebo comparator study)] [n = 74 (tranexamic acid comparator study)]	Decreased time to onset of symptom relief (4.6 h placebo *vs* 2.5 h, P = 0.142; 12 h tranexamic acid *vs* 2 h, P < 0.001)	Injection site reactions; common events include recurrent attacks, nausea, abdominal pain, headache, asthenia, rash	Precautions: caution should be used in patients with acute ischemic heart disease or unstable angina pectoris, and in patients in the weeks following a stroke
KALBITOR[40] Ecallantide (30 mg) SQ	Randomized, double-blind, placebo-controlled trial (n = 96)	Decreased symptom severity measured by Mean Symptom Complex Severity scores (-0.4 placebo *vs* -0.8, P = 0.010)	Headache, nausea, diarrhea, pyrexia, injection site reactions, nasopharyngitis	Warnings: hypersensitivity reactions, including anaphylaxis
KALBITOR[40] Ecallantide (30 mg) SQ	Randomized, double-blind, placebo-controlled trial (n = 72)	Improved symptom response to treatment measured by Treatment Outcome Scores (36 placebo *vs* 63, P = 0.045)		

AE: Adverse events; HAE: Hereditary angioedema; SQ: By subcutaneous route.

abdominal pain with intestinal wall edema on CT scan can be misinterpreted as ischemia and result in unnecessary laparotomy. Abdominal pain with intestinal obstruction in cases of severe edema may be interpreted as an "acute abdomen", resulting in unnecessary laparotomy. Abdominal pain with gallbladder distension may be interpreted as biliary colic, and result in cholecystectomy. Abdominal pain with transient ascites on CT scan or ultrasonography may misdirect the focus to the liver as the cause of abdominal symptoms. Surgical exploration of abdominal symptoms should be avoided in the absence of signs of acute abdomen (i.e. absence of fever, leukocytosis, or peritoneal signs; presence of bowel sounds)[23]. Alternate diagnoses for recurrent abdominal symptoms should be considered to avoid unnecessary medical procedures.

Without intervention, angioedema attacks typically last from 1 to 5 d, depending on the underlying cause. However, acute treatment is often necessary to prevent a fatal outcome when the respiratory system is involved, or to alleviate pain when the abdominal viscera are involved.

Acute treatment of angioedema varies by type. Airway integrity is the first priority. Establishing intravenous access early and initiating appropriate intravenous hydration is also essential. For acute allergic angioedema, adrenaline by the intramuscular or subcutaneous route, and intravenous diphenhydramine, will help reduce edema. Hydrocortisone or methylprednisolone can reduce the risk of relapse[32]. Allergen avoidance is the best prophylaxis for allergic angioedema[32]. The same principles are true for acute treatment of medication-induced angioedema. Clinicians need to consider the short-term risk of relapse despite the discontinuation of the culprit medication. Hospital admission may be necessary to ensure close observation following a medication-induced attack.

Table 3 reviews clinical trial data of agents for the treatment of patients with HAE. For HAE, C1 INH concentrate has shown efficacy in aborting acute attacks of angioedema and has been used in this capacity in Europe for over 20 years. In the emergency room or critical care setting, administration of C1 INH may be a necessary ur-

gent intervention in a patient with severe oropharyngeal, respiratory or abdominal symptoms who has a known diagnosis of HAE. Antihistamines, glucocorticoids or epinephrine typically do not improve acute HAE exacerbations[5]. Fresh frozen plasma (FFP) has also shown efficacy in aborting acute attacks of HAE, with only mild and transient adverse events[41]. However, because FFP contains contact-system proteins that could contribute to increased bradykinin production, there is a small risk of exacerbating an attack[18]. Although long available in Europe, the plasma-derived C1 INH, BERINERT® P [C1 esterase inhibitor (human); CSL Behring GmbH, Marburg, Germany] was approved by the United States Food and Drug Administration (FDA) in October 2009 [under the name BERINERT®, C1 esterase inhibitor (human); CSL Behring LLC, Kankakee, IL] for the treatment of acute abdominal and facial attacks of HAE in adolescents and adults in the United States[37]. Patients who received the recommended dose experienced a significantly shorter time to symptom relief compared with patients given placebo. Common potential side effects of BERINERT include subsequent HAE attack, headache, abdominal pain, nausea, muscle spasms, pain, diarrhea, and vomiting[37]. Rarely, a paradoxical increase in severity of pain associated with HAE may occur with BERINERT treatment[37].

Two agents that work using different mechanisms have recently become available for treating acute attacks of HAE. Icatibant (FIRAZYR®; Jerini AG, Berlin, Germany) is a bradykinin-2 receptor antagonist that was approved by the European Commission in July 2008 for the treatment of acute attacks of HAE in adults[38]. In December 2009, the kallikrein inhibitor, ecallantide (KALBITOR®; Dyax Corporation, Cambridge, MA), was approved by the FDA for the treatment of acute attacks of HAE in patients aged 16 and older[40]. A black box warning notes that ecallantide should only be administered by a healthcare professional with appropriate medical support due to a risk of anaphylaxis after administration of ecallantide.

In Europe, the C1 esterase inhibitor, CETOR® (Sanquin; Amsterdam, Netherlands) is approved for the treatment of acute attacks of AA[42]. No treatments are approved for acute attacks of AA in the United States. Acute treatment has typically paralleled treatment of acute attacks of HAE, with the use of FFP or C1 INH concentrate. Although patients with AA generally require higher doses of C1 INH, treatment of the underlying malignancy or lymphoproliferative disorder will best prevent recurrent symptoms and laboratory abnormalities. Additionally, plasmapheresis is sometimes necessary to decrease autoantibody levels in AA type 2.

ATTACK PROPHYLAXIS

Although patient safety and symptom resolution are the primary goals of acute angioedema treatment, routine and pre-procedure prophylaxis focus on preventing acute events and are particularly appropriate in HAE, AA, and idiopathic recurrent angioedema. For patients with idiopathic recurrent angioedema, routine prophylaxis includes avoidance of provoking factors and low sedation antihistamines, supplemented as necessary with sedating antihistamines at night[1].

In HAE and AA, pre-procedure prophylaxis increases the quantity of circulating C1 INH prior to invasive medical or surgical procedures, so as to reduce the risk of developing life-threatening acute angioedema during the peri-operative period. Thus, an accurate diagnosis of angioedema type is central to developing an appropriate long-term management strategy. Once a gastroenterologist has reached a diagnosis of HAE or AA, a physician specializing in allergy and immunology should be consulted to guide further management.

Attenuated androgens (e.g. danazol and stanozolol), C1 INH, and the antifibrinolytic, tranexamic acid, are used in Europe for prophylaxis[43]. In the United States, the nanofiltered C1 INH concentrate, CINRYZE™ [C1 esterase inhibitor (human); ViroPharma Incorporated, Exton, PA; approved by the FDA in October 2008 for intravenous administration] and oral attenuated androgens (e.g. danazol), are the only FDA-approved treatments for routine prophylaxis of HAE attacks[34,35]. Antifibrinolytics (e.g. tranexamic acid) have also been shown to be effective. However, the nanofiltered C1 INH concentrate has become the preferred therapy for attack prophylaxis due to the significant risk for adverse events associated with attenuated androgens and antifibrinolytics[5]. Biweekly administration of CINRYZE (every 3-4 d) has been shown to reduce angioedema attack rate, attack severity, and time to symptom resolution in patients with HAE[34]. Common potential side effects include upper respiratory infections, sinusitis, skin rash, and headache[34]. CINRYZE is approved for patient self-administration.

Prior to any surgical or dental procedure, or any invasive procedure associated with tissue trauma, patients with HAE should receive prophylaxis with either C1 INH concentrate 1 h prior to surgery with subsequent doses available, or oral attenuated androgens starting 5 d before and 2 d after the procedure, or FFP at least 1 h before surgery[16,28]. These measures will help prevent an acute HAE attack during the peri-operative period.

Since C1 INH is purified from human plasma, there exists a theoretical risk of transmission of viral infections or Creutzfeldt-Jakob agent[34,37]. To reduce the risk of viral transmission, both C1 INH products referenced in this paper start with plasma donor screening for human immunodeficiency virus, hepatitis B, and hepatitis C viruses. Further steps within the manufacturing process are designed to reduce the risk of viral transmission, with both products ultimately using different processes to this end. For CINRYZE, no transmission of disease has been reported[34]. For BERINERT, a few suspected cases of viral transmission have been reported[37]. C1 INH has been associated with a risk of thrombosis if used off-label at high doses[34,37]. Severe hypersensitivity reactions may rarely occur, and since such reactions are clinically indistinguishable from an acute HAE attack, epinephrine injection should

Table 4 Food and Drug Administration approved drugs for prophylaxis and treatment of hereditary angioedema attacks[18,34,35,37,38,40]

Drug	FDA approved indication	Usual adult dose	Range
Cinryze	Prophylaxis	1000 units IV	Every 3rd or 4th day
Danazol	Prophylaxis	200 mg/d	100 mg every 3rd day to 600 mg/d
Berinert	Acute attacks	20 units/kg body weight IV	Per attack
Icatibant	Acute attacks	30 mg SQ	Per attack
Ecallantide	Acute attacks	30 mg SQ	Per attack

FDA: Food and Drug Administration.

be available at all times during administration of C1 INH, for use if necessary[34,37]. Table 4 summarizes the FDA approved drugs for prophylaxis and treatment of HAE.

CONCLUSION

Patients experiencing angioedema with abdominal involvement often present to the gastroenterologist with a history of recurrent episodes of abdominal pain. A careful history and physical examination, imaging studies, laboratory assessments, and awareness of angioedema types can help the clinician order appropriate tests to explore the differential diagnosis. Suspicion may be raised from the patient or family history, but the physical examination, imaging studies, and laboratory tests may confirm a diagnosis. Several reports have shown that abdominal pain can sometimes be the only manifestation of HAE or drug-induced angioedema. Therefore, these differential diagnoses need to be considered even in the absence of a history of prior cutaneous, oropharyngeal or respiratory symptoms, because recurrent abdominal symptoms may predate these other presentations by several years. Once a correct diagnosis has been made, appropriate treatments can be considered.

It is this author's opinion that patients receiving ACE-Is who present with unexplained recurrent or chronic abdominal pain should be tested for HAE. However, since these drugs can have abdominal pain adverse effects independent of a diagnosis of HAE, *via* a pathway leading to excessive bradykinin concentration, negative tests for HAE should not completely remove these drugs from consideration as a cause of the patient's symptoms. Furthermore, the absence of classical intestinal thickening on imaging studies such as CT scan should not automatically absolve this drug class of blame in cases of unexplained abdominal pain, without a brief trial of drug withdrawal to see if symptoms improve.

If a diagnosis of HAE is made, appropriate immediate and short-term treatment with one of the approved agents should be undertaken. In addition, a plan for long-term management should be discussed with the patient and their family. Appropriate testing of family members, to identify those at risk, should be offered.

A multi-disciplinary approach to management of angioedema is necessary. A specialist in allergy and immunology should be engaged to guide additional evaluation and to provide the patient with long-term management.

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ORIGINAL ARTICLE

Anti-inflammatory effects of *Mangifera indica* L. extract in a model of colitis

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Abstract

AIM: To investigate the effect of aqueous extract from *Mangifera indica* L. (MIE) on dextran sulfate sodium (DSS)-induced colitis in rats.

METHODS: MIE (150 mg/kg) was administered in two different protocols: (1) rectally, over 7 d at the same time as DSS administration; and (2) once daily over 14 d (by oral gavage, 7 d before starting DSS, and rectally for 7 d during DSS administration). General observations of clinical signs were performed. Anti-inflammatory activity of MIE was assessed by myeloperoxidase (MPO) activity. Colonic lipid peroxidation was determined by measuring the levels of thiobarbituric acid reactive substances (TBARS). Reduced glutathione (GSH) levels, expression of inflammatory related mediators [inducible isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, respectively] and cytokines [tumor necrosis factor (TNF)-α and TNF receptors 1 and 2] in colonic tissue were also assessed. Interleukin (IL)-6 and TNF-α serum levels were also measured.

RESULTS: The results demonstrated that MIE has anti-inflammatory properties by improvement of clinical signs, reduction of ulceration and reduced MPO activity when administered before DSS. In addition, administration of MIE for 14 d resulted in an increase in GSH and reduction of TBARS levels and iNOS, COX-2, TNF-α and TNF R-2 expression in colonic tissue, and a decrease in IL-6 and TNF-α serum levels.

CONCLUSION: MIE has anti-inflammatory activity in a DSS-induced rat colitis model and preventive administration (prior to DSS) seems to be a more effective protocol.

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Key words: Oxidative stress; Ulcerative colitis; Inflammation; Polyphenols; *Mangifera indica*; Antioxidants

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease that is characterized by bloody diarrhea, colonic mucosal ulceration and, in severe cases, systemic symptoms. An abnormal immune response against antigens of the colonic microbiota in genetically predisposed individuals is suggested to be involved in the etiology of UC[1]. Several authors have proposed that such intestinal conditions are mediated by the activation of lymphocytes and non-lymphoid cells such as macrophages and neutrophils. Once a large number of neutrophils and macrophages are activated, these cells enter the injured mucosa of the large intestine, which leads to over-production of oxygen free radicals that can cause injury to target cells in inflamed tissue[2]. Many animal models have been designed to study pathogenic events during colitis development. The symptoms and colonic histopathology of the rodent colitis model induced by dextran sulfate sodium (DSS) salt resemble more human UC than other chemically induced colitis, and has become a research model for the pathogenesis of UC and for the development of new drugs[3].

In spite of several pharmacological treatments for UC, new therapies must be developed to increase the number and duration of remissions. In this regard, traditional medicine worldwide is nowadays being re-evaluated by extensive research on different plants and their therapeutic principles. Many plants produce antioxidant compounds to control the oxidative stress caused by sunbeams and oxygen, and represent a source of new compounds with antioxidant activity[4]. An aqueous stem bark extract from *Mangifera indica* (*M. indica*) L. (Anacardiaceae family) has been traditionally used as a nutritional supplement. The composition is a defined mixture of polyphenols, flavonoids, triterpenoids, steroids, phytosterols, fatty acids and microelements (mainly zinc, copper and selenium)[5]. The extract has been described as an antioxidant with anti-inflammatory and immunomodulatory activities in several experimental settings[6-8]. In addition, some experimental models have demonstrated that *M. indica* extract (MIE) improves its effects when it is given on various days before the induction of damage[9]. For example, when administered orally 1 h before lipopolysaccharide (LPS), MIE inhibited LPS-induced tumor necrosis factor (TNF)-α production in mice dose-dependently with ED_{50} 64.5 mg/kg. However, the extract inhibited the TNF serum levels but with ED_{50} 37.4 mg/kg when it was administered orally during 7 d before LPS challenge. The increasing evidence related to the positive effects of natural compounds with antioxidant and anti-inflammatory properties on UC prompted us to investigate whether MIE could protect colonic mucosa of rats from damage induced by oral administration of DSS, using two different treatment protocols, and to elucidate the possible mechanism(s) involved.

MATERIALS AND METHODS

Materials

Twenty-eight male outbred Wistar Hannover rats (HsdRccHan:Wist, from Harlan Spain), initially weighing 190-200 g, were housed five per cage and maintained in an animal holding room controlled at a constant temperature of 24 ± 2℃, with a relative humidity of 70% ± 5% and a 12-h light/dark cycle. Animals were fed a standard pellet chow with free access to fresh tap water. All experimental protocols followed the guidelines of the Animal Welfare Committee of the Universidad Complutense according to European legislation (2003/65/EC). Chemicals were from Sigma (Spain) or as indicated.

Extract preparation

M. indica L. was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code: 41722) were deposited at Herbarium of the Academy of Sciences, Institute of Ecology and Systematics, Ministry of Science, Technology and Environment, La Habana, Cuba. Stem bark extract was concentrated by evaporation and spray-dried to obtain a fine brown powder, which is used as the standardized active ingredient of MIE formulations. It melts at 210-215℃ with decomposition. The chemical composition of MIE has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry, nuclear magnetic resonance (NMR), and UV-V spectrophotometry (fully described in[5]). The elemental inorganic composition has been determined by inductively coupled plasma spectrometry[6]. Extracts were prepared by suspending powder in 0.5% carboxymethylcellulose for oral administration and in melted suppository vehicle (Witepsol H15; Sasol, Witten, Germany) for rectal administration.

Colitis model

The experiment lasted for 21 d. The rats were randomly divided into four groups (Table 1). Control, A and B groups received vehicle orally during 2 wk. Group C received MIE (150 mg/kg) orally once daily. At day 15, oral administration was stopped and colitis was induced by 4% DSS (MP Biomedicals) in drinking water during 7 d for groups A, B and C. The control group received water. At the same time, groups B and C were co-treated rectally with extract at an equal dose while the controls and group A received vehicle rectally.

Macroscopic assessments, including weight changes, visible fecal blood and stool consistency were determined. The severity of diarrhea was evaluated according to the following score: no diarrhea = 0; mild diarrhea = 2; severe watery diarrhea = 3; and severe watery diarrhea with blood = 4[10]. Seven days after DSS (or 21 d from the onset of the study), animals were sacrificed after terminal anesthesia with sodium pentobarbital, and the entire colon

Table 1 Experimental design of dextran sulfate sodium-induced colitis model

Group	Treatment regimens			*n*
	Week 1	Week 2	Week 3	
Control	Vehicle *po*	Vehicle *po*	DSS no + vehicle	4
A	Vehicle *po*	Vehicle *po*	DSS yes + vehicle	8
B	Vehicle *po*	Vehicle *po*	DSS yes + MIE 150 mg/kg rectal	8
C	MIE 150 mg/kg *po*	MIE 150 mg/kg *po*	DSS yes + MIE 150 mg/kg rectal	8

MIE: *Mangifera indica* L.; DSS: Dextran sulfate sodium.

was removed. The colon length was measured and colon samples were collected for biochemical determinations and histological assessment.

Histological assessment

Each removed colon was washed in saline solution and cut longitudinally. Distal fractions were immediately embedded in Tissue-Teck OCT (Sakura), frozen and cut in transverse sections (7 μm) in a microtome cryostat. Samples were mounted on glass slides, cleaned and stained with hematoxylin and eosin for histological evaluation. Each slide was coded and analyzed in a blinded fashion by two investigators who assigned to each sample a histological score based on mucosal injury, with particular attention paid to alterations of the colonic crypts and the presence of inflammation in the colon. Colonic epithelial damage was assessed as: grade 0, normal; grade 1, slight damage and a few inflammatory cells infiltrated in a small area of mucosa; grade 2, moderate damage in two or more areas of the mucosa, with slight bleeding of the submucosa and mild inflammatory infiltrate; and grade 3, severe damage of the mucosa that extended into the muscular mucosa, with loss of the epithelium, and a large inflammatory infiltrate[1].

Myeloperoxidase activity

Immediately after removal, colon samples were minced on ice and homogenized (glass/glass) in 0.5% hexadecyltrimethylammonium bromide, 0.5% Nonidet P40 (Boehringer, Mannheim, Germany) in 20 mmol/L phosphate buffer, pH 6.0. The homogenates were then centrifuged for 20 min at 12000 *g*. Tissue levels of myeloperoxidase (MPO) were determined in supernatants using hydrogen peroxide as a substrate for the enzyme. A unit of MPO activity was defined as that which converted 1 μmol hydrogen peroxide to water in 1 min at 40℃[11].

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde (MDA) following a previously described method[12] with some modifications. Colonic samples were homogenized (glass/glass) in 10 vol 50 mmol/L phosphate buffer and deproteinized with 40 % trichloroacetic acid and 5 mol/L HCl, followed by addition of 2 % (w/v) thiobarbituric acid in 0.5 mol/L NaOH. The reaction mixture was heated in a water bath at 90℃ for 15 min and centrifuged at 12000 *g* for 20 min. The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer. The results were expressed as nmol/mg protein.

Glutathione determination

Reduced glutathione (GSH) levels were determined in accordance with a procedure described by Kamencic *et al*[13]. Frozen colonic samples were homogenized (glass/glass) in 20 vol cold 50 mmol/L Tris buffer, pH 7.4. Homogenates were centrifuged at 12000 *g* for 20 min and the supernatants were collected. The samples were then treated with monochlorobimane (mCB) 100 μmol/L and glutathione-s-transferase 1 U/mL, and were incubated at room temperature for 30 min. The GSH-mCB adducts were measured in a Labsystems Fluoroskan reader with excitation at 380 nm and emission measured at 470 nm. Concentration of GSH in samples were calculated by standard curve of GSH and expressed as μg/mg protein.

Western blotting analysis

To determine the levels of inducible nitric oxide synthase (iNOS), inducible cyclooxygenase (COX)-2, TNF-α and its receptors TNF-R1, and TNF-R2, tissues were homogenized at 4℃ in 5 vol buffer that contained 320 mmol/L sucrose, 1 mmol/L, DL-dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 50 nmol/L Tris brought to pH 7.0, and supernatants after centrifugation at 12000 *g* for 20 min were used. The supernatants were diluted (Laemmli) and heated at 90℃ for 10 min. After loading (20 μg protein), proteins were sized-separated in 10% or 14% (for TNF-α analysis) SDS-PAGE (90 mV). The gels were processed against the antigens and after blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), they were incubated with specific goat polyclonal anti-rat COX-2 (1:1000), rabbit polyclonal anti-rat iNOS (1:1000), polyclonal rabbit anti-rat TNF-α (1:1000), polyclonal rabbit anti-rat TNF-R1 (1:500) and polyclonal rabbit anti-rat TNF-R2 (1:500) antibodies (all from Santa Cruz Biotechonology, Santa Cruz, CA, USA, except anti-rat TNF-α that was purchased from PeproTech EC). The correspondent peroxidase secondary antibody was used and proteins recognized by the antibody were visualized on X-ray film by chemiluminescence following the manufacturer's instructions (Amersham Ibérica, Madrid, Spain). Autoradiographs were quantified by densitometry (Software Total Lab Dynamics Ltd, Phoretix, Newcastle, UK), and several time expositions were analyzed to ensure the linearity of the band intensities.

Detection of serum TNF-α and interleukin-6

ABC-ELISAs of double antibodies sandwich were adopted for determination of the two cytokines (kits were obtained from R&D Corporation).

Statistical analysis

All results are presented as mean ± SE. Data were ana-

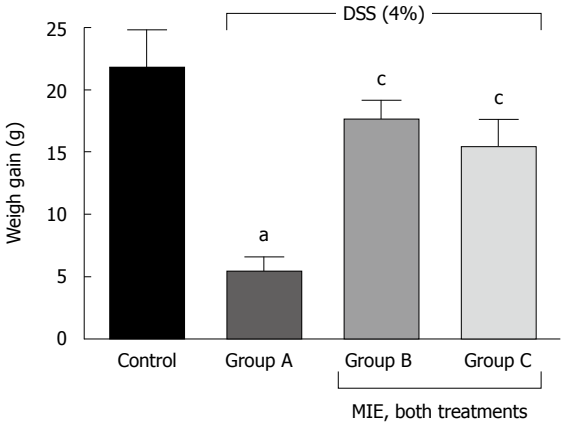

Figure 1 Effect of *Mangifera indica* L. on weight gain in dextran sulfate sodium-treated rat ulcerative colitis. Experimental colitis was induced by 4% dextran sulfate sodium (DSS) dissolved in drinking water for 7 d. Control group received common water; Group A was exposed to 4% DSS and vehicle; Group B was co-treated with rectal *Mangifera indica* L. (MIE) for 7 d, at the same time as DSS administration; Group C was treated with MIE (150 mg/kg) orally during 14 d prior to DSS administration, and co-administered rectally during DSS exposure. Each bar represents the difference between the weight at the beginning and ending of the experiment, and it was expressed as the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

lyzed using the Graph Pad Prism 4 statistical software. One-way analysis of variance followed by Newman-Keuls test were used for statistical evaluation of the parametric data. Non-parametric data were analyzed by Kruskal-Wallis one-way analysis followed by Dunn's test. $P < 0.05$ was considered as statistically significant.

RESULTS

General observations

None of the animals in the four experimental groups died throughout the experiment. The intake of drinking water in the three groups administered with DSS (A-C) decreased significantly from the beginning compared with that in the control group (data not shown). The weight gain of rats in the DSS group (A) was significantly lower than in the control group. Administration of MIE in the both pre/co-treated (C) and co-treated only (B) groups prevented this effect (Figure 1). On the other hand, all groups with DSS exhibited an increase in diarrhea and rectal bleeding from day 4 post-DSS until the end of experiment. However, in the case of group C (pre/co-treated group), diarrhea score was found to be less severe than in the DSS group (A) at days 4 and 5 post-DSS. Group B did not show any significant differences compared to the group that received DSS alone (Figure 2A). Colon length is a useful assessment of colitis and it is considered as a marker of inflammation. As shown in Figure 2B, 7 d after DSS administration, there was a significant shortening of the colon length in the group given DSS only (group A: 14.1 ± 0.1 cm) compared with the control group (17.4 ± 0.2 cm). In both pre-and co-treated groups (C and B), MIE significantly improved this inflammatory marker.

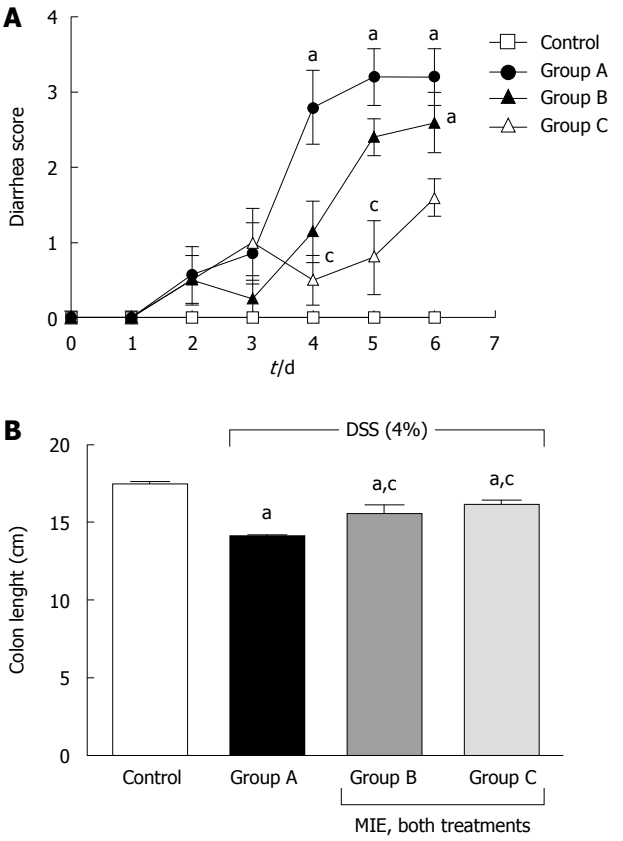

Figure 2 Effect of *Mangifera indica* L. on diarrhea and colon length in dextran sulfate sodium-treated rat ulcerative colitis. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. Changes in diarrhea score (A) and colon length (B) after 4% dextran sulfate sodium treatment in the presence or absence of *Mangifera indica* L. (MIE) are presented. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$. DSS: Dextran sulfate sodium.

Histological findings

The occurrence of UC was corroborated on the basis of histological damage and inflammatory infiltrate as shown in Figure 3. Figure 3D summarizes the microscopical damage scores from DSS rats and DSS rats treated with MIE. The control group exhibited normal mucosal morphology. Rats that received DSS and vehicle (group A) showed extensive mucosal damage with a large number of inflammatory cells, obtaining as a result, the highest score in the microscopic analysis. MIE in both treatment protocols (groups B and C) decreased the grade and number of ulcerations and diminished the inflammatory infiltrate.

Effect of MIE on MPO activity

DSS colitis was also characterized by increased MPO activity in colonic tissue, an indicator of polymorphonuclear leukocyte accumulation. The DSS group (A) showed a significant elevation of MPO levels in colonic tissue (21.1 ± 2.7 mU/mg), $P < 0.05$ *vs* the control group. The increase observed in the DSS group was clearly diminished by both treatments with MIE as shown in Figure 4A.

Effect of MIE on lipid peroxidation

The effect of MIE on lipid peroxidation - an indicator of

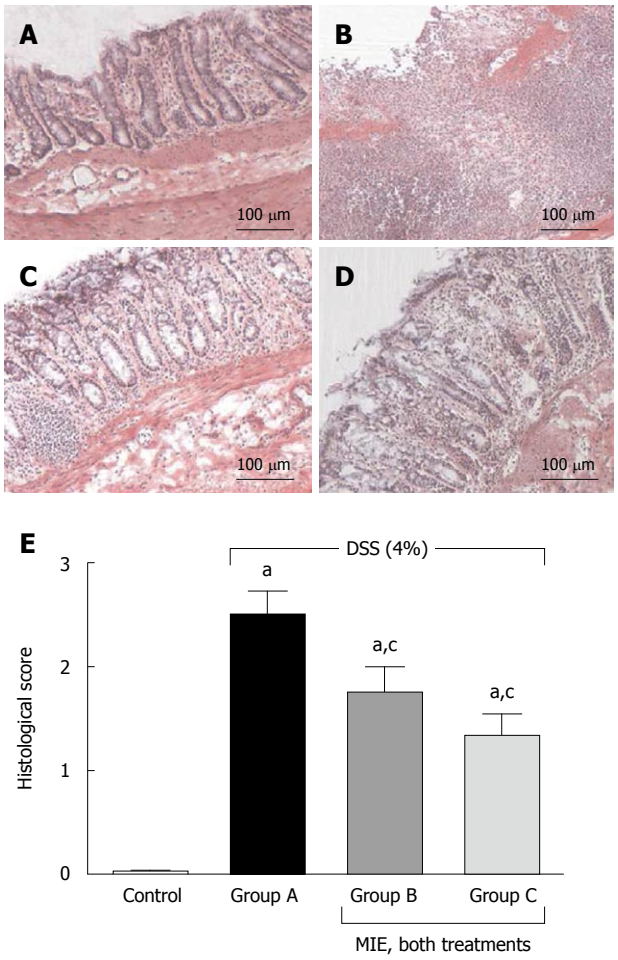

Figure 3 Hematoxylin and eosin staining of colons obtained from rats untreated or treated with dextran sulfate sodium (4%) and *Mangifera indica* L.. A: Control group: Received common water (100 ×); B: Exposed to 4% dextran sulfate sodium (DSS) and vehicle (100 ×); C: Co-treated with *Mangifera indica* L. (MIE) rectally (150 mg/kg) for 7 d, at the same time as DSS administration (100 ×); D: Treated with MIE (150 mg/kg) orally during 14 d prior to DSS administration, and co-administered rectally during DSS exposure (100 ×); E: Changes in histological score were assigned according to criteria defined in the Material and Methods. Each bar represents the mean ± SE of the different groups. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

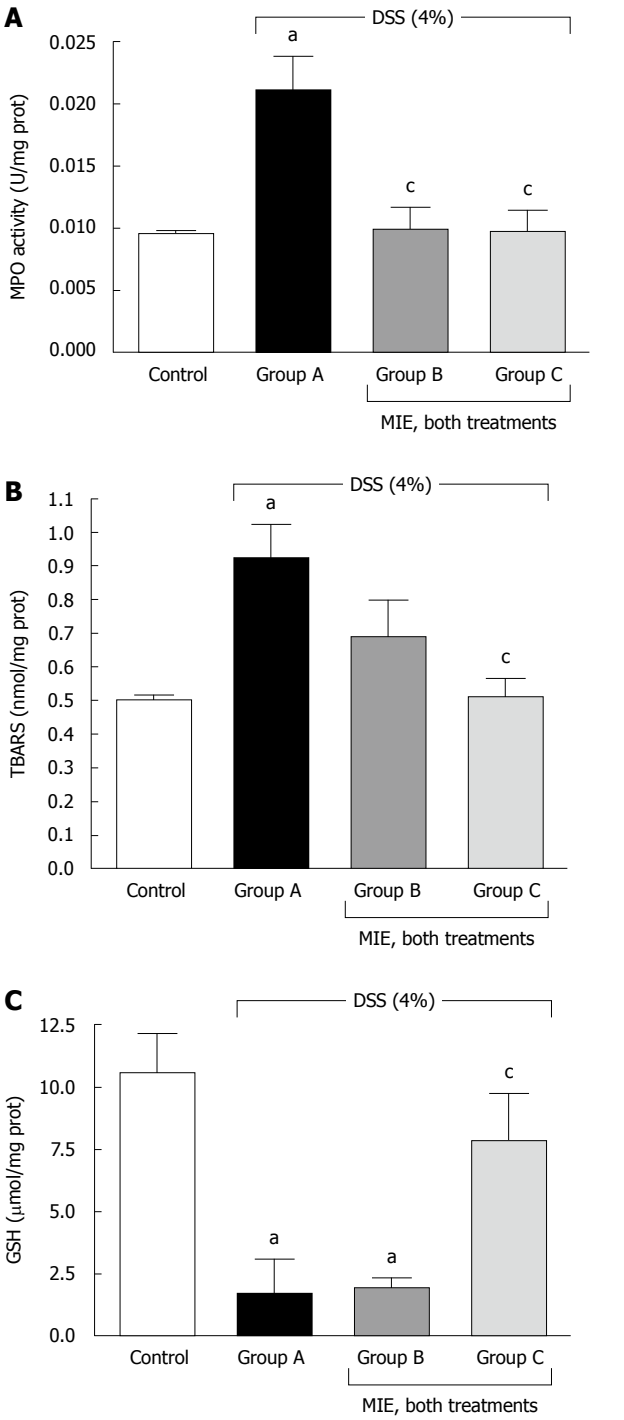

Figure 4 Effect of *Mangifera indica* L. on myeloperoxidase activity, lipid peroxidation and glutathione levels in dextran sulfate sodium-treated rat colon tissue. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. Each bar represents the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$. A: Myeloperoxidase (MPO) levels were determined; B: Lipid peroxidation was estimated according to the presence of thiobarbituric acid reactive substances (TBARS); C: Glutathione (GSH) levels were determined. MIE: *Mangifera indica* L.; DSS: Dextran sulfate sodium.

cell membrane damage as a result of oxidative toxicity - in rats treated with 4% DSS is shown in Figure 4B. In the DSS-induced colitis rats, the level of TBARS was significantly increased (0.71 ± 0.07 nmol/mg) when compared with the control group (0.41 ± 0.05 nmol/mg). Although previous administration of MIE resulted in a reduction in TBARS level (group C, 0.4 ± 0.04 nmol/mg), co-treatment with MIE (group B) did not decrease TBARS level (0.57 ± 0.16 nmol/mg) compared with that in the DSS-treated rats.

Effect of MIE on GSH levels

GSH is one of the most important endogenous antioxidants. Figure 4C shows a significant decrease of GSH in group A (1.68 ± 1.4 μg/mg) compared to the control group (10.55 ± 1.6 μg/mg). In this case, there were no significant differences between the DSS group and group B (co-treated but not pre-treated with 1.91 ± 0.4 μg/mg MIE). However, the administration of MIE prior to 4% DSS resulted in an increase in GSH level (7.80 ± 1.91 μg/mg) compared with that in the 4% DSS treatment group.

Effects of MIE on expression of iNOS and COX-2

When rats were treated with 4% DSS, the levels of inflam-

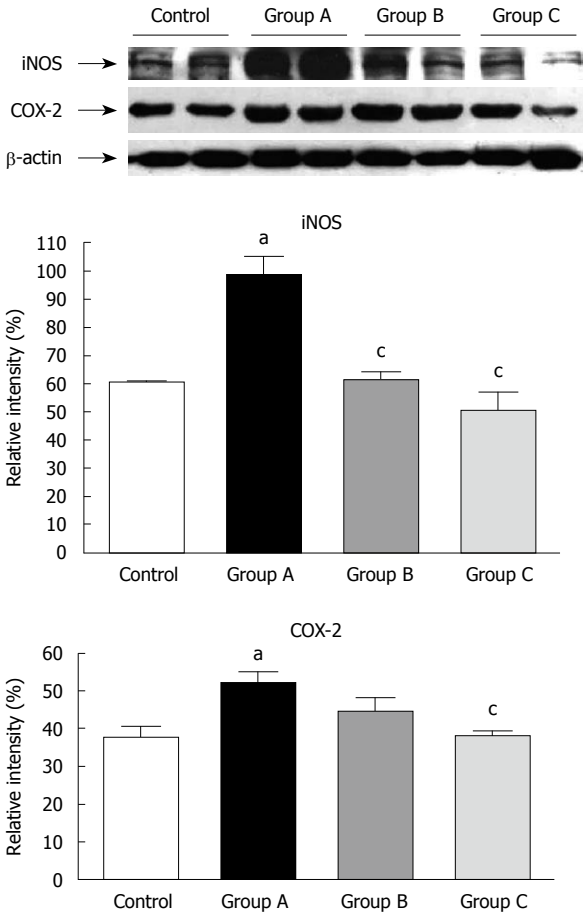

Figure 5 Effect of *Mangifera indica* L. on isoforms of nitric oxide synthase and cyclooxygenase-2 production in dextran sulfate sodium-treated rat colon tissue. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. The protein extracts were obtained as described in the Materials and Methods. β-actin was used as an internal control. Expression of isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 proteins were analyzed by Western blotting using iNOS and COX-2 polyclonal antibodies. The relative intensity was calculated using imaging software. Each bar represents the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

mation-related proteins (iNOS and COX-2) in colonic tissue were significantly increased (Figure 5). In the case of iNOS, both treatments with MIE resulted in a decrease of expression. However, for COX-2 expression, attenuation of band intensities was observed only in group C (pretreated group).

Effects of MIE on expression of TNF-α and TNF receptors

Administration of 4% DSS induced a significant increase in TNF-α (Figure 6, lanes 3-5) and TNF R-2 in inflamed tissue (Figure 6, lanes 3-6). Treatment with MIE 15 d before 4% DSS resulted in a gradual weakness of band intensities for TNF-α and TNF R-2. Relative band intensities of increased TNF-α and TNF R-2 expression caused by DSS were reduced by prior treatment with MIE (group C) in 17.8 and 22.8% respectively *vs* DSS. There were no significant differences between relative intensities in group B compared with the group treated with DSS alone. Expression of TNF R-1 was not affected by DSS supplementation.

Table 2 Serum levels of tumor necrosis factor-α and interleukin-6 in experimental groups

Group	TNF-α	IL-6
A	146.17 ± 13.1	118.15 ± 16.7
C	101.92 ± 9.3[a]	61.36 ± 18.8[a]

Results are presented as % of control group, mean ± SE of each group. [a]$P < 0.05$ ($n = 8$ in both groups). TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.

Effect of MIE on TNF-α and interleukin-6 serum levels

Based on the effects of MIE given before colitis induction (group C) on tissue cytokine expression, we tested the systemic levels of cytokines. Administration of 4% DSS produced an increase in TNF-α serum levels (46.2%), whereas interleukin (IL)-6 serum levels showed a tendency to elevation in group A (treated with DSS alone) but this was not statistically significant (18.1%). Treatment with MIE, before DSS intake, clearly decreased TNF-α levels by 44.3% and reduced IL-6 serum levels (down to control serum levels) by 58.8% (Table 2).

DISCUSSION

UC is a chronic, relapsing disease that causes inflammation and ulcerations of the colonic mucosa with a variable extent and severity. The etiology of UC remains essentially unknown but the results from many studies in humans and animal models suggest that it is related to an abnormal immune response in the gastrointestinal tract, possibly associated with genetic and environmental - mainly microbial - factors[14]. Aminosalicylates, glucocorticoids and immunosuppressive drugs have been mainly used for the treatment and maintenance of remission of UC, but the side effects or toxicity of these drugs represents a major clinical problem[15]. For these reasons, natural medicine has become an alternative therapy in addition to the conventional therapies that are used to treat UC[16].

In the present study, we demonstrated that MIE has an anti-inflammatory effect on colonic injury provoked by oral supplementation with DSS in rats, mainly when it is administered before the induction of damage. DSS-induced colitis is a well-established model that is phenotypically similar to UC in humans[17]. Oral administration of DSS for several days, leads to colonic epithelial lesions and acute inflammation characterized by the presence of neutrophils and macrophages within damaged segments. The reason for the deleterious effects of DSS is not well understood, however, epithelial cell permeability and macrophage activation have been proposed as potential mechanisms. We administered *M. indica* extract in two different protocols to evaluate the role of pretreatment with this product. The decrease in colitis induced by MIE was accompanied by a lower weight loss of rats and a partial

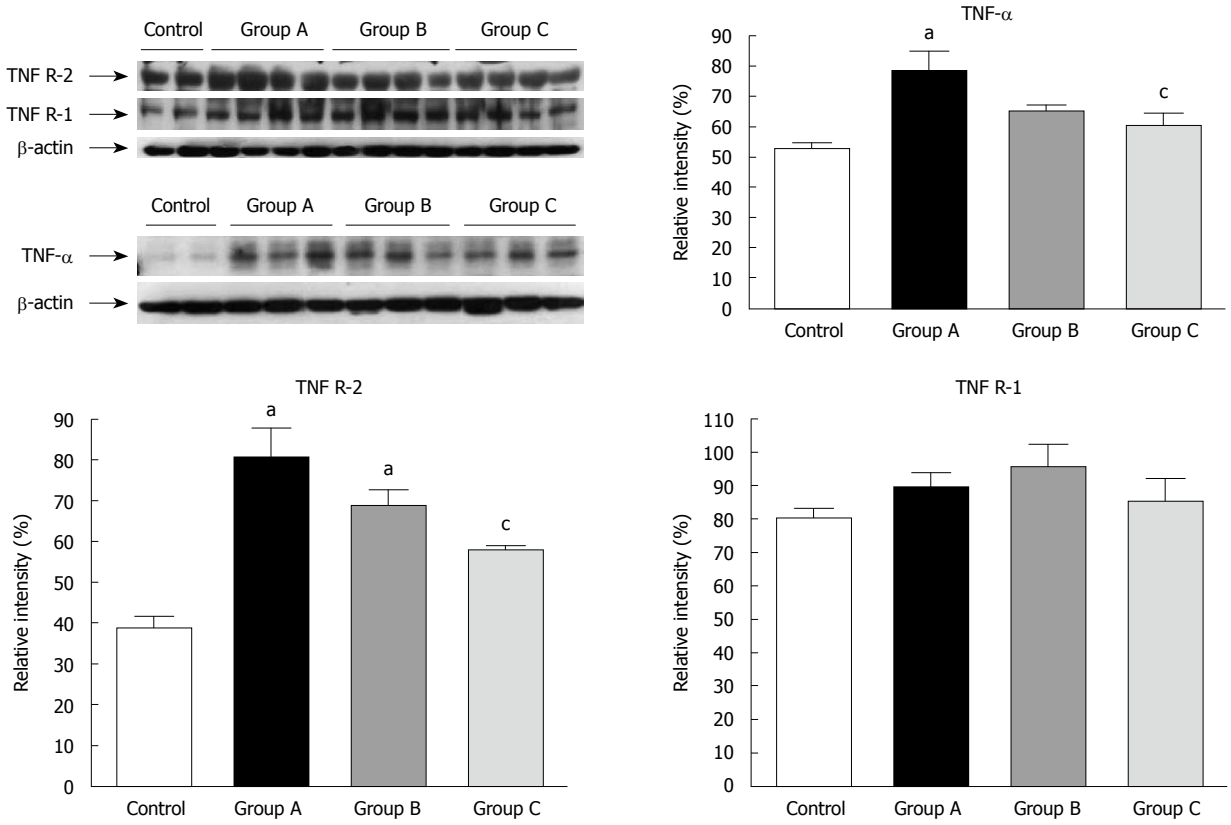

Figure 6 Effect of *Mangifera indica* L. on tumor necrosis factor-α and tumor necrosis factor receptor production in dextran sulfate sodium-treated rat colon tissue. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. The protein extracts were obtained as described in the Materials and Methods. β-actin was used as an internal control. Expression of tumor necrosis factor (TNF)-α and TNF receptor proteins was analyzed by western blotting using TNF-α, TNF R-1 and TNF R-2 polyclonal antibodies. The relative intensity was calculated using imaging software. Each bar represents the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

restoration of colon length, which is an indirect assessment of colon inflammation. However, a decrease in the occurrence of diarrhea was only observed when MIE was administered before DSS. Confirming clinical results, microscopic analysis established a protective action of MIE, which was measured as a decrease in ulceration, conservation of epithelial crypts, and a reduction in infiltrated cells. These effects were more evident in the pretreated group.

The infiltration of leukocytes into the mucosa contributes significantly to the tissue necrosis and mucosal dysfunction, as they represent a major source of reactive oxygen species (ROS)[18]. MPO is an enzyme that is found predominantly in neutrophils, and a good marker of inflammation and tissue injury. Therefore, the decrease of MPO activity can be explained through the reduction of neutrophil accumulation in inflamed tissue[19]. In addition, oxygen radicals and NO can interact and exert a cytotoxic effect by causing lipid peroxidation, which results in the formation of MDA[20]. Our results showed that MIE in both treatment protocols inhibited MPO activity, whereas the decrease in MDA production was only observed when animals received MIE before DSS administration. A decrease of MPO activity with MIE treatment in different experimental models of inflammation (ear and paw edema) has been described[21]. In addition, several studies have established the high antioxidant capacity of the extract by blocking oxygen radical formation[22,23]. The mechanism involved is associated with the antioxidant activity reported for mangiferin, which has a low redox potential that proves its ROS scavenger ability[24]. Therefore, the antioxidant capacity of the extract, administered prior to colitis development, probably leads to a decrease of lipid peroxidation and MPO activity. However, the results presented here indicated that co-treatment with MIE was not sufficient to reduce MDA levels. This could be related to the necessary oxidative pre-conditioning that has been described for many antioxidants[25]. We hypothesize that MIE could be useful in the prevention of relapse in patients with quiescent UC.

Furthermore, the increased generation of highly toxic ROS in UC exceeds the limited intestinal antioxidant defense system, thereby contributing to intestinal oxidative injury. Glutathione, as the most abundant cellular antioxidant system in animal cells, plays an essential role in modulating cell responses to redox changes[26]. GSH deficiency predisposes animals to organ failure and death after an otherwise nonlethal period of hypotension[27,28]. GSH deficiency is associated with severe injury such as inflammation and sepsis, therefore, treatment strategies that maintain GSH stores might decrease the incidence of organ failure. Our findings demonstrated that MIE administered before colitis induction produced a significant increase in GSH levels, which were probably associated with the radical scavenger capacity of the extract

and the protection of thiol groups described by numerous polyphenols[29]. Polyphenols are the main constituent of MIE (around 50%)[5].

Moreover, pathological invasion of inflammatory cells into the mucosa produces increased concentrations of inflammatory cytokines such as interleukins, TNF-α and interferon-γ[30]. Pro-inflammatory cytokines induce the expression of genes associated with inflammation, such as iNOS, and stimulate iNOS activity, which increases the production of the free radical NO[31]. Studies in knockout mice have demonstrated that iNOS plays an important role in the pathogenesis of colitis[32], and the role of iNOS in the pathogenesis of human UC has been previously suggested[33]. In the present study, MIE inhibited iNOS expression, as described in other inflammatory experimental settings[34].

In addition to iNOS, DSS-induced expression of COX-2 was also inhibited by prior administration of MIE. Previous studies in endotoxin-stimulated macrophages also have demonstrated that MIE inhibits COX-2 protein and mRNA levels, but at doses higher than those required for iNOS inhibition, which suggests that longer treatments or higher doses of MIE than those needed for inhibition of COX-2[34] are necessary. This might explain the lack of effect when the extract was administered only in the co-treatment regimen. The synthesis and activity of iNOS and COX-2 are induced by almost the same pro-inflammatory stimuli and are associated with inflammatory conditions. Therefore, it is possible that inhibition of iNOS and COX-2 induced by prior treatment with MIE could provide the most potent anti-inflammatory effect.

On the other hand, TNF-α has been described as a key molecule in UC pathogenesis, and a monoclonal antibody against this molecule, such as infliximab, has proven to be effective in the treatment of moderate to severe UC[35]. This cytokine, by interaction with its receptors I and Ⅱ, recruits leukocytes to inflammatory sites, stimulates monocytes and vascular endothelial cells to express cytokines, induces the cascade effects for other cytokines, and finally results in inflammatory lesions in tissues[36,37]. Our results demonstrated that prior administration of MIE inhibits DSS-induced increased TNF-α and TNF R-Ⅱ expression. TNF R-Ⅰ is expressed constitutively, whereas TNF R Ⅱ is induced by diverse stimuli and plays a key role in the local inflammatory response[38]. Previous *in vivo* and *in vitro* studies have appointed MIE as a potent TNF-α inhibitor[9] and some polyphenols structurally related to those present in MIE inhibit lymphocyte proliferation and cytokine production[39,40]. Moreover, the reduction of TNF R-Ⅱ receptor expression seems to enhance the inhibitory action of the extract on the TNF-α signaling system.

The reduction of inflammatory enzymes iNOS and COX-2, TNF-α and TNF R-Ⅱ expression induced by MIE can be correlated with its antioxidant properties. The effects of antioxidant agents have been ascribed by some authors to inhibition of activation of the nuclear transcription factor nuclear factor (NF)-κB, which is activated by ROS with the subsequent induction and expression of various cytokines (such as TNF-α) and enzymes (i.e. iNOS and COX-2)[41,42] that are involved in the induction and development of UC. Although *in vitro* studies have demonstrated that MIE inhibits NF-κB in macrophages[43], further research is necessary to demonstrate that MIE exerts an inhibitory effect on NF-κB signaling pathways.

In addition, TNF-α and IL-6 serum levels were determined in our study. Administration of DSS produced an increase in systemic TNF-α levels, which was reversed by prior administration of MIE. This fact is probably associated with the molecular changes found in the local inflammatory focus. Although several studies have establish an increase in IL-6 serum levels after DSS supplementation[3,44], our results demonstrated a non-significant tendency to increase IL-6 levels in serum. Nevertheless, prior administration of MIE produced a significant decrease in this cytokine. A previous study has demonstrated the ability of MIE to modulate macrophage function through inhibition of chemotaxis and phagocytosis[43]. Macrophages are one of the main sources of cytokines (i.e IL-6 and TNF-α), therefore, a possible modulation of macrophage activity by MIE could influence the decrease in cytokine production. This result suggests an important role for MIE as a modulator of the immune system and should be taken into account for future investigations.

In conclusion, the results showed that MIE administered in co-treatment regimens is able to prevent body weight loss and colon shortness, as well as modulate MPO activity and reduce iNOS expression levels. However, when MIE is administered before DSS damage, its protective effects are broader and enhanced, as demonstrated by a decrease in diarrhea and lipid peroxidation; an increase in GSH levels; a decrease in iNOS, COX-2, TNF-α and TNF R-Ⅱ expression levels, as well as a reduction in TNF-α and IL-6 serum levels.

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COMMENTS

Background

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) that is characterized by bloody diarrhea, colonic mucosal ulceration and, in severe cases, systemic symptoms. An exaggerated immune response against antigens of the colonic microbiota in genetically predisposed individuals is suggested to be involved in the etiology of UC. Current available treatment includes anti-inflammatory and immunosuppressive agents, all of which have many adverse reactions after long-term treatment to prevent remissions of the disease.

Research frontiers

New therapies must be developed to increase the number and duration of remissions. In this vein, traditional medicine around the world is now being re-evaluated by extensive research on different plants and their therapeutic principles. Many plants produce antioxidant compounds to control oxidative stress. An aqueous stem bark extract from *Mangifera indic*a L. (MIE, Anacardiaceae family), has been traditionally used as a nutritional supplement. The composition is a defined mixture of polyphenols, flavonoids, triterpenoids, steroids, phytosterols, fatty acids

and microelements (mainly zinc, copper and selenium). The extract has been described as an antioxidant with anti-inflammatory and immunomodulatory activities in several experimental settings.

Innovations and breakthroughs

MIE has an anti-inflammatory effect on colonic injury in a rat model of UC, mainly when it is administered before the induction of damage. The decrease in colitis induced by MIE was accompanied by a lower weight loss of rats and a partial restoration of colon length, which is an indirect assessment of colon inflammation. Furthermore, a decrease was also observed in occurrence of diarrhea, which is the main clinical finding. By confirming the clinical results, microscopic analysis established protective activity of MIE, as measured by a decrease in ulceration, conservation of epithelial cells, and a reduction in infiltrating cells. Finally, MIE modulated most of the inflammatory mediators in colitis: inducible nitric oxide synthase (NOS), inducible cyclooxygenase (COX), and consequent lipid peroxidation. MIE inhibited two of the main inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-6.

Applications

MIE is able to prevent body weight loss and colon shortness, as well as decrease some of the intra- and intercellular mechanisms of inflammatory damage in the colon, in an animal model of UC. In this way, this study might represent a future strategy for therapeutic intervention in the preventive management of patients with UC.

Terminology

NOS and COX are two enzymatic sources of inflammatory mediators, and their activation leads to an increase in reactive oxygen species, which can damage cells. Peroxidation of lipid components of the cell membranes is the result of this damage. Cytokines are a family of pleiotropic intercellular proteins, mainly in immunological cells, and most of them are pro-inflammatory, such as TNF-α and IL-6.

Peer review

This is a novel and interesting study that demonstrates the anti-inflammatory effects of MIE on colonic mucosa in a DSS colitis model in rats. The results are important and potentially relevant for designing therapy in IBD. The results have are well presented and support the authors' conclusions. However, the addition of another colitis model would improve the paper.

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ORIGINAL ARTICLE

HBx-induced reactive oxygen species activates hepatocellular carcinogenesis *via* dysregulation of PTEN/Akt pathway

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Abstract

AIM: To investigate the role of hepatitis B virus X-protein (HBx)-induced reactive oxygen species (ROS) on liver carcinogenesis in HBx transgenic mice and HepG2-HBx cells.

METHODS: Cell growth rate was analyzed, and through western blotting, mitogenic signaling was observed. Endogenous ROS from wild and HBx transgenic mice and HepG2-Mock and HBx cells were assayed by FACScalibur. Identification of oxidized and reduced phosphatase and tensin homolog (PTEN) was analyzed through N-ethylmaleimide alkylation, nonreducing electrophoresis.

RESULTS: We observed that the cell-proliferation-related phosphoinositide 3-kinase/Akt pathway is activated by HBx *in vivo* and *in vitro*. Increased ROS were detected by HBx. Tumor suppressor PTEN, *via* dephosphorylation of Akt, was oxidized and inactivated by increased ROS. Increased oxidized PTEN activated the mitogenic pathway through over-activated Akt. However, treatment with ROS scavenger N-acetyl cysteine can reverse PTEN to a reduced form. Endogenously produced ROS also stimulated HBx expression.

CONCLUSION: HBx induced ROS promoted Akt pathways *via* oxidized inactive PTEN. HBx and ROS maintained a positive regulatory loop, which aggravated carcinogenesis.

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Key words: Hepatitis B virus X protein; Hepatocellular carcinoma; Akt; Reactive oxygen species; Phosphatase and tensin homolog

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Among other risk factors (including alcohol abuse, cirrhosis, and aflatoxin B1), chronic hepatitis B virus (HBV) infection plays a central role in the etiology of

HCC[1]. About 53% of HCC cases are related to HBV, and the risk of HCC in chronic HBV carriers is approximately 100 times greater than in uninfected individuals[2]. Among the four proteins encoded by the HBV genome, X protein (HBx) is a multifunctional regulatory protein that is closely linked to HCC, but its role in tumor growth has not been fully clarified. Prior work from this laboratory has shown that HBx induces liver cancer in transgenic mice[3]. HBx does not bind directly to DNA, but affects transcriptional activation through interaction with nuclear transcription factors and by cytoplasmic modulation of signal transduction pathways[4]. HBx also mediates the activation of the Ras/Raf/extracellular signal-regulated kinase and mitogen-activated protein kinase kinase kinase-1/c-Jun NH_2-terminal kinase cascades, which leads to the induction of activator protein-1 and nuclear factor κB[5,6]. One of the most well-known pathways activated by HBx is phosphoinositide 3-kinase (PI3K)/Akt, which is associated with anti-apoptotic activity and cell proliferation[7-9]. Therefore, HBx is thought to be associated with the development of human HCC, but the precise function of HBx in the tumorigenic transformation of liver cells remains unclear.

Previous studies have indicated that HBx protein directly interacts with the membrane proteins of mitochondria, the major site of reactive oxygen species (ROS) production, and alters the mitochondrial membrane potential in a hepatoma cell line. HBx also increases the level of mitochondrial ROS and lipid peroxide production[10]. The results of many previous studies have shown that normal cells exposed to low levels of H_2O_2 can increase their proliferation[11]. In this context, many types of cancer cells manifest increased production of H_2O_2[12].

Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Together with tyrosine kinases, PTPs regulate the phosphorylation state of many important signaling molecules. They have been suggested to be direct targets of H_2O_2[13,14]. In general, PTPs exert an inhibitory effect on cancer signaling by opposing the tyrosine phosphorylation initiated by activated receptor kinases. Cell stimulation induces the transient activation of class-I PI3K, and the subsequent production of PI 3,4,5-trisphosphate (PIP_3) which is important for the activation of a variety of downstream signaling molecules, including the protein kinase Akt, that mediate promotion of cell proliferation and survival[15]. The reaction catalyzed by PI3K is reversed by phosphatase and tensin homolog (PTEN), which functions as a PIP_3 3-phosphatase. Indeed, by negatively modulating the PI3K signaling pathway, PTEN acts as a tumor suppressor. PTEN is also a member of the PTP family. It has been previously demonstrated that Cys-124 in the catalytic site of human PTEN is readily oxidized by exogenous H_2O_2 to form a disulfide with Cys-71[16].

In the present study, we attempted to determine the effect of HBx on the activated Akt pathways. We showed that HBx-produced H_2O_2 induces reversible inactivation of PTEN and activation of Akt. We suggest that scavenging H_2O_2 could be a therapeutic target for abnormal cell signaling to reactivate PTEN.

MATERIALS AND METHODS

Transgenic mice

The production of HBx transgenic mice used in this study has been reported previously[3]. HBx homozygous (+/+) transgenic mice were produced by mating HBx heterozygous transgenic mice with each other. To generate HBx homozygous transgenic mice on a mixed background of C57BL/6 and CBA strains, HBx homozygous mice with C57BL/6 backgrounds were crossed with CBA wild-type mice. The heterozygous transgenic offspring with a mixed background of C57BL/6 and CBA strains were cross mated. Among their offspring, HBx homozygous transgenic mice were selected by genotyping the next generation. Selected mice were then crossed up to F12, which is applicable for the study as an inbred strain with a mixed genetic background (C57BL/6 and CBA). In the current study, these F12 mice were used for *in vivo* analyses. HBx (+/+) transgenic mice were verified by polymerase chain reaction (PCR) analysis. The PCR primers used were as follows: one set was sense primer 5'-TTCTCATCTGCCGGTCCGTG-3' and antisense primer 5'-GGGTCAATGTCCATGCCCCA-3', and another set was sense primer 5'-GAAAACACACTCACTGTTCAGAG-3' and antisense primer 5'-GTAAGCCGCTTTCTCTTATGCAG-3'. The wild-type mice were derived from littermates between HBx heterozygous transgenic male and female mice, with a mixed genetic background (C57BL/6 and CBA). Mice were housed in a specific pathogen-free environment. Mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

Cell lines and cell culture conditions

HepG2-HBx cells derived from HepG2 cells were stably transfected and expressed HBx. HepG2 cells were grown in an atmosphere that contained 5% CO_2 at 37℃ in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin.

Proliferation assay

Cell proliferation was determined by the crystal violet staining method, as described previously[17].

Western blotting analysis

Proteins (20 μg/sample) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blotted at 4℃ overnight with primary antibodies. The membranes were washed five times with 10 mmol/L Tris-HCl (pH 7.5) plus 150 mmol/L NaCl (Tris-buffered saline; TBS) that contained 0.2% Tween-20, and incubated with horseradish peroxidase (HRP)-conjugated IgG. After the removal of excess antibodies by washing with TBS, specific binding was detected using a chemiluminescence detection system (Amersham, Berks, UK) according to the manufacturer's in-

structions. Mouse monoclonal antibody to PTEN was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to phospho-Akt (Ser-473), Akt, and monoclonal antibodies to cyclin D1, were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies to GAPDH were from Lab Frontier (Seoul, Korea), and HRP-conjugated goat antibodies to mouse or rabbit IgG were from Amersham and Sigma.

RNA isolation and quantitative-PCR analysis

Total RNA was isolated from the HepG2-HBx cells, or liver tissues from HBx transgenic mice, using TRIzol reagent (Invitrogen, Seoul, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final elutes was determined by nano-drop. Total RNA was converted into single-strand cDNA using a cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). Amplification of the target genes by real-time reverse transcriptase (RT)-PCR was conducted using SYBR Green (Takara, Otsu, Shiga, Japan) followed by analysis using the ExicyclerTM 96 Real-Time Quantitative Thermal block (Bioneer, Daejeon, Korea). Relative gene expression was calculated using the comparative Ct (2-ΔΔCt) method.

Identification of reduced and oxidized PTEN by immunoblot analysis

Cells were harvested, washed once with PBS, and resuspended in 0.2 mL 100 mmol/L Tris-HCl (pH 6.8) that contained 2% SDS and 40 mmol/L N-ethylmaleimide (Sigma). Protein (20 μg/sample) was loaded and subjected to SDS-PAGE under nonreducing conditions. The separated proteins were then transferred to nitrocellulose membranes and immunoblotted with a mouse anti-PTEN antibody. Binding was detected by an HRP-conjugated anti-mouse Ig (1:10 000, Sigma) and enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Isolation of primary hepatocytes

Hepatocytes were isolated using the same methods as previously reported[18].

ROS detection

Cells treated with 500 μmol/L H_2O_2 and 10 mmol/L N-acetylcysteine (NAC) were stained for 15 min with 5 μmol/L H_2O_2-sensitive fluorescent dye dichlorofluorescein diacetate (DCFDA, FL-1; Molecular Probes, Eugene, OR, USA) at 37℃ in the dark, washed three times with PBS, and subsequently assayed by FACSCalibur (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Comparisons were analyzed for statistical significance by unpaired or paired Student's *t* test using Microsoft Excel software. $P < 0.001$ was considered as significant. All data are reported as mean ± SD.

RESULTS

HBx promotes tumor formation

The HBx protein is considered to be closely associated with the development of HCC. HBx transgenic mice, previously developed in this laboratory[3], developed dysplasia around 4 wk of age, and hepatic tumors developed from 6 mo of age[19]. Several studies have shown that HBx stimulates cell proliferation and growth through the activation of signal transduction pathways such as Akt. To study the role of the HBx protein in cancer generation at the cellular level, HepG2-HBx cells were obtained by stably transfecting HepG2 cells with an HBx expression plasmid. The growth rate of the HepG2-HBx cells was approximately double that of the HepG2 control cells (Figure 1A and B). There were differences not only in cell growth, but also in morphology. HepG2-HBx cells showed aberrant actin bundling. Taken together, these results show that HBx has a role in the development of the liver tumor by activating proliferation and changing cell characteristics.

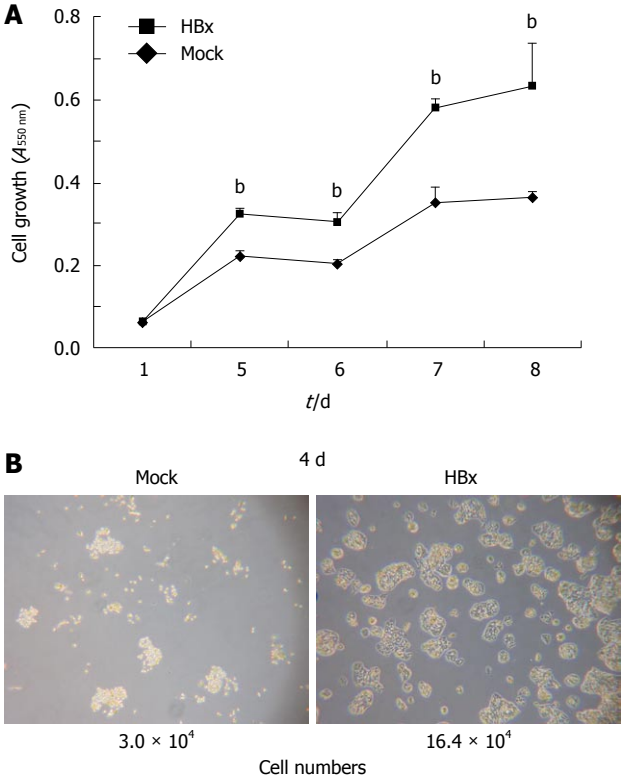

Figure 1 Effect of hepatitis B virus X-protein on induction of aberrant cell growth. A: Cell growth analysis by crystal violet staining and $A_{550\ nm}$ detection. 10^4 cells were seeded, and Stained at 1, 5, 6, 7 and 8 d after seeding. Values represent mean ± SD ($n = 3$). $^bP \leq 0.001$ compared with mock transfectants; B: Morphology of the cells was observed by optical microscopy. HBx: Hepatitis B virus X-protein.

Tumorigenesis in HBx transgenic mice and HepG2-HBx cells through activation of the Akt pathway

The PI3K/Akt signaling pathway is crucial to many aspects of cell growth and survival. To determine whether HBx-associated HCC is also accompanied by activation of the Akt pathway, lysates from the mouse liver tissue and cells transfected with HBx or an empty vector were used. As expected, the livers of HBx transgenic mice and HepG2-HBx cells displayed an activated Akt pathway. Accumulated β-catenin, phosphorylated Akt, and increased cyclin D1 were detected (Figure 2A). Even though cancer

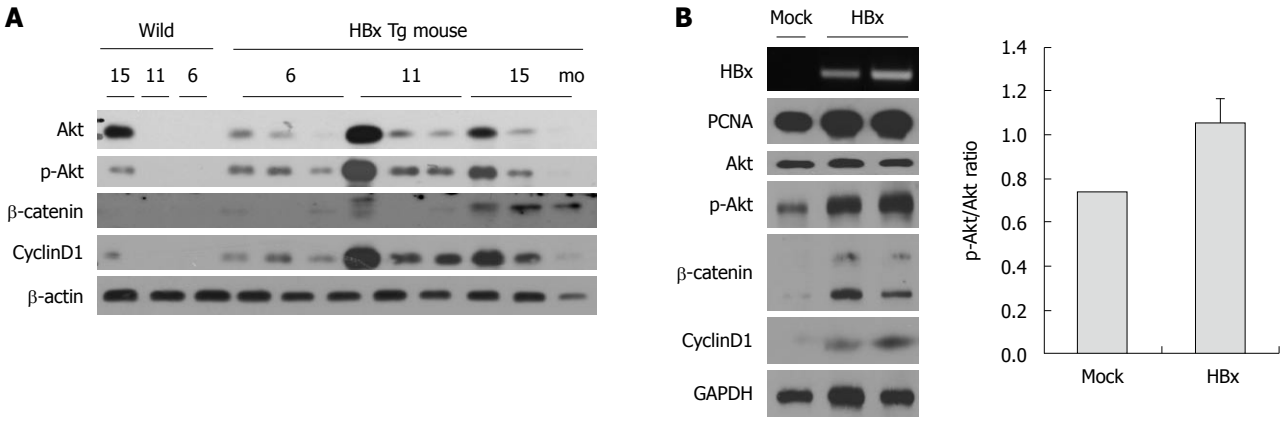

Figure 2 Effect of hepatitis B virus X-protein on activation of the Akt pathway. A: Activation of Akt pathway was examined by Western blotting with liver tissue extracts from 6-, 11- and 13-mo-old hepatitis B virus X-protein (HBx) transgenic and wild-type mice; B: Western blotting was also performed on extracts from stable HepG2-Mock and HBx cell lines.

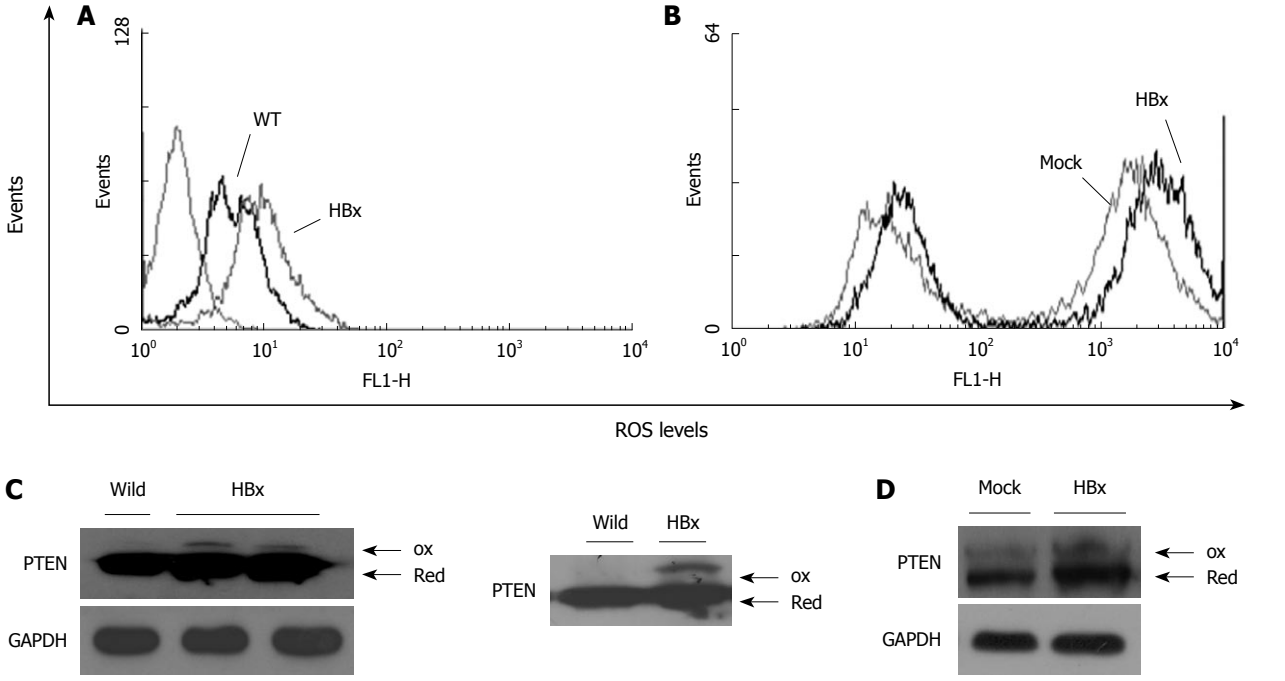

Figure 3 Effect of hepatitis B virus X-protein-induced endogenous reactive oxygen species and phosphatase and tensin homolog oxidation. Endogenous reactive oxygen species (ROS) level was examined by flow cytometry. A, B: Increased production of ROS in hepatitis B virus X-protein (HBx) primary hepatocytes compared to the wild-type hepatocytes and HepG2-HBx compared to the Mock cells. Oxidized phosphatase and tensin homolog (PTEN) was detected by N-ethylmaleimide alkylation, and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis; C: In the upper panel, 20 μg protein was loaded, and for the lower panel, 50 μg was loaded; D: Parallel experiments were performed with extracts from HepG2-Mock and HBx cell lines.

cell lines might have activated Akt, total Akt per p-Akt of HepG2 HBx cells was increased 1.4-fold compared with the HepG2 control cells (Figure 2B).

HBx-induced endogenous ROS cause PTEN inactivation via cysteine oxidation

Peroxides are known to modify PTPs by oxidation. PTEN is also known to be inactivated through H_2O_2-mediated oxidation[20]. FACS analysis was used to verify HBx-induced ROS in mice and HepG2 cells. Primary hepatocytes were isolated from HBx transgenic and wild-type mice at the same age. ROS levels were significantly increased in HBx transgenic hepatocytes and HepG2-HBx cells compared to controls (Figure 3A and B). HBx expression was also associated with decreased mitochondrial membrane potential (data not shown). To examine the effect of HBx-induced ROS on PTEN inactivation, a PTEN oxidation assay was performed. HBx-expressing cells had higher ROS levels, and showed higher levels of oxidized PTEN when evaluated in primary hepatocytes and in HepG2 cells. HBx-induced ROS inactivated PTEN by promoting oxidation of cysteine residues within PTEN, thereby inactivating PTEN and promoting the function of Akt.

Inactivated PTEN correlates with upregulation of the PI3 kinase/ Akt pathway

To investigate the activation of Akt in the presence of ROS-inactivated PTEN, we examined the Akt pathway

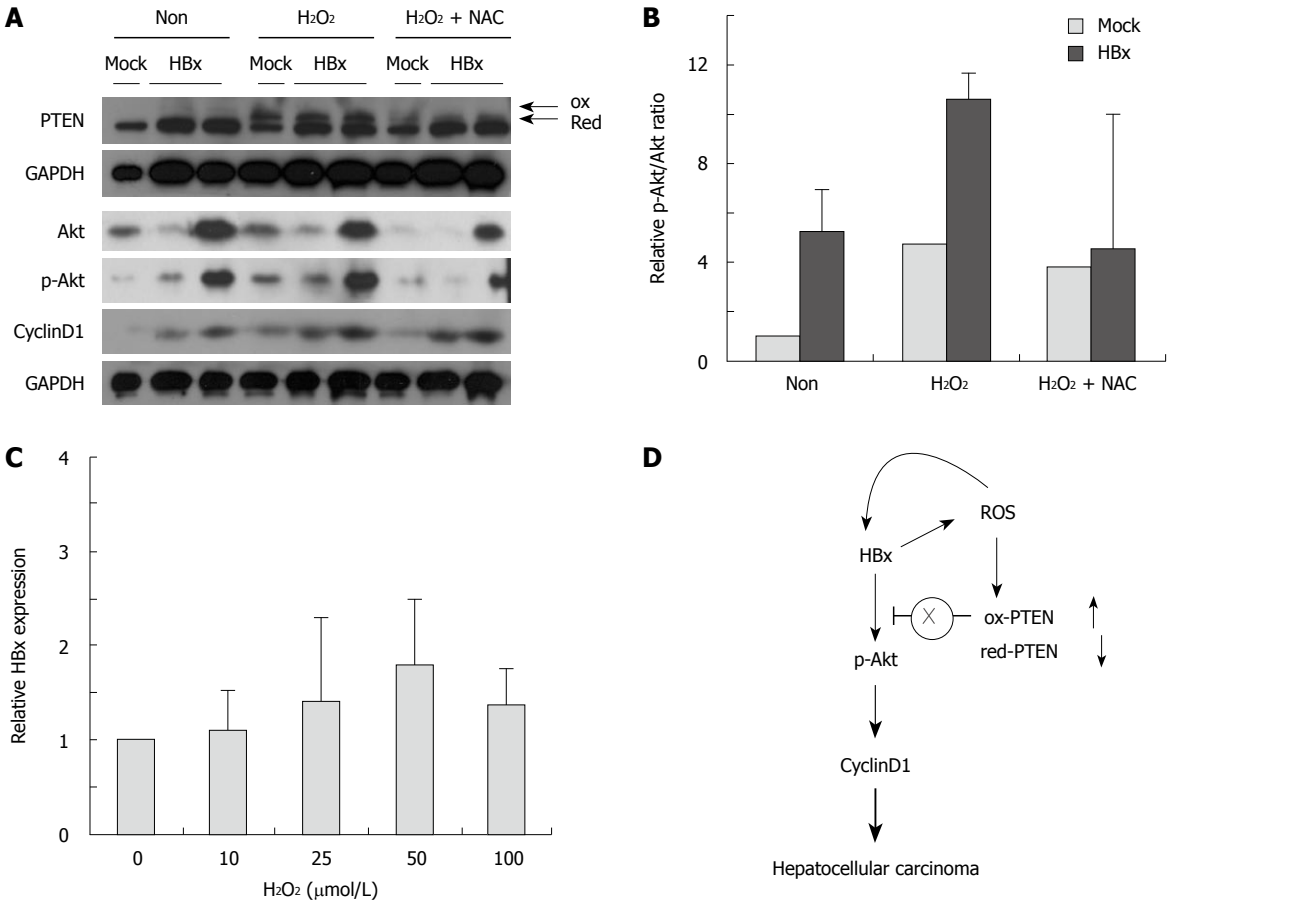

Figure 4 Effect of reactive oxygen species on phosphatase and tensin homolog oxidation, Akt pathway and hepatitis B virus X-protein expression. A, B: H_2O_2 treatment induced phosphatase and tensin homolog (PTEN) oxidation and activation of Akt pathway (increased relative p-Akt/total Akt ratio, cyclin D1 expression). Reactive oxygen species (ROS) scavenging through N-acetylcysteine (NAC) treatment reduced PTEN oxidation and Akt pathway; C: ROS effect on hepatitis B virus X-protein (HBx) expression, by quantitative reverse transcriptase polymerase chain reaction; D: Proposed scheme for ROS effect for activating Akt pathway *via* PTEN oxidation in HBx-induced hepatocarcinogenesis.

activity, which was detected in 0 and 500 μmol/L H_2O_2. Increases in oxidized PTEN were associated with a higher p-Akt/total Akt ratio and increased cyclin D1 expression. To investigate further whether induced ROS is required for activation of the Akt pathway, HepG2 cells were treated with H_2O_2 in the presence or absence of NAC, a ROS quencher. Scavenging ROS through NAC were able to block the Akt pathway (Figure 4A and B). These observations are consistent with the hypothesis that HBx-mediated generation of ROS inactivates PTEN, thereby activating the Akt pathway in carcinogenesis. In addition, elevated ROS was also associated with elevated levels of HBx (Figure 4C).

DISCUSSION

One of the HBV-encoded proteins, HBx, is considered to be a major risk factor for HCC. It is well known that HBx activates cell signal transduction pathways, such as PI3K. Mutations or inactivation of the tumor suppressor, PTEN, regulates Akt activation[21]. This is considered one of the reasons for activation of Akt signaling in cancer. For example, endogenously produced H_2O_2 has been shown to inactivate PTEN in a macrophage cell line and cancer cell lines[16,22]. In this study, HBx-triggered ROS were associated with the oxidation and functional inactivation of PTEN. Although quantification of the extent of PTEN oxidation in the cells was not possible, the level of oxidized, inactivated PTEN was associated with several factors, such as Akt activation and accelerated HepG2 cell growth, and thus might be associated with hepatocarcinogenesis in HBx transgenic mice. Both cell growth and abnormal actin filaments were observed in HepG2-HBx cells. It has been reported that reorganization of actin filaments can cause loss of focal adhesions and cell-cell contact, which leads to an epithelial-mesenchymal transition that consequently disrupts monolayer integrity[23]. The HBx-induced ROS appear to stimulate HBx expression further, which suggests the existence of a positive feedback loop. Such feedback would be expected to cause a rapid increase in the abundance of H_2O_2. This localized H_2O_2 accumulation would be expected to result in the oxidation of only those PTEN molecules located nearby, possibly explaining the small proportion of PTEN molecules that undergo oxidative inactivation in HepG2-HBx cells and mouse livers.

The scheme presented in Figure 4D represents the HBx-induced generation of H_2O_2. H_2O_2 participates in

intracellular signaling by targeting PTEN, and regulation of HBx gene expression, depending on the concentration. The results of the present study suggest that the HBx-mediated activation of Akt is regulated, at least in part, by the effects of HBx-induced ROS upon PTEN.

In summary, these studies further strengthen the case for a close relationship between oxidative stress and tumorigenesis. The studies reported herein have shown that HBx-induced generation of ROS can promote cellular transformation signaling by altering the function of PTEN. H_2O_2-oxidized PTEN leads to the activation of Akt. This is significant from a mechanistic as well as therapeutic point of view. Hence, drugs that scavenge endogenous ROS might slow down progression to HBx-induced liver cancer.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. HCC is closely associated with hepatitis B virus (HBV) infection, especially in Asia. Among the HBV-encoding proteins, X protein (HBx) is a potential candidate for involvement in HBV-related HCC. One of the best-known pathways activated by HBx is phosphoinositide 3-kinase (PI3K)/Akt, which is associated with anti-apoptotic activity and cell proliferation. The reaction catalyzed by PI3K is reversed by phosphatase and tensin homolog (PTEN), which functions as a PI 3,4,5-trisphosphate 3-phosphatase. Indeed, by negatively modulating the PI3K signaling pathway, PTEN acts as a tumor suppressor.

Research frontiers

HCC is one of the cancers with poor prognosis. HBV carriers are approximately 100 times greater than in uninfected individuals. Finding a diagnostic marker and preventing severe liver damage are important areas in liver cancer research.

Innovations and breakthroughs

There have been several studies about HBx-induced reactive oxygen species (ROS). However, most of the studies have used *in vitro* models. This is believed to be the first study of HBx-induced ROS in mice and HepG2 cells, and the increased ROS promoted Akt pathways *via* oxidized inactive PTEN.

Applications

The suggestions in this study are significant not only from a mechanistic point of view - HBx-induced ROS activate the Akt pathway - but also from a therapeutic point of view - prevention of overactivation of the Akt pathway by scavenging ROS.

Peer review

In this experimental study, the molecular pathway of HBx-associated HCC tumorigenesis *via* PI3K/Akt was addressed. The authors demonstrated an important role for ROS as HBx-dependent tumorigenesis mediators. This paper is well written and concise.

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BRIEF ARTICLE

Peri-nuclear antibodies correlate with survival in Greek primary biliary cirrhosis patients

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Abstract

AIM: To investigate possible associations of anti-nuclear envelope antibody (ANEA) with disease severity and survival in Greek primary biliary cirrhosis (PBC) patients.

METHODS: Serum samples were collected at diagnosis from 147 PBC patients (85% female), who were followed-up for a median 89.5 mo (range 1-240). ANEA were detected with indirect immunofluorescence on 1% formaldehyde fixed Hep2 cells, and anti-gp210 antibodies were detected using an enzyme linked immunosorbent assay. Findings were correlated with clinical data, histology, and survival.

RESULTS: ANEA were detected in 69/147 (46.9%) patients and 31/147 (21%) were also anti-gp210 positive. The ANEA positive patients were at a more advanced histological stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 56.5%/43.5% *vs* 74.4%/25.6%, $P = 0.005$) compared to the ANEA negative ones. They had a higher antimitochondrial antibodies (AMA) titer (≤ 1:160/> 1:160 50.7%/49.3% *vs* 71.8%/28.2%, $P = 0.001$) and a lower survival time (91.7 ± 50.7 mo *vs* 101.8 ± 55 mo, $P = 0.043$). Moreover, they had more advanced fibrosis, portal inflammation, interface hepatitis, and proliferation of bile ductules ($P = 0.008$, $P = 0.008$, $P = 0.019$, and $P = 0.027$, respectively). They also died more frequently of hepatic failure and/or hepatocellular carcinoma ($P = 0.016$). ANEA positive, anti-gp210 positive patients had a difference in stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 54.8%/45.2% *vs* 74.4%/25.6%, $P = 0.006$), AMA titer (≤ 1:160/> 1:160 51.6%/48.4% *vs* 71.8%/28.2%, $P = 0.009$), survival (91.1 ± 52.9 mo *vs* 101.8 ± 55 mo, $P = 0.009$), and Mayo risk score (5.5 ± 1.9 *vs* 5.04 ± 1.3, $P = 0.04$) compared to the ANEA negative patients. ANEA positive, anti-gp210 negative patients had a difference in AMA titer (≤ 1:160/> 1:160 50%/50% *vs* 71.8%/28.2%, $P = 0.002$), stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 57.9%/42.1% *vs* 74.4%/25.6%, $P = 0.033$), fibrosis ($P = 0.009$), portal inflammation ($P = 0.018$), interface hepatitis ($P = 0.032$), and proliferation of bile ductules ($P = 0.031$). Anti-gp210 positive patients had a worse Mayo risk score (5.5 ± 1.9 *vs* 4.9 ± 1.7, $P = 0.038$) than the anti-gp210 negative ones.

CONCLUSION: The presence of ANEA and anti-gp210 identifies a subgroup of PBC patients with advanced disease severity and poor prognosis.

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Key words: Primary biliary cirrhosis; Antimitochondrial antibodies; Antinuclear antibodies; Antibodies against nuclear envelope antigens; Anti-gp210 antibodies

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic, progressive, cholestatic liver disease of probable autoimmune etiology, characterized by destruction of small intrahepatic bile ducts, portal inflammation, and, eventually, development of liver cirrhosis and hepatic failure. Elevated cholestatic enzymes, compatible liver histology, and detectable antimitochondrial antibodies (AMA), at titers higher than 1:40, are the three diagnostic criteria used for the diagnosis of PBC. Two of the three criteria suffice for making a definite diagnosis of PBC[1].

In fact, AMA are the specific marker of PBC, detected in 90%-95% of patients[2]. Antinuclear antibodies (ANA) are also present in 30% to 50% of patients[3-6], and are detected by indirect immunofluorescence (IIF) with various patterns. The peri-nuclear pattern is the most common IIF pattern of ANA, which detects nucleoporin 62 (p62)[7,8] and gp210[4] (nuclear pore complexes, NPC), as well as the lamin B receptor and lamin A/C[9,10], all antigens of the nuclear envelope. The multiple nuclear dot IIF pattern of ANA, is due to nuclear antigens such as sp100, SUMO, and PML. Another ANA IIF pattern found in PBC patients is the anti-centromere (ACA) one[11,12].

Although AMA are not associated with disease progression[1], ACA and anti-nuclear envelope antibody (ANEA) (ANA against the nuclear envelope antigens) seem to be associated with disease severity[4,13]. In fact, anti-NPC presence has been associated with more active and severe disease[7,13]; anti-gp210 and ACA positive patients have been reported to be associated with either hepatic failure or portal hypertension respectively[4,13,14]. However a study on Spanish and Greek patients did not confirmed these findings[15].

The purpose of the present study is to examine associations between the presence of serum ANEA and the severity and survival in a homogeneous cohort of Greek patients with AMA positive and negative PBC, in a single referral centre.

MATERIALS AND METHODS

Patients

From January 1989 to June 2009, 232 PBC patients were diagnosed by standard criteria and followed up at the Department of Gastroenterology of the University Hospital of Heraklion, Crete. Patients that had frozen (-80℃) serum collected at diagnosis, had negative hepatitis viral markers, were regularly followed up and gave their consent, were included in the study. These criteria were fulfilled by 147 patients. They were followed-up for 1-240 mo (mean 96.1 ± 55.8 mo, median 89.5 mo) after the initial diagnosis of PBC. Nineteen (12.9%) patients were AMA negative and 128 (87.1%) were AMA positive, with a titer > 1/40, M2 positive. Twenty-two patients (15%) were males and 125 (85%) were females. The mean age at diagnosis was 59.2 ± 10.9 years (median 60, range 31-80). According to the Ludwig classification, 97 patients (66%) were at an early stage (Ⅰ-Ⅱ), 50 (34%) were at a late stage (Ⅲ-Ⅳ), of whom 32/50 were at stage Ⅳ. The mean Mayo risk score at the time of diagnosis was 5.1 ± 1.6. All patients were treated with ursodeoxycholic acid (UDCA) at a dose of 13-15 mg/kg, starting after the time of serum collection. Other coexisting autoimmune diseases were: Sjogren syndrome in 12 patients, Raynaud phenomenon in two, psoriasis in one, sarcoidosis in one, discoid lupus erythematosus in one, autoimmune atrophic gastritis in two, and vitiligo in one.

During the follow-up period 14 patients developed variceal bleeding, 15 developed ascites, two developed hepatic encephalopathy, and six developed hepatocellular carcinoma. Four patients underwent orthotopic liver transplantation. Thirty patients died during follow-up (five from liver unrelated causes). The remaining 117 patients are alive and are still being followed up at our Department at the end of the study. The study was approved by the Ethics Committee of the Hospital. All patients have given a written, informed consent.

Methods

The Autoantibodies studied were correlated with clinical data, histology at diagnosis, the major events occurring during the follow-up period, and survival.

Ninety-eight biopsy specimens with more than three portal tracts were reexamined and graded by a single pathologist. The histological variables analyzed included: fibrosis (1-3) (1 = mild, 2 = moderate, 3 = severe), interface hepatitis (0-3) (0 = absent, 1 = mild, 2 = moderate, 3 = severe), portal inflammation (1-3) (1 = mild, 2 = moderate, 3 = severe), intralobular inflammation (0-2) (0 = absent, 1 = mild, 2 = moderate), epithelioid granulomas (0-1) (0 = absent, 1 = present), and proliferation of bile ductules (0-1) (0 = absent, 1 = present).

Cell culture

Human Hep2 cells (larynx and cervical carcinoma) were used. The cells were cultured in Minimum Essential Medium supplied with 10% Fetal Bovine Serum 100 U/mL penicillin/streptomycin and 1% non-essential amino acids.

They were maintained in humidified atmosphere at 37℃ and 5% CO_2. All the culture media were from Gibco (Invitrogen, UK)

IIF for serum autoantibody analysis

An IIF assay of Nova Lite™ (IFA) ANA plus Mouse Kidney & Stomach (Inova Diagnostics, San Diego CA, Inc) was used for screening and semi-quantitative determination of AMA IgG antibodies, according to the manufacture's instructions.

IIF was performed for detection of ANEA, as previously described[16]. Briefly, Hep2 cells were grown overnight on coverslips and washed with PBS (Gibco, Invitrogen, UK).

The cells were fixed with 1% and 4% formaldehyde (Sigma-Aldrich, Germany) for 10 min. The cells were washed with PBS. To quench auto-fluorescence and enhance antigenicity, the fixed cells were incubated with 20 mmol/L glycine (Sigma-Aldrich, Germany) in PBS for 5 min. After blocking with PBS containing 0.2% TritonX-100, 2 mmol/L $MgCl_2$, and 1% gelatin from cold water fish skin (Sigma-Aldrich, Germany) for 10 min, cells were incubated with serum (dilution 1:80) in blocking buffer for 45 min. Subsequently, the coverslips were washed with blocking buffer for 10 min and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (dilution 1:500, H+L secondary antibody, Chemicon, Millipore, Germany) for 45 nin. Finally, cells were rinsed in PBS and mounted with mounting medium containing Dapi (Santa Cruz Biotechnology, Inc, Germany). All steps were performed at room temperature. Fluorescence was observed under a Leica SP confocal microscope.

Enzyme linked immunosorbent assay for gp210

For the assessment of anti-gp210 antibodies, a Quanta lite™ enzyme linked immunosorbent assay (ELISA) (Inova Diagnostics, San Diego CA, Inc) kit was used according to the manufacture's instructions.

Statistical analysis

Data are presented as percentages (%) or as mean ± SD, unless otherwise stated. Differences between autoantibody positive and negative patients for various clinical, histological, and serological measurements were compared using multivariate regression analysis. The survival time was estimated by the Kaplan-Meier method, and compared by the Breslow test. Fisher's exact test was used to compare causes of death between ANEA positive and negative and gp-210 positive and negative patients. A P value < 0.05 was considered significant. Statistical analyses were performed using SPSS v.15.0 and Excel 2003 software.

RESULTS

Fixation was important in visualization of ANEA by immunofluorescence, 1% fixation allowed for much better discrimination of antinuclear antibodies (Figure 1).

Parameters used in multivariate analysis are shown in Tables 1-3. The ANEA were detected by IIF on Hep2 cells giving a typical peri-nuclear staining pattern (Figure 1). ANEA were detected in 69 (46.9%) of 147 patients. Comparisons between ANEA positive and negative patients are shown in Tables 1 and 2. Although there was no significant difference in the number of alive/dead between positive and negative ANEA patients [51 (77.3%)/15 (22.7%) *vs* 66 (86.8%)/10 (13.2%), NS], there was a statistical significance in survival period between the two groups (91.7 ± 50.7 mo *vs* 101.8 ± 55 mo, P = 0.043) (Table 1 and Figure 2). Moreover, causes of death were significantly different between ANEA positive and negative patients (Figure 3).

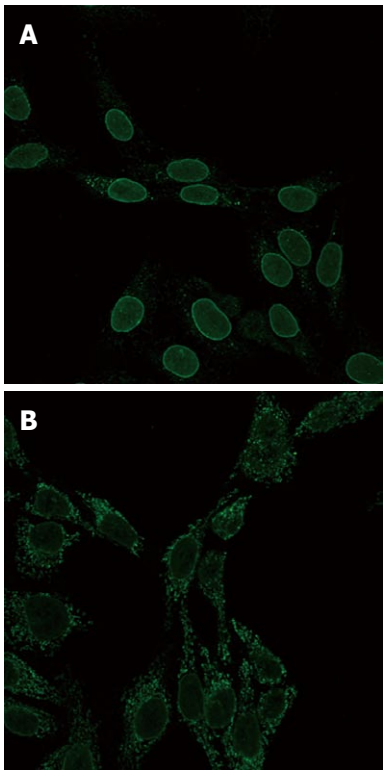

Figure 1 Typical peri-nuclear staining showing anti-nuclear envelope antibody positive sera in indirect immunofluorescence. A: Cells fixed with 1% formaldehyde; B: Cells fixed with 4% formaldehyde.

Table 1 Comparison of clinical parameters between anti-nuclear envelope antibody positive and anti-nuclear envelope antibody negative patients (mean ± SD) *n* (%)

	ANEA		*P* value
	Positive	Negative	
Patients	69 (46.9)	78 (53.1)	
Age (yr)	59.3 ± 11.8	59.1 ± 10.1	0.330
AMA titer ≤ 1:160/ > 1:160[1]	35 (50.7)/34 (49.3)	56 (71.8)/22 (28.2)	0.001
Stage Ⅰ-Ⅱ/ stage Ⅲ-Ⅳ	39 (56.5)/30 (43.5)	58 (74.4)/20 (25.6)	0.005
Alive/dead	51 (77.3)/15 (22.7)	66 (86.8)/10 (13.2)	0.162
Survival	91.7 ± 50.7	101.8 ± 55	0.043
Mayo risk score	5.19 ± 1.8	5.04 ± 1.3	0.239

[1]1/160 is the median antimitochondrial antibodies titer. ANEA: Anti-nuclear envelope antibody.

Table 2 Comparison of histological parameters between anti-nuclear envelope antibody positive and anti-nuclear envelope antibody negative patients *n* (%)

	Histological parameters		*P* value
	ANEA positive (*n* = 69)	ANEA negative (*n* = 78)	
Fibrosis			
1	10 (22.7)	22 (40.7)	0.008
2	17 (38.6)	22 (40.7)	
3	17 (38.6)	10 (18.6)	
Portal inflammation			
1	8 (18.2)	22 (40.7)	0.008
2 + 3	36 (81.8)	32 (59.3)	
Interface hepatitis			
0 + 1	16 (36.4)	31 (57.4)	0.019
2 + 3	28 (63.6)	23 (42.6)	
Intralobular inflammation			
0	11 (25)	9 (16.7)	0.359
1	19 (43.2)	35 (64.8)	
2	14 (31.8)	10 (18.5)	
Proliferation of bile ductules			
0	12 (27.3)	25 (46.3)	0.027
1	32 (72.7)	29 (53.7)	
Epithelioid granuloma			
0	31 (70.5)	39 (72.2)	0.425
1	13 (29.5)	15 (27.8)	

ANEA: Anti-nuclear envelope antibody.

Table 3 Comparison of histological parameters between anti-nuclear envelope antibody positive, gp210 negative, and anti-nuclear envelope antibody negative patients *n* (%)

	Histological parameters		*P* value
	Gp210 negative (*n* = 38)	ANEA negative (*n* = 78)	
Fibrosis			
1	7 (25.0)	22 (40.7)	0.009
2	8 (28.6)	22 (40.7)	
3	13 (48.4)	10 (18.6)	
Portal inflammation			
1	5 (17.9)	22 (40.7)	0.018
2 + 3	23 (82.1)	32 (59.3)	
Interface hepatitis			
0 + 1	10 (35.7)	31 (57.4)	0.032
2 + 3	18 (64.3)	23 (42.6)	
Intralobular inflammation			
0	8 (28.6)	9 (16.7)	0.274
1	14 (50.0)	35 (64.8)	
2	6 (21.4)	10 (18.5)	
Proliferation of bile ductules			
0	7 (25.0)	25 (46.3)	0.031
1	21 (75.0)	29 (53.7)	
Epithelioid granuloma			
0	19 (67.9)	39 (72.2)	0.342
1	9 (32.1)	15 (27.8)	

ANEA: Anti-nuclear envelope antibody.

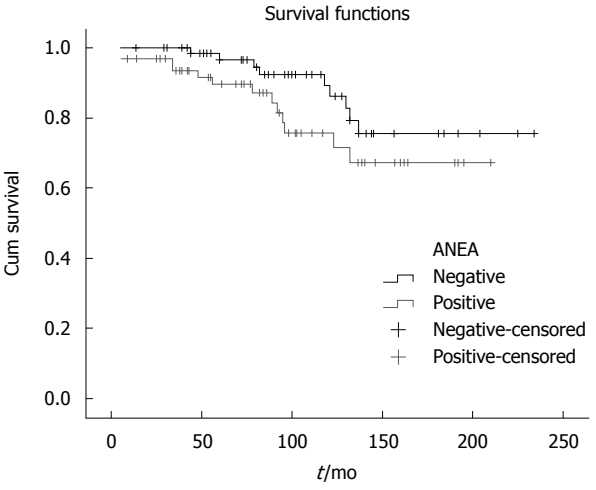

Figure 2 Kaplan-Meier curve of survival between anti-nuclear envelope antibody negative and anti-nuclear envelope antibody positive patients (*P* = 0.043 by Breslow test). ANEA: Anti-nuclear envelope antibody.

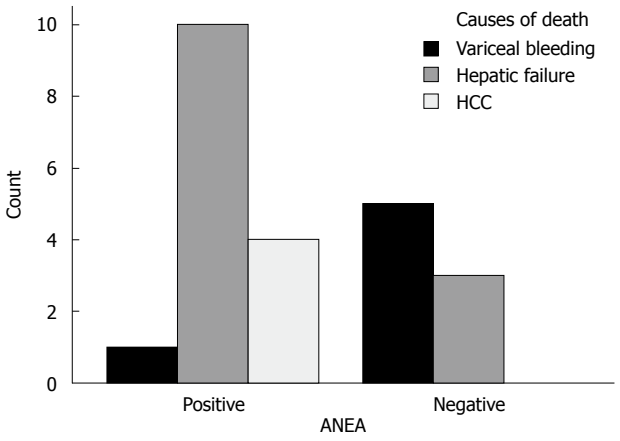

Figure 3 Causes of death between anti-nuclear envelope antibody positive and anti-nuclear envelope antibody negative patients (*P* = 0.016). Anti-nuclear envelope antibody (ANEA) positive patients died more frequently of hepatic failure and/or hepatocellular carcinoma (HCC), while ANEA negative patients died more frequently as a result of variceal bleeding.

AMA titers

AMA titers were not associated with disease severity. Kaplan-Meier analysis showed $P > 0.7$ when AMA titers were examined in relation to patient survival.

Anti-Gp210

We tested all 69 ANEA positive patients (18 dead, five from liver unrelated death) for the anti-gp210 antibodies by ELISA and found 38 (55.1%) negative and 31 (44.9%) positive, representing 21% of all studied patients.

Comparing the anti-gp210 positive patients ($n = 31$) with the ANEA negative patients ($n = 78$) we found significantly higher AMA titer (≤ 1:160/> 1:160 51.6%/48.4% *vs* 71.8%/28.2%, $P = 0.009$), more late stages (Ⅰ-Ⅱ/Ⅲ-Ⅳ 54.8%/45.2% *vs* 74.4%/25.6%, $P = 0.006$), higher Mayo risk score (5.5 ± 1.9 *vs* 5.04 ± 1.3, $P = 0.04$) and shorter survival period (91.1 ± 52.9 mo *vs* 101.8 ± 55 mo, $P = 0.009$) (Figure 4).

Comparing the 38 ANEA positive-gp210 negative patients with the 78 ANEA negative patients, we found that the ANEA negative ones had lower AMA titers (≤ 1:160/> 1:160 50%/50% *vs* 71.8%/28.2%, $P = 0.002$), earlier stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 57.9%/42.1% *vs* 74.4%/25.6%, $P = 0.033$), less severe fibrosis, portal inflammation, in-

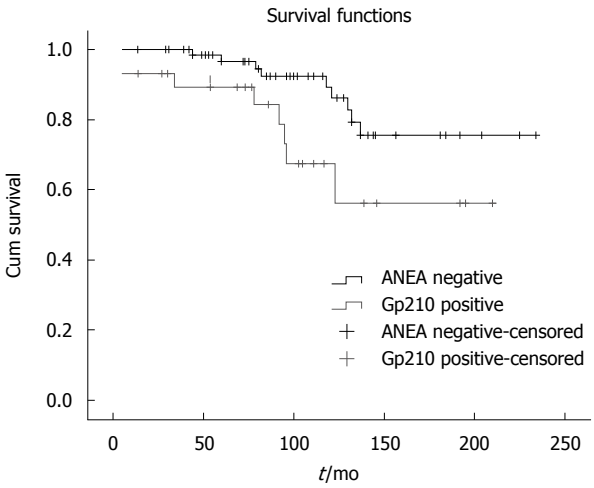

Figure 4 Kaplan-Meier curve of survival between anti-gp210 positive and anti-nuclear envelope antibody negative patients (P = 0.009 by Breslow test). ANEA: Anti-nuclear envelope antibody.

terface hepatitis, and proliferation of bile ductules (P = 0.009, P = 0.018, P = 0.032 and P = 0.031, respectively) (Table 3).

Between anti-gp210 positive (n = 31) and ANEA positive, anti-gp210 negative (n = 38) patients the only parameter that differed was Mayo risk score (5.5 ± 1.9 *vs* 4.9 ± 1.7, P = 0.038). No difference between the two groups was found for any of the other clinical, demographic, histological parameters or for survival (mean 91.1 ± 52.9 and 92 ± 49.6 mo respectively).

Causes of death were no different between gp-210 positive and negative patients.

DISCUSSION

In recent years, significant steps have been made in the clarification of the pathogenesis of PBC, although definitive data have not yet been provided[17,18]. Moreover, the mechanisms controlling disease severity and, therefore, prognosis in this clinically heterogeneous disease are still not well established. In recent years, more patients have been diagnosed in at the presymptomatic or asymptomatic stage than before. Some of these patients undergo a benign course and others a more progressive one, with early appearance of symptoms and rapid deterioration, leading to liver transplantation or death[1,19,20].

Prognostic scores based on clinical parameters (Mayo risk score and bilirubin) have been developed in patients with advanced disease, but they have not been validated in presymptomatic or asymptomatic patients.

Earlier studies failed to demonstrate an association of disease severity and progression, with ANEA[21,22]. However in 2001, Invernizzi *et al*[7] reported an association of antibodies to NPC with disease activity and severity, which was confirmed, in particular for anti-gp210, in Italian PBC patients two years later[23]. In American and Canadian patients, ANEA were associated with increased risk of liver failure[24].

Nakamura *et al*[4] in 276 Japanese PBC patients reported a prevalence of 26% for anti-gp210. In that study, anti-gp210 presence correlated with survival and a hepatic failure pattern of disease progression. By contrast, in a cohort of 170 Spanish and 162 Greek patients, only 10.4% of patients were anti-gp210 positive. In that study, there was no correlation with survival or histological severity, although correlations with Mayo risk score, ALP, and bilirubin were reported[15]. The authors stated that the low prevalence could be an ethnic or geographic variation and that, although anti-gp210 represents a disease severity marker, it is not a prognostic one. However, our results of anti-gp210 prevalence, using the same ELISA kits, in a homogeneous Greek population from the island of Crete, are similar to the Japanese report.

Similarly to the Japanese report, our ANEA positive patients died more frequently of hepatic failure and/or hepatocellular carcinoma, while ANEA negative patients died more frequently as a result of variceal bleeding.

Indeed, we found that 46.9% of the patients were ANEA positive and 21% anti-gp210 positive, a similar percentage to the Japanese patients (26%). The ANEA positive patients at the time of diagnosis were at later histological stages, with more severe fibrosis, portal inflammation, bile ductular proliferation, and interface hepatitis. They had higher AMA titers and, as expected, shorter survival periods than the ANEA negative patients. The anti-gp210 positive patients differed from the rest of the ANEA positive patients only in their higher Mayo risk scores. It should be noted that, with the usual 4% formaldehyde fixation used with Hep2 cells, there might be a difficulty in discrimination between peri-nuclear and cytoplasmic fluorescence caused by the presence of high titers of antimitochondrial antibodies. By contrast, our slight modification using 1% fixation instead allowed for much better visualization of peri-nuclear staining; therefore, we strongly recommend this fixation for further use.

In conclusion, our data confirm that, in Greek PBC patients, there is a correlation between the presence of ANEA antibodies and disease severity and shorter survival. The presence of anti-gp210 seems to be an additional factor, reducing survival. Therefore, we suggest that presence of ANEA and anti-gp210 should be routinely checked, because their presence identifies a subgroup of PBC patients with poor prognosis. The mechanism underlying the association of ANEA with prognosis requires further elucidation.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is an autoimmune disease of unknown etiology, where genetic and environmental factors have roles in disease pathogenesis. The clinical course of the disease is variable, and no specific serological markers can predict disease progression. Peri-nuclear antibodies have not been adequately evaluated as predictive markers and the results so far are contradictory.

Research frontiers

Peri-nuclear antibodies were evaluated using a modification of the immunofluorescent identification technique, which allows for better visualization of these antibodies and avoids a possible confusion with antimitochondrial antibodies. The presence of anti-nuclear envelope antibody (ANEA), was associated with decreased patient survival and causes of death.

Innovations and breakthroughs

The modified technique might explain the reported differences with other European patient cohorts. Moreover, the patient population in this study is racially homogeneous, thus excluding possible racial differences as a factor of genetic influence in the results.

Applications

The findings of the present study suggest that identification of the presence of ANEA should be included in the routine work up of patients with PBC, because they identify a subgroup with worse prognosis, for whom a more intense follow up scheme should be applied.

Peer review

In this manuscript, the authors describe the apparent association between antinuclear antibodies, especially ANEA, and the severity and survival of 147 patients with PBC in a single-center cohort in Greek. They examined ANEA and anti-gp210 antibodies in sera at diagnosis and found that ANEA positivity, as well as anti-gp210 positivity, could identify a subgroup of PBC patients with poor prognosis. These results are coincident with previous results from Japan and look very interesting.

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BRIEF ARTICLE

Pancreatic function, quality of life and costs at long-term follow-up after acute pancreatitis

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Abstract

AIM: To evaluate long-term endocrine and exocrine pancreatic function, quality of life and health care costs after mild acute pancreatitis and severe acute pancreatitis (SAP).

METHODS: Patients prospectively included in 2001-2005 were followed-up after 42 (36-53) mo. Pancreatic function was evaluated with laboratory tests, the oral glucose tolerance test (OGTT), fecal elastase-1 and a questionnaire. Short Form (SF)-36, was completed.

RESULTS: Fourteen patients with a history of SAP and 26 with mild acute pancreatitis were included. Plasma glucose after OGTT was higher after SAP (9.2 mmol/L *vs* 7.0 mmol/L, $P = 0.044$). Diabetes mellitus or impaired glucose tolerance in fasting plasma glucose and/or 120 min plasma glucose were more common in SAP patients (11/14 *vs* 11/25, $P = 0.037$). Sick leave, time until the patients could take up recreational activities and time until they had recovered were all longer after SAP ($P < 0.001$). No significant differences in SF-36 were seen between the groups, or when comparing with age and gender matched reference groups. Total hospital costs, including primary care, follow-up and treatment of complications, were higher after SAP (median €16 572 *vs* €5000, $P < 0.001$).

CONCLUSION: Endocrine pancreatic function was affected, especially after severe disease. SAP requires greater resource use with long recovery, but most patients regained a good quality of life.

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Key words: Acute pancreatitis; Endocrine function; Exocrine function; Quality of life; Cost

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INTRODUCTION

Acute pancreatitis is associated with a mild and uneventful course in 80% of cases. However, severe acute pancreatitis (SAP) is associated with the risk of complications both in and around the pancreas, and eventually has a fatal outcome in up to 15%-20% of cases[1]. Exocrine and endocrine pancreatic function has mainly been studied after SAP, with divergent results[2-9]. In mild, edematous-interstitial cases of acute pancreatitis, the pancreas can recover completely. After SAP, however, morphological changes may often remain and functional recovery is not always complete[3,4,9]. Impairment of exocrine pancreatic function with steatorrhea has been reported to diminish over time[8,10]. Damaged pancreatic acinar cells may recover with improvement in pancreatic function[4]. However,

endocrine dysfunction with diabetes mellitus (DM) seems to be more common over time[9,11].

Until recently, results of treatment strategies were determined only in terms of cure, impairment of pancreatic functions, disability or death. However, there is a need for critical assessment of outcome measures that also include quality of life and health economical aspects. In some studies, SAP patients have been reported to regain a satisfactory quality of life[12-15], while others report a clear impairment[16,17].

Severe disease requires prolonged hospitalization, frequently including a stay in the intensive care unit (ICU). Sometimes there is also a need for multiple radiological and surgical interventions. Long rehabilitation of survivors can be expected, resulting in not only a great challenge for the individual but also a substantial need for resources together with high associated costs for society. There have only been a few previous cost analyses performed for acute pancreatitis[11,15,18-21].

The aim of the present study was to evaluate both pancreatic endocrine and exocrine function, as well as general recovery, quality of life and costs in patients who had recovered from mild acute pancreatitis, as well as from SAP, and to compare these groups at long-term follow-up.

MATERIALS AND METHODS

Study population

Case records from patients with mild acute pancreatitis and SAP, previously evaluated for participation in 2 nutritional studies[22,23], were examined. Severity definition was performed according to the Atlanta classification[24]. Exclusion criteria were dementia, malignancy, an additional history of SAP and chronic pancreatitis. Two out of the 20 SAP patients had died, one had dementia and 2 had developed chronic pancreatitis. In total, 15 patients with SAP, and a group of 30 gender- and age-matched patients from the group with mild disease were asked to participate in this follow-up survey. Invitation was performed by letter followed by a telephone call, offering an appointment at the outpatient clinic. Five patients, one of whom was from the SAP group, declined to participate. Finally, 14 patients with a history of SAP and 26 with a history of mild disease were included. Thus, 40 patients participated in this long-term evaluation for a median of 42 (36-53) mo after the episode of acute pancreatitis. APACHE Ⅱ, scored in all patients at initial admission to hospital, was a median of 7 (6-10) in patients who later developed severe disease and 6 (5-7) in the group of patients with mild disease ($P = 0.012$). Five out of 14 patients in the SAP group had an APACHE Ⅱ < 8 and 3/26 in the group with mild acute pancreatitis had a score ≥ 8.

All patients were seen by the same surgeon at the outpatient clinic. A thorough physical and physiological investigation was performed. Blood samples were taken after fasting and during an oral glucose tolerance test (OGTT), and a fecal sample was collected. All patients completed a questionnaire examining current pancreatic function, medication, abdominal surgical interventions, eating and drinking habits, readmissions for pancreatitis, ability to return to normal daily activity and time until they had recovered from the acute pancreatitis episode. Actual working capacity (including retirement/early retirement/sick leave/still working) was evaluated. Body weight and height was measured. Quality of life forms were completed. Several aspects of the patients' current condition were evaluated, using a visual analogue scale (VAS: 0-100).

Exocrine pancreatic function

Fecal elastase-1 concentration, a specific human protease synthesized by the acinar cells, was measured in stool samples using a commercial enzyme-linked immunosorbent assay, (ScheBo Biotech, Giessen, Germany). It is non-invasive, stable and correlates well with exocrine pancreatic function tests[25,26]. A value > 200 μg elastase/g stool is considered normal. Subjective pancreatic function was evaluated *via* a questionnaire, including questions about the incidence of abdominal discomfort, bowel habit including frequency of defecation, presence of diarrhea and steatorrhea, intolerance to fat and other food, unintentional weight loss and use of pancreatic enzyme supplementation.

Endocrine pancreatic function

Fasting plasma (FP) glucose, C-peptide and insulin were measured in all patients. In non-diabetic patients ($n = 39$), a 75 g, 2 h OGTT was performed to detect impaired glucose tolerance (IGT) and DM. Glucose and C-peptide were measured in venous plasma at 0, 15, 30, 60 and 120 min. Insulin was determined at 0 and 120 min. The guidelines and definitions established by the World Health Organisation were followed[27]. FP glucose $\geq$ 7.0 mmol/L met the criteria for DM and 6.1-6.9 mmol/L for IGT. OGTT plasma glucose values $\geq$ 11.1 mmol/L at 2 h were defined as DM and values $\geq$ 7.8 and $<$ 11.1 mmol/L as IGT. Measurements of baseline and stimulated insulin and C-peptide values allowed the differentiation of DM induced by insulin resistance or beta cell failure. The homeostasis model assessment (HOMA) for evaluating insulin resistance [HOMA IR = fasting insulin (mIE/mL) × FP glucose (mmol/L)/22.5] was calculated[28]. Fasting glycosylated hemoglobin A1c (HBA1c) was measured for assessing long-term glucose homeostasis.

Quality of life

The Swedish version of Standard Short Form 36 (SF-36), a widely used general quality-of-life questionnaire that has been validated in a variety of medical settings, was used[29]. The SF-36 examines 8 areas consisting of social and physical function, physical and emotional well-being, bodily pain, vitality, mental health and overall general health perception. Swedish normative data of age-matched controls were used for comparison.

Costs

Costs were calculated as total hospital costs per patient at the primary hospital stay, including expenses on the ward,

ICU stay, anesthesia and operating costs, radiological and clinical physiology expenses, and costs for laboratory analysis and blood products. Subsequent costs, both for in-hospital stay and outpatient care, directly related to the primary acute pancreatitis episode, were also calculated. Sick leave days were retrieved from the patient's medical records and from the patients at follow-up. All costs are given in 2008 price levels, inflated using the Swedish consumer price index. The costs have been converted from Swedish krona (SEK) to Euros (€) using the yearly average exchange rate for 2008 (9.6055 SEK to €1).

Statistical analysis

Continuous variables are presented as medians with 25th and 75th percentiles. Categorical variables are given as frequencies and percentages. Univariate analysis for continuous variables was conducted with the Wilcoxon test. Categorical variables were analyzed by the χ^2-test, except when expected frequencies were less than 5, in which case Fisher's exact test was used. The Kaplan-Meier estimate was used to calculate time to event. The log-rank test was used to compare the difference between the groups. Data were analyzed using Hmisc, Survival and Design packages of the R software (R Foundation for Statistical Computing, Vienna, Austria), version 2.8.1. The level of significance was set at $P < 0.05$. The study was approved by the Human Ethics Committee, Lund University.

RESULTS

Of the 40 patients finally included in the study, 16 (40%) were men. The median age was 61 (48-68) years at follow-up. Body mass index was 28 (26-31) kg/m^2, with no difference between patients with severe or mild acute pancreatitis. Patient data from the episode with acute pancreatitis are presented in Table 1. There was no difference in follow-up time between the groups. Most routine laboratory parameters, such as hemoglobin, creatinine, calcium, bilirubin and lipase showed no difference at follow-up, though pancreas-specific amylase was lower in patients with a history of SAP, and alanine aminotransferase was higher (Table 2). Sick leave, time until the patients could regain recreational activities and time until recovery were all significantly longer after SAP (Table 2). A total of 7 patients from both groups did not feel that they had fully recovered at the time of follow-up, a median 39 (33-49) mo after the episode of acute pancreatitis (Figure 1). There was no difference between the groups when using the subjective VAS for expressing abdominal pain, fatigue, nausea, anxiety, working capacity and energy for recreational activities. The question whether actual difficulties were thought to be late effects of the acute pancreatitis episode also showed no difference comparing SAP and mild acute pancreatitis, 3 (2-43) *vs* 3 (0-13), $P = 0.33$. Additional hospital visits, including both scheduled follow-ups and emergency visits due to abdominal pain, excluding any additional acute pancreatitis episodes, were more common after severe disease. In SAP, 12 (86%) patients had one or more visits and in the mild group, 7 (27%) had only one and none any additional visits, $P < 0.001$. At follow-up, no patient in either the SAP or the mild group used medication regularly for abdominal pain.

Table 1 Patient characteristics and parameters during the acute care for pancreatitis, divided into mild and severe acute pancreatitis cases

Parameter	Mild acute pancreatitis	Severe acute pancreatitis	All patients	Difference between groups (*P*)
Gender, male	10	6	16	0.79
Etiology				
Gallstone	16 (62)	4 (29)	20 (50)	0.096
Alcohol	5 (19)	5 (36)	10 (25)	0.278
Post ERCP	0	3 (21)	3 (8)	0.037
Unknown	5 (19)	2 (14)	7 (18)	1.0
Other diseases	13 (50)	5 (36)	18 (45)	0.39
ASA class[1]	1.5 (1-2)	1 (1-1.75)	1 (1-2)	0.14
APACHE II[1]	6 (5-7)	7 (6-10)	6 (5-7)	0.012
Weight loss	13 (50)	11 (79)	24 (60)	0.079
Weight loss in kg[1]	5 (1.5-6)	9 (7-10)	6.4 ± 4.7 6 (3-9)	0.003
Pancreatic surgery	0	4 (29)	4 (10)	0.004
Pancreatic pseudocysts	0	7 (50)	7 (18)	< 0.001
Hospital stay in days[1]	7.5 (4-9)	18 (16-24)	10 (6-16)	< 0.001
ICU stay	0	8 (57)	8 (20)	< 0.001

Values are median and in parentheses are percentages or [1]interquartile range. ERCP: Endoscopic retrograde cholangiopancreatography; ASA: American Society of Anesthesiologists; APACHE Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; ICU: Intensive care unit.

Table 2 Patient characteristics and parameters at follow-up after mild and severe acute pancreatitis

Parameter	Mild acute pancreatitis	Severe acute pancreatitis	All patients	Difference between groups (*P*)
Time to follow-up (mo)	41 (35-50)	47 (37-63)	42 (36-53)	0.14
Age (yr)	61 (51-70)	58 (45-67)	61 (48-68)	0.72
BMI (kg/m^2)	27 (25-32)	29 (26-31)	28 (26-31)	0.82
ASA	2 (1-2)	1 (1-2)	1.5 (1-2)	0.68
Serum pancreas amylase (μkat/L)	0.45 (0.37-0.53)	0.27 (0.18-0.43)	0.043 (0.27-0.52)	0.007
P-ALAT (μkat/L)	0.30 (0.23-0.47)	0.39 (0.33-0.54)	0.34 (0.29-0.50)	0.035
Sick leave days	14 (7-30)	120 (70-165)	30 (14-97)	< 0.001
Time to activity (d)	10.5 (0-21)	90 (60-365)	21 (2-60)	< 0.001
Time to recovery (d)	21 (14-60)	270 (180-[1])	60 (14-365)	0.005

[1]Less than 75% of the patients had recovered at time of follow-up. Values are given as median (interquartile range). BMI: Body mass index; ASA: American Society of Anesthesiologists; P-ALAT: Plasma alanine aminotransferase.

Exocrine pancreatic function

There were no statistically significant differences between the severe and mild groups concerning incidence of steatorrhea (1/14 *vs* 2/26), change in bowel habits (4/14 *vs* 10/25) or the need for pancreatic enzyme supplementa-

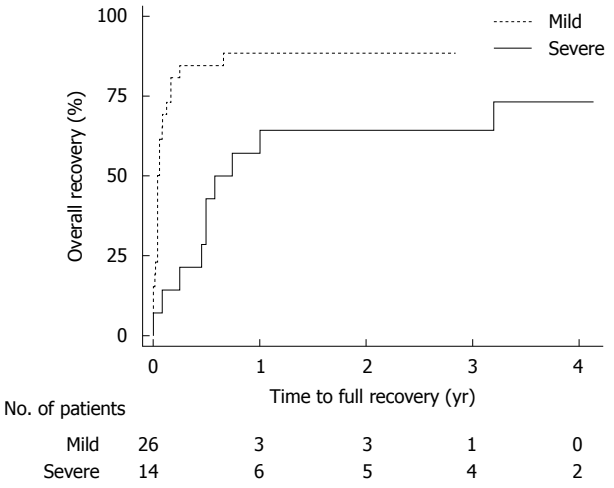

Figure 1 Log-rank test for time to recovery after acute pancreatitis in mild and severe acute pancreatitis patients.

tion (2/14 *vs* 1/26). Most patients had weight loss during the acute disease. At the time of follow-up, 4/14 *vs* 5/26 still had a decreased weight compared to body weight before the disease, with no difference between the groups. A change in diet was more common after SAP (9/14 *vs* 6/26, $P = 0.01$). Fecal elastase-1 was found to be decreased only in one of the patients (with SAP). Plasma albumin did not differ between the groups.

Endocrine pancreatic function

One patient with a history of mild acute pancreatitis was excluded from this part of the follow-up because of type 2 DM, already treated with insulin before the acute pancreatitis episode. Another patient had a history of insulin treatment starting immediately after the SAP episode, but had no treatment for DM for the 9 mo prior to the acute episode. FP glucose was 5.2 mmol/L and the patient was included in the OGTT. FP glucose had a tendency to be higher and plasma glucose was higher after the OGTT in patients with a history of SAP as compared to those with mild acute pancreatitis ($P = 0.055$ and $P = 0.044$, respectively; Figure 2). A higher level was also registered for HBA1C ($P = 0.041$). Patients with a history of SAP more frequently fulfilled the criteria for DM and/or IGT in either FP glucose or 120 min plasma glucose, or both, (11/14 *vs* 11/25, $P = 0.037$). There was no significant difference in the incidence of DM when comparing different etiologies of acute pancreatitis; 4/10 with alcohol as the etiological factor and 3/19 with underlying gallstone disease had DM ($P = 0.193$). The 4 patients subjected to pancreatic surgery all fulfilled the criteria for DM or IGT. Plasma C-peptide was higher in patients fulfilling the criteria for DM, both fasting and after the OGTT, and a significant difference was also seen for serum insulin (Table 3). Fasting C-peptide as well as HOMA-IR had a tendency, although not significant, to be lower in patients with DM and/or IGT after severe as compared to mild disease.

Quality of life

An exact gender- and age-matched reference group (n = 84) was randomly selected for the SAP group from the Swedish SF-36 norm database (n = 8930). Six control subjects were used for each patient (quota = 6:1). The numbers of reference subjects were decided from the lowest number representing one study patient (female, 83 years old). The corresponding figures for mild acute pancreatitis had a reference group of 182 persons, and a quota = 7:1 decided from the lowest number representing one study patient (male, 79 years old). No significant differences were

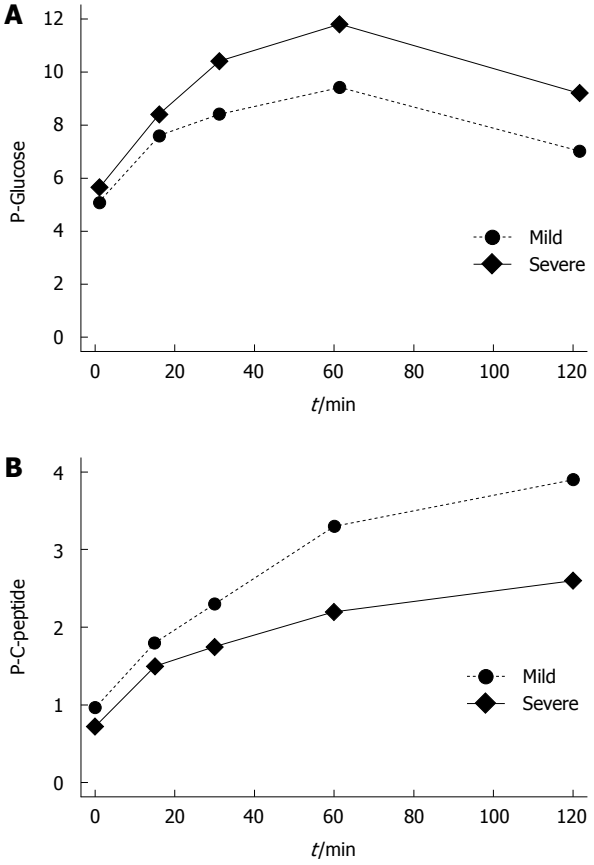

Figure 2 Relationship between mild and severe acute pancreatitis patients in the oral glucose tolerance test evaluating plasma glucose (mmol/L, A) and C-peptide values (nmol/L, B).

Table 3 Endocrine parameters in patients classified as having diabetes according to the World Health Organization definition and patients not fulfilling the criteria

Parameter	Diabetic patients (n = 9)	Non-diabetic patients (n = 30)	Difference between groups (P)
Fasting plasma glucose, 0 min[1]	6.9 (6.0-7.3)	5.1 (4.6-5.5)	< 0.001
Plasma glucose 120 min[1]	13 (12-14)	6.8 (5.5-8.2)	< 0.001
Plasma C-peptide 0 min[2]	1.7 (1.3-2)	0.72 (0.6-1.0)	< 0.001
Plasma C-peptide 120 min[2]	4.8 (4.1-5.9)	2.9 (2.2-4.4)	0.024
Serum insulin 0 min[3]	16 (13-17)	6 (4-9)	0.001
Serum insulin 120 min[3]	103 (79-126)	42 (28-60)	0.001
HOMA-insulin resistance	4.2 (3.7-5.4)	1.3 (0.9-2.2)	< 0.001
HBA1c	5.3 (5.0- 5.6)	4.6 (4.5-4.8)	< 0.001

[1]mmol/L; [2]nmol/L; [3]mIE/L; Values are given in median (interquartile range). HOMA: Homeostasis model assessment; HBA1c: Fasting glycosylated hemoglobin.

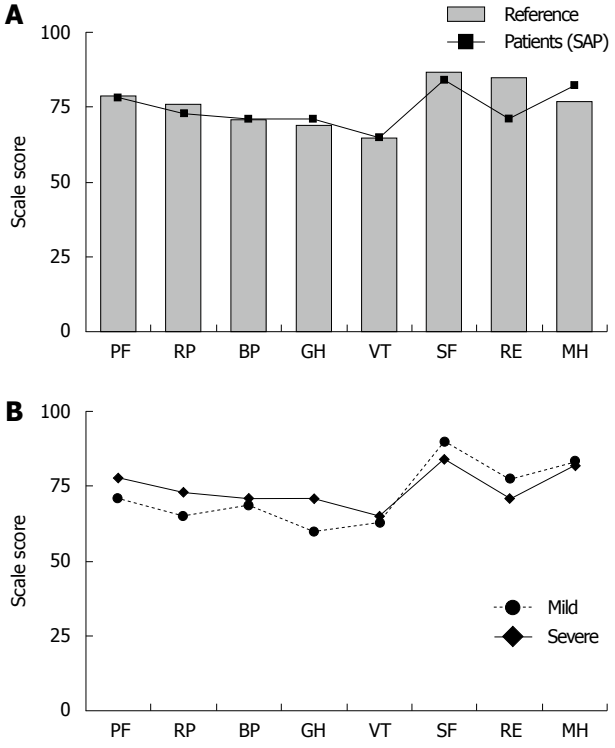

Figure 3 The graphs shows quality of life, measured by Short Form-36, in mild and severe acute pancreatitis patients, comparing age- and gender-matched control group. A: Mean values for patients after mild (black squares) acute pancreatitis and severe (black circles) acute pancreatitis in the 8 Short Form-36 domains; B: Mean value for patients after severe (black squares) acute pancreatitis compared with an age- and gender-matched control group (grey bars) in the 8 SF-36 domains. SAP: Severe acute pancreatitis; PF: Physical function; RP: Role physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role emotional; MH: Mental health.

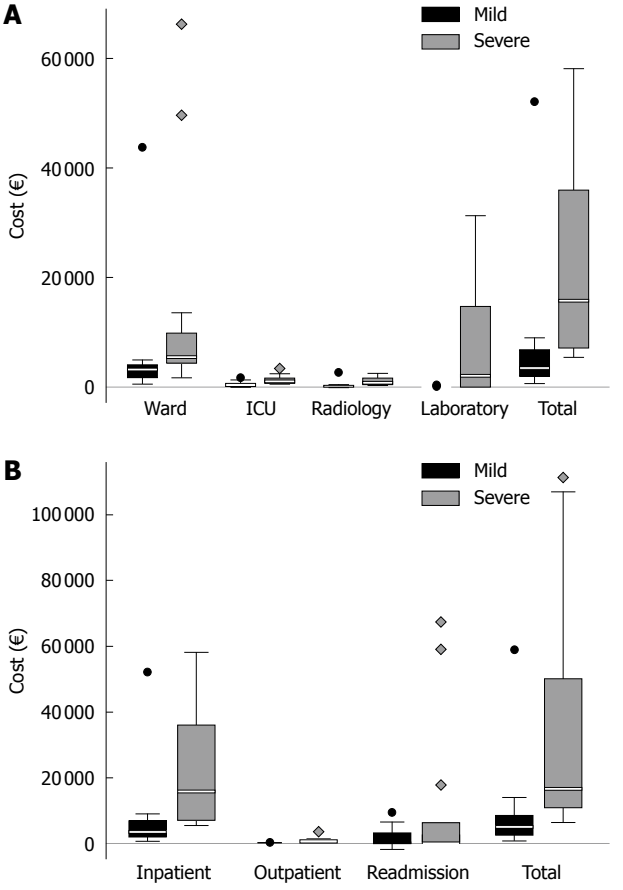

Figure 4 Box-plots showing the specified hospital costs in acute pancreatitis patients. A: In-hospital costs during primary care for acute pancreatitis, in mild and severe acute pancreatitis patients; B: Costs at follow-up, including cost for primary care and all subsequent inpatient and outpatient costs, in mild and severe acute pancreatitis patients. ICU: Intensive care unit.

seen between the groups with a history of severe and mild disease in the 8 SF-36 domains (Figure 3A). When comparing patients with SAP with their respective reference group, no significant differences could be found (Figure 3B).

Costs

There was a pronounced difference between severe and mild acute pancreatitis when comparing costs for the primary hospital stay, a median of €15 774 (€7455-€35 960) in SAP *vs* €3480 (€2049-€6662) in mild acute pancreatitis and a mean of €23 592 ± €18 821 *vs* €5908 ± €9740, ($P < 0.001$, Figure 4A). When including total hospital costs, adding costs for follow-up, both including in-hospital stay and outpatient care, the difference between severe and mild disease was a median of €16 572 (€11 017-€45 619) *vs* €5000 (€2562-€8384) and a mean of €35 427 ± €36 790 *vs* €7536 ± €11 228 ($P < 0.001$, Figure 4B). This means that the severe cases were about 3.3 times more expensive regarding the hospital costs. For the subgroup of SAP patients requiring stay in the ICU ($n = 8$), the total hospital costs, including care for late complications was a median of €30 026 (€17 636-€84 323) and a mean of €49 894 ± €41 905.

DISCUSSION

At the Marseilles symposium on pancreatitis in 1963 it was stated that after recovery complete restitution of the pancreas was the norm[30]. Since then several studies have been performed, mostly on SAP patients, with different follow-up times and measured parameters, with somewhat divergent results. In 1983 Mitchell and coworkers published a study including patients with both mild acute pancreatitis and SAP. All had initial exocrine insufficiency and 80% recovered after 12 mo[8]. The 1984 Marseilles Symposium acknowledged that both exocrine and endocrine function of the pancreas can be disrupted to varying degrees and duration following an acute attack of pancreatitis[31]. There is thus a dysfunction during the initial stage after acute pancreatitis, but functional recovery of the gland is controversial. Full recovery has been described[6], but some dysfunction is the usual scenario, especially after severe disease with necrosis[2,4,9,15].

After surgical treatment, a persistent exocrine insufficiency has been noted in up to 80%-85% of patients[3,4]. Acute pancreatitis with alcohol as an etiological factor may more likely be associated with exocrine impairment[32], though this has not been confirmed by others[9]. Exocrine insufficiency may not necessarily be clinically relevant and evaluation is difficult, particularly when non-invasive methods are used. Invasive tests also have their drawbacks. Furthermore, pancreatic insufficiency is often not

obvious until 90% of the gland has been destroyed. In the present study, only one patient had an objective exocrine insufficiency, as measured by fecal elastase-1. The evaluation of this test has been proven as a highly sensitive and specific pancreatic function tests, even comparable with oral pancreatic function tests, such as the pancreaolauryl test[26,33]. When analyzing the patient's answers regarding change in stool habits, including frequency, mild impairment was common, with a fluctuation over time, and constant steatorrhea at follow-up was uncommon. Medication with pancreatic enzyme supplements was used by a very limited number of patients. Change in diet was also a common answer, which can be due to a number of factors. Thus, there is a grey area concerning when and to what extent an exocrine dysfunction is present and of clinical relevance.

Endocrine dysfunction with glucosuria and elevated blood sugar levels is common during the acute phase of pancreatitis, but is usually self-limiting and resolves. Endocrine dysfunction with DM and IGT is, however, more common over time[9]. DM overall is also known to occur more often after operative treatment[11]. The 4 patients subjected to pancreatic surgery in the present survey all fulfilled the criteria for DM or IGT. We further found DM or IGT in 79% after SAP and in 42% after mild acute pancreatitis. Loss of β-cell function is expected after severe necrotizing disease, and the patients in the present study also had lower C-peptide and insulin levels after SAP as compared with mild disease. Fasting C-peptide and insulin levels were, however, higher in the patients with DM, as compared with the other patients, indicating that insulin resistance is an additional and important explanation for the development of DM. The insulin resistance was obvious, not only with hyperinsulinemia, but also with an elevated HOMA-IR in both groups, with a tendency to be more pronounced after mild disease. The present rate of DM and IGT found at follow-up in acute pancreatitis patients is much higher than expected in the population. Insulin resistance is furthermore associated with different conditions such as obesity, liver failure and inflammatory conditions, though patients in the present survey did not fulfill any of these criteria. Insulin resistance is a prominent feature in patients after pancreatic resection[34], implying the coexistence of pancreatic damage and hyperinsulinemia[5,35]. The pathophysiological mechanisms involved are, however, not clarified. The result in the present study indicates that the insufficiency after acute pancreatitis may be an underestimated problem. When taking the risk for untreated DM patients with poor metabolic control into consideration, a follow-up of these patients may be more important than hitherto proposed.

In follow-up studies, the focus has been on pancreatic dysfunction and less attention has been paid to other aspects of recovery and long-term detriment. With an increasing number of patients surviving SAP, more attention has been directed towards quality of life and long-term outcomes. Until recently, results of treatment strategies were determined only in terms of death, disability and cure. Quality of life as an outcome measure has only sporadically been reported, with a tendency for, or a statistically significant, reduced quality of life after acute pancreatitis[12,15-17]. In the present study, SF-36 was used, which in 36 questions, divided into 8 scales, estimates both function and wellbeing, and can be summarized as health-related quality of life. In a Finnish study, a difference was seen in the SF-36 general health domain, but the conclusion was that there were no clinically significant differences in quality of life compared with that of the normal population[14]. This is in accordance with our results, where no difference was noted between groups, nor between these and the normal reference population. After debridement for pancreatic necrosis, quality of life has been shown to far exceed that noted in patients with other severe medical diseases, such as congestive heart failure and severe hypertension[12]. It may be that experiencing critical illness, such as SAP, might change the opinion about what is really important in life, contributing to explaining why some patients feel well, despite possible persisting restrictions in daily life activities and not being entirely recovered.

Only a few reports on cost analyses in acute pancreatitis have been made, mainly focusing on severe cases[15,20]. One study has estimated hospital costs for acute pancreatitis patients, regardless of severity grade, with figures obtained from a large national hospital database, reporting a cost of $9870 per hospitalization and a good long-term outcome[19]. In the present study, we calculated not only costs for the primary admission but also additional hospital costs directly related to the pancreatitis episode. Data on total hospital costs has not previously been presented. Expressed as percentages, the increase was highest in the mild group due to subsequent treatment of gallstone disease. This is an important aspect that can possibly be optimized with less resource use. The additional costs after SAP showed a wide spread with a few patients requiring a great deal of resources. Reports on when the patients return to daily activity and work are also limited. Doepel *et al*[11] found that 84% of patients who were working the year before the onset of SAP returned to work. In the present study, corresponding figures are higher, but still the return to work in many cases took a long time after SAP, with the possibility that some patients, despite surviving the acute disease, are never able to return to a normal life and work again. The time until patients felt recovered varied between patients and groups, but overall it took a long time, with several patients not being subjectively recovered, but back in full time work. Patients not recovered at the time of follow-up were mainly, but not exclusively, found in the group recovering from SAP.

Generally, reports in the literature concerning follow-up after acute pancreatitis have limitations and have led to contradictory results. This is due to a number of factors existing in almost all studies, e.g. a limited total number of patients, absence of agreement on a classification system describing the severity of the disease, differences in the proportions concerning e.g. etiology, different criteria for IGT and DM, different tests used to evaluate exocrine

insufficiency and different instruments to evaluate quality of life. The follow-up time also varied widely[36].

In the present study, most of the patients with a history of SAP had a major need to talk and discuss what actually had happened during their hospital stay and during the follow-up period. In the group with mild acute pancreatitis, a number of patients even had to remind themselves that they really had been ill a few years previously. A structured follow-up plan for patients that have undergone SAP, dealing with physiological aspects, including information on signs of exocrine insufficiency and the possible benefits of controlling blood glucose levels, could be of great benefit.

A weakness in the present study is the limited number of patients. A strength, however, is the attempt to make a complete follow-up including several important factors, and that a strict definition of SAP has been used. Another strength is that the same surgeon evaluated all patients at the follow-up in the outpatient clinic.

In conclusion, the present study presents a thorough long-term evaluation concerning several aspects associated with severe and mild acute pancreatitis. The results point at an impairment in endocrine function and also a subtle exocrine dysfunction. Sick leave and time until the patients recover can be long and the disease is associated with high costs for society, especially after SAP. The quality of life both after severe and mild acute pancreatitis is, however, as good as in the normal population years after the disease.

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COMMENTS

Background

Acute pancreatitis is associated with a mild and uneventful course in 80% of patients. However, severe acute pancreatitis is associated with the risk of complications both in and around the pancreas. Long-term exocrine and endocrine pancreatic function has been studied with divergent results. Morphological changes may often remain and functional recovery is not always complete. Additional important outcome measures are quality of life and health economical aspects.

Research frontiers

Long-term evaluation of patients after acute pancreatitis has mainly been focused on the severe disease with divergent results concerning pancreatic function and quality of life. Endocrine and exocrine dysfunction usually occurs. Only a few cost analyses have been published hitherto.

Innovations and breakthroughs

In this study, the authors found that the incidence of endocrine dysfunction was high but clinically important exocrine dysfunction limited after both severe and mild acute pancreatitis. The recovery was long especially after the severe form, but the quality of life was excellent. The hospital costs were high after severe acute pancreatitis, with a large spread within the group.

Applications

It is important to be aware of the high incidence of endocrine dysfunction, and a structured follow-up plan can be of importance. A good long time quality of life is important information for the patients and relatives during the demanding severe disease.

Peer review

The present paper is interesting and well written. Number of admitted patients are not so numerous but well selected and stratified.

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BRIEF ARTICLE

Pulmonary involvement in inflammatory bowel disease

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Abstract

AIM: To determine the relationship of pulmonary abnormalities and bowel disease activity in inflammatory bowel disease (IBD).

METHODS: Thirty ulcerative colitis (UC) and nine Crohn's disease patients, and 20 control subjects were enrolled in this prospective study. Detailed clinical information was obtained. Extent and activity of the bowel disease were established endoscopically. Each patient underwent pulmonary function tests and high-resolution computed tomography (HRCT). Blood samples for measurement of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), angiotensin converting enzyme and total IgE were delivered by the patients.

RESULTS: Ten (25.6%) patients had respiratory symptoms. A pulmonary function abnormality was present in 22 of 39 patients. Among all patients, the most prevalent abnormalities in lung functions were a decrease in forced expiratory volume in 1 s (FEV1), FEV1/forced vital capacity (FVC), forced expiratory flow (FEF) 25%-75%, transfer coefficient for carbon monoxide (DLCO), DLCO/alveolar volume. Increased respiratory symptoms score was associated with high endoscopic activity index in UC patients. Endoscopic and clinical activities in UC patients were correlated with FEV1, FEV1/FVC, and FEF 25%-75%. Smoking status, duration of disease and medication were not correlated with pulmonary physiological test results, HRCT abnormalities, clinical/endoscopic disease activity, CRP, ESR or total IgE level or body mass index.

CONCLUSION: It is important that respiratory manifestations are recognized and treated early in IBD. Otherwise, they can lead to destructive and irreversible changes in the airway wall.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; High-resolution computed tomography; Pulmonary function tests; Lung diseases

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that commonly involves the gastrointes-

tinal tract, and it is of unknown etiology. Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of chronic IBD. Extraintestinal manifestations are very common: dermatological manifestations, erythema nodosum and pyoderma gangrenosum; ocular manifestations, uveitis and episcleritis; hepatobiliary manifestations, primary sclerosing cholangitis and autoimmune hepatitis; musculoskeletal manifestations, peripheral arthritis and axial arthropathy[1]. In contrast, pulmonary involvement is rare. A relationship between pulmonary disease and IBD was suggested 40 years ago. Respiratory involvement in IBD is disclosed with some pathophysiological mechanisms: both the colonic and respiratory epithelia share embryonic origin from the primitive foregut, and both types of epithelial cells include goblet cells and submucosal glands; and the lungs and gastrointestinal tract contain submucosal lymphoid tissue and play crucial roles in host mucosal defense. The similarity in the mucosal immune system causes the same pathogenetic changes. The aberrations in both innate and acquired immunity that are involved in the pathogenesis of IBD are complex and still incompletely understood[2]. The patterns of involvement in IBD are[2,3]: (1) upper airway: glottic/subglottic stenosis, tracheal inflammation and stenosis; (2) bronchi: chronic bronchitis, bronchiectasis, and chronic bronchial suppuration; (3) small airways: bronchiolitis obliterans, bronchiolitis, and diffuse pan-bronchiolitis; (4) lung parenchyma: bronchiolitis obliterans-organizing pneumonia, nonspecific interstitial pneumonia, granulomatous interstitial lung disease, desquamative interstitial pneumonitis, pulmonary infiltrates and eosinophilia, and sterile necrobiotic nodules; (5) sarcoidosis, α1 antitrypsin deficiency; (6) pulmonary vascular disease; Wegener's granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis, and pulmonary vasculitis; (7) venous thromboembolism; and (8) serositis: pleural and pericardial manifestations.

The aim of present study was to evaluate pulmonary involvement in IBD. For this, we examined frequency of respiratory symptoms, pulmonary function tests, bronchial hyperreactivity, high-resolution computed tomography (HRCT), serum angiotensin-converting enzyme (ACE), C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR).

MATERIALS AND METHODS

During a 2-year period from January 2007 to December 2009, 39 consecutive patients with the diagnosis of IBD, who were seen in a gastroenterology clinic, were referred to our outpatient clinic. Subjects with the following characteristics were included: age $\geqslant$ 18 years old, CD and UC with endoscopic examination performed in a week.

Subjects with the following characteristics were excluded: lack of compliance in performing lung function tests, age < 18 years old, history of previous lung disease, history of atopy or familial atopy, peripheral eosinophilia, and obesity [body mass index (BMI) > 30 kg/m^2].

Thirty UC and nine CD patients were enrolled in this prospective study. Age- and sex-matched normal controls (20 subjects) were recruited from healthy volunteers. The detailed anamnesis of the subjects (age, sex, cigarette pack/years, family history, occupational history) was gathered. Duration of disease from the date of first endoscopic diagnosis and maximal extent of endoscopic diagnosis were recorded. The extent of the bowel disease was defined as pancolitis when the entire colon was involved; left-side colitis when the bowel from the hepatic flexure to the rectum was involved; and distal colitis when the sigmoid colon and rectum were involved. Patients with CD were classified with colon involvement, small bowel involvement, or ileocecal involvement. In patients with UC, the clinical activity of the disease was assessed using the Truelove score[4]: mild was considered to be in remission, and patients with moderate and severe indices had active disease. Endoscopic activity was assessed by videocolonoscopy (Fujinon EC 450/WL, Tokyo, Japan). All colonoscopic examinations were performed by an experienced investigator. The Rachmilewitz endoscopic activity index for UC was used to assess disease activity[5]. CD activity was assessed on the basis of clinical and endoscopic features[6]. Smoking habit was also recorded, however, most of our patients were nonsmokers or former smokers. Symptoms of cough, sputum, wheezing and breathlessness were scored out of a maximum of 2: 0 = no symptoms; 1 = intermittent symptoms; and 2 = regular symptoms. The total symptom score (maximum of 8) for each patient was derived from the sum of the individual symptom scores. A total symptom score of $\geqslant$ 3 points was assessed as "respiratory symptom is present" or "symptomatic"[7]. Blood samples for measurement of CRP, ESR, ACE and total IgE were delivered by the patients prior to endoscopy.

Pulmonary function testing

Each patient underwent standard pulmonary function tests for forced expiratory volume in 1 s (FEV1), vital capacity, forced vital capacity (FVC), and transfer coefficient for carbon monoxide (DLCO) measured by means of the single-breath test. Account was also taken of the hemoglobin value when calculating the DLCO. The results were compared with those of age- and sex-matched controls and expressed as a percentage of predicted values. Pulmonary function test indices were measured with a Sensormedics V max 229 (Sensormedics, Yonda Linda, CA, USA) series flow-sensitive spirometer. The limitation of our study was that lung volumes could not be measured. Bronchial hyperresponsiveness (BHR) (PD_{20}, dose of methacholine that caused a 20% fall in FEV_1) was measured in the morning with the methacholine challenge test using the dosimeter method according to ERS task force in all IBD patients[8]. In patients with high IgE level, the existence of an atopic state was evaluated by skin prick test using common allergen extracts (grass, tree and weed pollens; house dust mites; molds; cat and dog extracts), and reactions at least 3 mm greater then negative control test were regarded as positive (Stallergenes, Antony cedex, France). Histamine was used as a positive control.

HRCT

All CT scans were obtained with a scanner (Siemens Somatom Emotion, Germany). Images were acquired during inspiration. CT scans were evaluated by an independent investigator who was blinded to the results of the pulmonary function tests and clinical data. The individual features evaluated included the following: bronchiectasis, bronchial wall thickening, ground-glass opacification, emphysema and cysts.

Ethical considerations

Informed consent was obtained from all patients and control subjects and the study was approved by the local ethical committee.

RESULTS

Patient description

The characteristics of the control group and the 39 patients with UC and CD are shown in Table 1. Twenty-three male and 16 female (59%, 41%) patients, as well as 20 healthy controls, with mean ages of 44.28 ± 12.85 years and 39.50 ± 12.47 years, respectively, were recruited to the study. The mean duration of disease was 39.07 ± 29.38 mo. Thirty individuals were never smokers, five were ex-smokers and four were smokers. Control patients were nonsmokers. None of our patients had an occupational history or family history of respiratory disease and atopy. Fifteen (50%) UC and four (44.4%) CD patients had clinically active bowel disease at the time of the study. Of the 39 patients, 33 were receiving sulfasalazine, one azathioprine, and five sulfasalazine plus azathioprine. None of the patients had extraintestinal manifestations other than pulmonary involvement.

Twenty-five (64.10%) patients had HRCT abnormalities (Table 2). Ten (25.6%) patients had respiratory symptoms. In 16 (41%) patients, CRP level was elevated, and in 26 (66.7%), ESR was increased. Four (10.3%) patients had high levels of total IgE, and in these patients, skin prick tests were negative, and in one patient, weak BHR was observed.

Pulmonary function tests

Three (7.69%) patients had obstructive dysfunction and small airway obstruction was reported in 17 (43.58%). Two patients (5.12%) had restrictive dysfunction. When comparing all IBD patients with controls, we found statistically significant differences for FEV1, FEV1/FVC, FEF 25%-75%, DLCO and DLCO/alveolar volume (VA) ($P < 0.05$) (Table 3).

Correlation between pulmonary function parameters, clinical characteristics and HRCT features

The correlation of pulmonary function and endoscopic and clinical disease activity is shown in Tables 4 and 5. The most prevalent abnormality was a decrease in FEF 25%-75% in patients with CD and endoscopically and clinically active UC. The impairment in FEV1 and FEV1/FVC was significant and more pronounced in patients with active UC *vs* controls. In 10 (33.3%) patients with UC, the endoscopic activity index was high and correlated significantly with pulmonary symptom scores ($P < 0.05$). There was no significant correlation between smoking status and pulmonary physiological test results, HRCT abnormalities or clinical/endoscopic disease activity. Also, no relationship was found between disease activity and HRCT abnormalities, respiratory symptoms, CRP, total IgE level, ESR or BMI. There was no relationship between duration of disease and pulmonary physiological test results, HRCT abnormalities, CRP, total IgE level or ESR. There was no correlation between BMI and pulmonary function.

Table 1 Characteristics of the control and patient groups with inflammatory bowel disease

	UC	CD	Controls
n	30	9	20
Sex (M/F)	22/8	1/8	10/10
Mean age (yr)	43 ± 3	46 ± 2	39.50 ± 12.47
Smoking (smoker/never/ex-smoker)	4/22/4	-	-
Duration and range of bowel disease (yr) (mean ± SD)	3 ± 0.5 (0-9)	3 ± 0.5 (0.5-4)	
Respiratory symptom (present/absent)	9/21	1/8	0/20
Disease activity (active/remission)	14/16	5/4	

UC: Ulcerative colitis; CD: Crohn's disease.

Table 2 Findings on high-resolution computed tomography in patients with inflammatory bowel disease

Findings on HRCT	*n*
Normal	14
Peribronchial thickness	15
Bronchiectasis	2
Ground-glass opacity	8
Emphysema	9
Air cysts	1
Reticulonodular opacity	1

HRCT: High-resolution computed tomography.

Table 3 Correlations of pulmonary function tests with inflammatory bowel disease and controls

	UC (*n* = 30)	CD (*n* = 9)	Controls (*n* = 20)
FEV1	86.87 ± 15.09[a]	85.89 ± 13.75	95.75 ± 11.56
FVC	88.37 ± 15.80	93.22 ± 8.90	96.40 ± 10.00
FEV1/FVC	79.67 ± 8.98[a]	78.56 ± 11.11	84.15 ± 4.21
FEF 25%-75%	73.93 ± 21.38	64.89 ± 21.23[a]	85.00 ± 11.85
DLCO	96.43 ± 12.84[a]	90.67 ± 19.88[a]	103.5 ± 11.90
DLCO/VA	104.83 ± 16.99	93.00 ± 17.85[a]	112.95 ± 10.22

[a]$P < 0.05$ *vs* control group statistically significant. UC: Ulcerative colitis; CD: Crohn's disease; FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; FEF: Forced expiratory flow; DLCO: Transfer coefficient for carbon monoxide; VA: Alveolar volume.

Table 4 Correlation of pulmonary function tests between endoscopically and clinically active Crohn's disease with controls

	Endoscopically active (n = 5)	inactive(n = 4)	Clinically active (n = 4)	inactive (n = 5)	Control (n = 20)
FEV1	82 ± 12.28	90.75 ± 7.04	81.25 ± 19.50	89.60 ± 7.37	95.75 ± 11.56
FVC	91.20 ± 7.33	95.75 ± 11.15	92.25 ± 7.68	94.00 ± 10.61	96.40 ± 10.00
FEV1/FVC	75.80 ± 14.60	82 ± 4.08	74.25 ± 16.38	82 ± 3.54	84.15 ± 4.21
FEF 25%-75%	61.80 ± 28.84	68.75 ± 7.54	58.50 ± 32.07	70 ± 7.52	85 ± 11.85[a,c]
DLCO	91.20 ± 15.83	90 ± 26.81	103 ± 13.64	80.80 ± 19.42	103.5 ± 11.90[c]
DLCO/VA	95.40 ± 20.38	90 ± 16.55	102.25 ± 16.15	85.60 ± 16.95	112.95 ± 10.22[a,c]

[a]P < 0.05 comparison between active and control groups statistically significant; [c]P < 0.05 comparison between inactive and control groups statistically significant. FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; FEF: Forced expiratory flow; DLCO: Transfer coefficient for carbon monoxide; VA: Alveolar volume.

Table 5 Correlation of pulmonary function tests between endoscopically and clinically active ulcerative colitis with controls

	Clinically active (n = 15)	inactive (n = 15)	Endoscopically active (n = 20)	inactive (n = 10)	Control (n = 20)
FEV1	86.53 ± 13.15	87.20 ± 17.28	78.10 ± 19.58	91.25 ± 10.26[c]	95.75 ± 11.56[a]
FVC	91.60 ± 14.07	85.13 ± 17.22	86.70 ± 16.49	89.20 ± 15.81	96.40 ± 10.00
FEV1/FVC	78.07 ± 6.22	81.27 ± 11.08	72.70 ± 10.95	83.15 ± 5.28[c]	84.15 ± 4.21[a]
FEF 25%-75%	67.73 ± 19.00	80.13 ± 22.43[c]	53.80 ± 22.30	84 ± 11.93[c]	85 ± 11.85[a,e]
DLCO	97.93 ± 12.88	94.93 ± 13.07	96 ± 13.42	96.65 ± 12.89	103.5 ± 11.90[e]
DLCO/VA	107.67 ± 14.25	102 ± 19.44	106.50 ± 17.28	104 ± 17.24	112.95 ± 10.22[e]

[a]P < 0.05 comparison between active and control groups statistically significant; [c]P < 0.05 comparison between active and inactive groups statistically significant; [e]P < 0.05 comparison between inactive and control groups statistically significant. FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; FEF: Forced expiratory flow; DLCO: Transfer coefficient for carbon monoxide; VA: Alveolar volume.

DISCUSSION

Extraintestinal manifestations of IBD are increasing in developed countries. In 1976, Kraft *et al*[9] described six patients in whom chronic bronchial suppuration had appeared between 3 and 13 years after the onset of IBD. Since then, all respiratory complaints in IBD patients that cannot be explained by other causes have been defined as pulmonary manifestations of the disease. Furthermore, reports of pulmonary manifestations of the disease are increasingly present in the literature. In our patient group, among all patients, the most prevalent abnormalities in lung functions were a decrease in FEV1, FEV1/FVC, FEF 25%-75%, DLCO, and DLCO/VA. Increased respiratory symptom score was associated with high endoscopic activity index in UC patients. The most prevalent abnormality was a decrease in FEF 25%-75% in patients with CD and endoscopically and clinically active UC. The impairment in FEV1 and FEV1/FVC was significant and more pronounced in patients with active UC compared with the controls.

Godet *et al*[10] have studied patients with UC, and pulmonary function test abnormalities were found in 55%, 15/66 subjects had an obstructive pattern, 19 had abnormal diffusion, one had a restrictive pattern, and five had both an obstructive pattern and abnormal diffusion; these alterations could not be predicted by current or past smoking status, family history of respiratory disease, occupational history or current medication use. In our study, 3/39 (7.69%) patients had obstructive dysfunction, two (5.12%) had restrictive dysfunction, and five (12.8%) had abnormal diffusion. These results were not correlated with smoking status. None of our patients had a family or occupational history of respiratory disease.

The influence of disease activity was studied. In a recent study with UC patients, small airway obstruction (as demonstrated by diminished FEF 25%-75%) was reported in the 15 patients (57.6%), restrictive dysfunction in eight (30.7%) and obstructive dysfunction in three (11.5%), and the impairment in pulmonary function tests was significant and more pronounced in patients with active UC compared with the controls[11]. In our study, the most prevalent abnormality was a decrease in FEF 25%-75%, and FEV1/FVC and FEF 25%-75% were significantly lower in patients with active UC. In 10 (33.3%) patients with UC, the endoscopic activity index was high and correlated significantly with pulmonary symptom scores (P < 0.05). These findings suggest a direct pathogenic link with IBD. Tzanakis *et al*[12] have found small airway dysfunction in patients with CD and UC despite their normal baseline spirometric values, and there was no difference between active and nonactive disease.

Chest radiography is often normal in patients with respiratory symptoms and IBD. Bronchiectasis is the classic pulmonary manifestation of IBD, and is noted in 66% of cases of IBD that involve the large airways[2]. Mahadeva *et al*[7] have found bronchiectasis in 13 of 17 patients with IBD, in whom sputum production was present in 10. In contrast, bronchiectasis was identified in only two patients in the present study. In our study, the most frequent find-

ing on HRCT was peribronchial thickness. The most common respiratory association of IBD is inflammation of the airways. Biopsy shows either severe nonspecific chronic inflammation or non-caseating tuberculoid granulomas. These appearances have been associated with those in the bowel, and it is possible that the gut and the lung are both affected because they share common antigens[13]. This inflammation is perceived on HRCT as an increase in bronchial wall thickness or an increase in diameter of pulmonary artery branches. In these patients, bronchial dilatation is commonly present and results from traction by fibrous tissue on the bronchial walls and results in bronchiectasis[14]. Consequently, peribronchial thickness might reflect inflammation, which usually responds well to steroids[15]. In this way, bronchiectasis can be prevented. This finding suggests a direct pathogenic link to IBD as well.

The expiratory HRCT seems to be a limitation in our study and air trapping could have been underestimated. In our series, nine patients had upper lobe emphysema, which was probably related to smoking but there was no significant correlation between smoking status and pulmonary physiological test results, HRCT abnormalities or clinical/endoscopic disease activity.

It is important to consider whether therapy with sulfasalazine or mesalazine could have been responsible for the pulmonary changes. The most common abnormality described in association with sulfasalazine therapy is upper lobe peripheral opacity, although lower lobe opacity, eosinophilic pneumonia, interstitial pneumonitis, bronchiolitis obliterans organizing pneumonia and cavitating nodules have also been reported[16,17]. None of our patients had peripheral blood eosinophilia, which is usually present in lung disease caused by sulfasalazine.

Kuzela *et al*[18] have identified a high incidence of pulmonary function abnormalities (suspicious of interstitial lung disorder) in patients with IBD, despite the lack of radiological abnormalities; 56.7% of patients with UC and 57.7% of those with CD had reduced lung transfer factor. Tzanakis *et al*[12] have shown that DLCO is significantly lower among IBD patients with active gastrointestinal disease than those in remission. Marvisi *et al*[19] have studied 32 patients with UC and found a mild reduction in DLCO and FEF 25%-75%, and the incidence was higher in patients with active disease despite the lack of radiological alterations and pulmonary symptoms. Also, significant differences in mean FVC, FEV1, total lung capacity and FEV1\FVC values were found between patients with active and inactive UC. In our study, DLCO and DLCO/VA were significantly lower among IBD patients, but not correlated with disease activity.

Douglas *et al*[20] have studied 44 IBD patients and found that 48% had unspecified respiratory symptoms. Songür *et al*[21] have found that 16 of 36 IBD patients (44%) in a gastroenterology clinic had symptoms of wheezing, cough, sputum production, or breathlessness. In our study, 25.6% of 39 IBD patients had respiratory symptoms.

The true prevalence of airway inflammation and respiratory and atopic symptoms in IBD remains obscure. Ceyhan *et al*[22] have studied 30 consecutive IBD subjects; allergic symptoms were seen in 14 IBD patients, respiratory symptoms were found in 15, asthma and antiasthmatic drug treatment were noted in three, and BHR was determined in four. They have concluded that allergic symptoms, respiratory symptoms, abnormal lung function tests and skin prick test positivity are more common among IBD patients in comparison with controls, and airway dysfunction is accompanied by atopy. Louis *et al*[23] have shown no correlation between BHR and airway inflammation in IBD patients, in contrast to asthma. In our study, we excluded subjects with atopy and a familial history of atopy, subjects with peripheral eosinophilia. Four (10.3%) patients had high levels of total IgE, and in these patients, skin prick tests were negative and in one patient, weak BHR was observed.

Nutritional status has been shown to have a significant influence on the overall pulmonary function in patients with IBD. Christie and Hill have demonstrated a 35% loss of body protein stores and associated 40% physiological impairment (FEV1, FVC and maximal voluntary ventilation) in patients with acute exacerbations of CD, compared to controls. There was a significant immediate and delayed improvement in these parameters after 2 wk nutritional supplementation, and further improvement on restoration of body proteins during convalescence[24]. Similarly, BMI has been examined as an index of nutritional status in patients with UC and in controls, and a significant positive correlation has been found between BMI and pulmonary function[10]. In our study, 19 patients were overweight (BMI: 25-29.9 kg/m^2), and there was no correlation between BMI and pulmonary function.

In conclusion, both the colonic and respiratory epithelia share embryonic origin from the primitive foregut. The inflammatory lesions seen beneath the bronchial epithelium are similar to those observed beneath the colonic epithelium in IBD. This means that there is inflammation that can be detected early by HRCT and pulmonary function tests. Although most patients have subclinical disease, the pulmonologist must be aware of the multiple potential pulmonary manifestations that can occur in a patient with IBD. Otherwise, they tend to generate persistent and annoying symptoms, and can lead to destructive and irreversible changes in the airway wall, or the "end-stage lung"[3].

COMMENTS

Background

Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of chronic inflammatory bowel disease (IBD), with unknown etiology. Extraintestinal manifestations such as dermatological, ocular, hepatobiliary and musculoskeletal diseases are very common. In contrast, pulmonary involvement is rare.

Research frontiers

Respiratory involvement in IBD is seen with some pathophysiological mechanisms: both the colonic and respiratory epithelia share embryonic origin from the primitive foregut, both types of epithelial cells include goblet cells and submucosal glands, and both the lungs and gastrointestinal tract contain submucosal lymphoid tissue and play crucial roles in host mucosal defense. The similarity in the mucosal immune system causes similar pathogenic changes. The aberrations in both innate and acquired immunity that are involved in the pathogenesis of IBD are complex and still incompletely understood. In this study, pulmonary involvement in IBD was evaluated.

Innovations and breakthroughs

The most prevalent abnormalities in lung functions are a decrease in forced expiratory volume in 1 s (FEV1), FEV1/forced vital capacity, forced expiratory flow 25%-75%, transfer coefficient for carbon monoxide (DLCO), DLCO/alveolar volume. The most frequent finding on high-resolution computed tomography, unlike previous studies, was peribronchial thickness. The most common respiratory association of IBD is inflammation of the airways. Biopsy shows either severe nonspecific chronic inflammation or non-caseating tuberculoid granulomas. These appearances are associated with those in the bowel, and it is possible that the gut and the lung are both affected because they share common antigens. This inflammation is perceived on high-resolution computed tomography as an increase in bronchial wall thickness or an increase in diameter of pulmonary artery branches. In these patients, bronchial dilatation is commonly present and results from traction by fibrous tissue on the bronchial walls, which results in bronchiectasis. Consequently, peribronchial thickness might reflect inflammation, which usually responds well to steroids. In this way, bronchiectasis can be prevented.

Applications

Various pulmonary manifestations can occur in IBD. It is important that respiratory manifestations are recognized and treated early. Otherwise, they might lead to destructive and irreversible changes in the airway wall, or the "end-stage lung".

Peer review

This is a very interesting study and gives us further insight into a disease that may manifest in various systems. Pulmonary disease in IBD has not been extensively studied because the problem is often treated only by gastroenterologists. This is a timely study and could lead to further studies that will help us understand more about this disease.

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BRIEF ARTICLE

Association of p53/p21 expression with cigarette smoking and prognosis in esophageal squamous cell carcinoma patients

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Abstract

AIM: To investigate the expression of p53 and p21 and associations with possible risk factors, such as cigarette smoking, in esophageal squamous cell carcinoma (ESCC) in northeastern Iran, a region with a high incidence of ESCC.

METHODS: The expression of p53 and p21 proteins was investigated immunohistochemically in tumor tissue from 80 ESCC patients and in 60 available paraffin-embedded blocks of adjacent normal specimens from the cases, along with normal esophageal tissue from 80 healthy subjects.

RESULTS: Positive expression of p53 protein was detected in 56.2% (45/80) of ESCC cases, and in none of the normal esophageal tissue of the control group ($P < 0.001$). Furthermore, 73.8% (59/80) of ESCC cases and 43.8% (35/80) of controls had positive expression of p21 protein ($P < 0.001$). Cigarette smoking was significantly associated with p53 over-expression in ESCC cases ($P = 0.010$, OR = 3.64; 95% CI: 1.32-10.02). p21 over-expression was associated with poorer clinical outcome among the ESCC patients ($P = 0.009$).

CONCLUSION: Over-expression of p53 in association with cigarette smoking may play a critical role in ESCC carcinogenesis among this high-risk population of northeastern Iran. Furthermore, p21 over-expression was found to be associated with poor prognosis, specifically in the operable ESCC patients.

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Key words: Esophageal squamous cell carcinoma; p53; p21; Immunohistochemistry; Survival; Smoking

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INTRODUCTION

Esophageal cancer has been reported as the eighth most

common cancer worldwide, with a great variation in the incidence all around the world[1]. A high-risk area for this cancer is known as the so-called "Asian esophageal cancer belt", which stretches from north central China into northern Iran, where esophageal squamous cell carcinoma (ESCC) predominates[2-4].

High incidence and mortality rates of ESCC have been reported in northeastern Iran, due to some distinct, but not well-known, environmental and genetic factors; however the complex network of molecular alterations underlying the development and progression of ESCC have not been clearly elucidated in this region[5,6].

Several studies have revealed that esophageal cancer, as with many other malignancies, is associated with cigarette smoking. However, the specific molecular targets affected by cigarette-derived carcinogens have not been thoroughly identified[7].

It has been shown that the p53 tumor suppressor gene is involved in the control of the cell cycle[8], and it is employed to protect cells exposed to DNA-damaging agents such as environmental risk factors including cigarette smoking[9]. The p53 inactivation in human cancer may result through binding to viral proteins, as a result of *MDM2* or *p19ARF* gene alteration or indirectly by p53 protein localization in the cytoplasm[10]. Furthermore, it has been shown that *p53* mutation is the most common aberration in human cancers, including esophageal carcinoma[11]. These mutations can lead to an increase in the stability of the protein, so it can accumulate in nuclei and be detected by immunohistochemistry methods. Therefore, it has been suggested that all cancer cells have some *p53* aberrations resulting in over-expression of p53[12].

Furthermore, it has been shown that p53 over-expression appears to have a central role in the progression of esophageal cancer in patients who have a positive history of tobacco consumption[7,13-15].

It is generally accepted that esophageal cancer develops through a multi-step process of genetic and epigenetic changes leading to a sequence of histological changes in the epithelia, including esophagitis, basal cell hypertrophia, dysplasia, carcinoma *in situ*, and finally advanced ESCC[13,16-19].

In normal cells, wild-type *p53* up-regulates the expression of several downstream genes to arrest the cell cycle so that damaged DNA either is repaired or apoptosis is promoted in response to DNA damaging agents[20,21]. The product of the mutated *p53* gene has a much longer half-life compared to the wild-type protein. Because of some conformational changes, it is more stable; thus, the accumulation of this protein in the early steps of carcinogenesis is easily detected by immunohistochemical techniques. Previous reports have shown a significant association between *p53* mutations and immunohistochemical p53 nuclear reactivity[19,22-24].

The p21 protein, which is encoded by the *p21*$^{WAF1/Cip1}$ gene, is regulated by wild type, not the mutant, *p53*[25,26]. It inhibits DNA synthesis, as well as the G1/S phase transition, by forming a complex with proliferating cell nuclear antigen and cyclin dependent kinase[27,28]. However, in addition to p53-dependent expression, p21 can be regulated in a p53-independent manner[29,30]. Unlike p53, mutation of the *p21* gene is a rare event in human cancers; therefore, alterations of this gene, involved in carcinogenesis, may be due to some abnormal changes at the expression level rather than genetic coding and epigenetic alterations[31]. Aberrant expression of p21, detected by immunohistochemical staining, has been shown in several cancers, including esophageal cancer, in which both the decrease and increase in p21 expression are reported to be associated with poor prognosis[32-34]. Concerning the clinical relevance of p21 and p53 expression in cancer patients, several studies have indicated that analyzing the combined immunohistochemical expression of these proteins may be more useful in interpreting the favorable and unfavorable clinical outcome than investigating each of them separately[35,36].

The current study was conducted to investigate the immunohistochemical expression of p53 and p21 in 80 ESCC patients in relation to possible risk factors, such as cigarette smoking, and to evaluate whether their expression is a prognostic factor with regard to p53-dependent and -independent pathways.

MATERIALS AND METHODS

Study population

A total of 80 consecutive patients with histologically-confirmed invasive squamous cell carcinoma of the esophagus (45 males and 35 females; mean age 61.39 ± 11.42 years, ranging from 35 to 83 years) were recruited from the two main referral oncology centers in northeastern Iran: Atrak clinic, the main specialized center for upper gastrointestinal (GI) disorders in Golestan province, and Omid Oncology Hospital, referral oncology hospital of northeastern Iran. All eligible subjects were recruited between September 2006 and September 2007 and written informed consents were obtained. The study was approved by the Ethics Committee of Tehran University of Medical Sciences.

The eligibility criteria for the enrolled ESCC patients were: (1) presence of a primary ESCC with no history of concurrent cancer in other organs or history of previous cancer in any organ; and (2) recent diagnosis of ESCC in the patients. Patients who had received any adjuvant therapy (radiotherapy or chemotherapy) were excluded.

Eighty eligible healthy subjects were randomly selected among individuals who were referred to Atrak clinic for upper GI health examination and diagnosed as normal based on physical examination and were histologically proven not to have a cancerous lesion. They were genetically unrelated to the cases and they had no previous cancer history. The control group was matched to the case group by age (± 6 years) and gender. According to a standard questionnaire, the demographic data of each patient and information about social habits, including cigarette smoking and opium use, were collected by an expert member of the research group.

With respect to social habits, never-users were defined as subjects who never or rarely used cigarettes or opium, and ever-users were defined as subjects who had used cigarettes or opium at least weekly for a period of 6 mo or more.

All patients were staged with radiological contrast (barium swallow) and computed tomography (CT) scans of the chest, abdomen, and pelvis. Endoscopic ultrasound and magnetic resonance imaging were performed when available. After the initial staging, patients with potentially operable conditions (defined as stages Ⅱ or ⅢA) proceeded directly to esophageal resection. The rest were categorized as an inoperable subgroup of patients based on their clinicopathological conditions. For those patients who were candidates for surgical resection, pathological stage was determined by histopathology at the time of esophagectomy, on the basis of the Union International Cancer TNM classification guidelines[37].

All 80 ESCC cases were followed up every 3 mo by office visits for clinical evaluation or *via* telephone contact. Deaths caused by ESCC were taken as outcome events, whereas others were considered censored. Survival duration was defined as the time interval from diagnosis to either death or the time of the last clinical evaluation of the patients. The cause of death was determined from the patient's records and death certificate.

Tissue collection

Tumor tissue and corresponding adjacent normal esophageal tissue specimens were obtained from the ESCC patients. All untreated specimen-proven carcinoma of the esophagus in ESCC cases, as well as esophageal normal epithelia of healthy controls, were obtained by esophagectomy or endoscopy procedure. All specimens were fixed and stored in 70% ethanol and embedded in paraffin. Esophageal squamous tumors were comprised of > 70% malignant cells with minimal necrosis, and normal esophageal specimens with no contaminating tumor cells were confirmed as noncancerous tissue by histological examination of a representative hematoxylin and eosin stained slide. Tumors were histologically verified as ESCC and sub-typed based on the grade of differentiation as well differentiated, moderately differentiated or poorly differentiated. Tumor tissue samples were selected so that all adjacent normal esophageal tissues were obtained from the macroscopically normal esophageal epithelium, distant from the cancerous lesion.

Immunohistochemical staining

Tissue sections 4-μm in thickness were obtained from archival alcohol-fixed paraffin-embedded tissues of the esophageal squamous tumor and normal esophageal specimens and mounted on poly-L-lysine-coated slides for immunohistochemistry study. After being dewaxed in xylene and rehydrated in a series of graded alcohols, they were placed in 10 mmol/L citrate buffer pH 6.0 to unmask the epitopes. After microwave antigen retrieval (20 min, 120 W; 3 × 5 min, 450 W), the sections were allowed to cool down to room temperature (approximately 20 min), and then incubated with 3% H_2O_2 for 10 min to quench the endogenous peroxidase activity. After blocking the nonspecific protein binding with serum-free protein block (Dako, Inc.) for 5 min, slides were incubated for 45 min at 37℃ with either anti-human $p21^{waf1/cip1}$ monoclonal antibody (clone SX118, DAKO, CA, USA; dilution 1:50) or anti-p53 monoclonal antibody (clone DO-7; DAKO, CA, USA; dilution 1:50) DO-7 which was raised against an epitope between amino acids 1 and 45 in the C-terminal domain of human wild-type and mutant *p53* recognizing both mutant and wild-type p53 protein, followed by phosphate buffered saline wash. Finally the primary antibody was detected, using EnVision™ + System/HRP, rabbit/mouse (DAB+) (Dako, Denmark), a secondary antibody. Staining was visualized using 3,3'-diaminobenzidine chromogen for 10 min, followed by acidified hematoxylin counterstaining for 1 min. Thereafter, the sections were mounted with mounting medium.

Control sections of known p53-positive and p21-positive cases of ESCC were included in each run, and the negative control section was carried out by omitting the primary antibody. Two expert pathologists who were blinded to the clinical and molecular results evaluated the tissue slides, independently. The final result was obtained through the consensus between the pathologists. Only staining of the cell nucleus was considered as a positive reaction for both $p21^{WAF1/CIP1}$ and p53 proteins (Figures 1 and 2). For p21 protein, the expression of p21 was graded as negative staining, < 10%; intermediate staining or low-expression, 10%-49%; high staining or over-expression, ⩾ 50%. The p53-negative expression was defined as less than 5% of p53 immunoreactivity, and p53-positive expression was classified into two groups according to the percentage of positive nuclei (5%-49%, intermediate staining or low-expression; strong staining or over-expression, ⩾ 50%). The median value for each p53 or p21 immunostaining (50%) was used as the cut off point for over-expression[24,38-40]. We also considered the adjacent non-neoplastic squamous epithelia to compare the positive staining in tumors.

Statistical analysis

The Statistical Package for the Social Sciences software version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The associations between p53 or p21 expression, clinicopathological parameters, and related risk factors were evaluated by the χ^2 test and Fisher's exact test in univariate analysis, and by logistic regression modeling in multivariate analysis. Prognostic factors were evaluated at the univariate level using the Kaplan-Meier method with log-rank test, and in multivariate analysis using the Cox's proportional hazards model of relevant prognostic variables. A 2-sided P value < 0.05 was considered as significant statistically.

RESULTS

A total of 80 ESCC cases (45 males and 35 females; mean age 61.39 ± 11.42 years, ranging from 35 to 83 years) and 80 healthy controls (48 males and 32 females; mean age

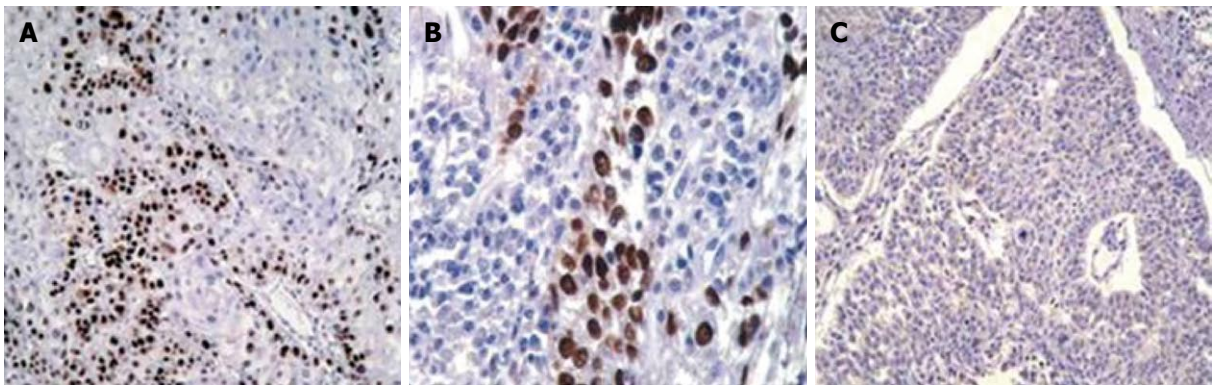

Figure 1 Immunohistochemical staining for *p53* in an esophageal squamous cell carcinoma exhibiting expression. A: With primary antibody, showing reactivity (brown nuclear staining of some tumor cells) (× 100); B: With primary antibody, showing reactivity (× 400); C: With primary antibody, no reactivity (× 100). Sections were counter-stained with hematoxylin.

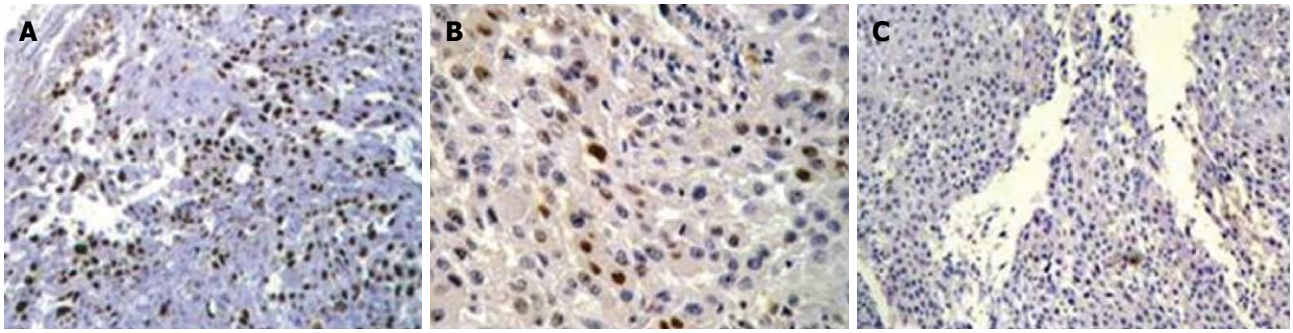

Figure 2 Immunohistochemical staining for *p21* in an esophageal squamous cell carcinoma exhibiting expression. A: With primary antibody, showing reactivity (brown nuclear staining of some tumor cells) (× 100); B: With primary antibody, showing reactivity (× 400); C: With primary antibody, no reactivity (× 100). Sections were counter-stained with hematoxylin.

Table 1 Distribution of the demographic variables for esophageal squamous cell carcinoma cases and healthy subjects *n* (%)

	Case	Control	*P* value[1]
Age (yr)			
< 60	36 (45)	27 (33.8)	NS
⩾ 60	44 (55)	53 (66.2)	
Gender			
Male	45 (56.2)	48 (60)	NS
Female	35 (43.8)	32 (40)	
Smoking			
Never	52 (65)	28 (35)	0.03
Ever	64 (80)	16 (20)	
Opium use			
Ever	21 (26.2)	18 (22.5)	NS
Never	59 (73.8)	62 (77.5)	
p53 expression			
Positive	45 (56.2)	0 (0)	< 0.001
Negative	35 (43.8)	80 (100)	
p21 expression			
Positive	59 (73.8)	37 (46.2)	< 0.001
Negative	21 (26.2)	43 (53.8)	

[1]Not statistically significant by χ^2 test. NS: Not significant.

62.81 ± 10.36 years, ranging from 32 to 84 years) were examined in this study. The distribution of demographic variables for the cases and controls are summarized in Table 1.

Analysis of protein expression by immunohistochemical staining

Expression of p53 protein in ESCC: Positive expression of p53 protein (Figure 1) was detected in 56.2% (45/80) of ESCC cases, and in none of the normal esophageal tissues of the control group ($P < 0.001$). Among the 35 ESCC tumors with p53-negative expression, 31 tumors showed no expression at all. The percentage of p53-positive cells ranged from 0% to 100%, with a mean of 54.6% and a median value of 50%. Of the p53-positive specimens, low-expression of p53 was detected in 21 of 80 cases (26.2%) and p53 over-expression was found in 24 of 80 cases (30%).

In the group of normal adjacent tissue, positive expression of p53 was observed only in the nuclei of basal cells. p53-negative expression was detected in 90% (54/60) of the normal adjacent tumor tissues; whereas, we detected the positive expression of p53 in 10% (6/60) of the normal adjacent tumor tissue samples including 50% (3/6) with dysplastic lesions, one graded as esophageal tissue with moderate to severe esophagitis, and the remaining two were histopathologically normal adjacent tissues.

There was no significant association between tumor and normal adjacent tissues based on p53-positive expression.

Expression of p21 protein in ESCC: Of the 80 ESCC

Table 2 Correlation between p53 and p21 expression in esophageal squamous cell carcinoma cases *n* (%)

p53 expression	p21 expression			Total number
	Negative (< 10%)	Positive		
		Low-expression (10%-49%)	Over-expression (≥ 50%)	
Negative (< 5%)	10/80 (12.5)	15/80 (18.7)	9/80 (11.2)	34
Positive				
Low-expression (5%-49%)	7/80 (8.75)	7/80 (13.6)	9/80 (11.2)	23
Over-expression (≥ 50%)	4/80 (5)	7/80 (13.6)	12/80 (15)	
Total number	21	29	30	80

There was no significant association between p53 and p21 expression among esophageal squamous cell carcinoma cases.

cases assessed in this study, positive expression of p21 protein (Figure 2) was detected in 73.8% (59/80) of ESCC cases, whereas only 43.8% (35/80) of controls had positive expression for p21 protein ($P < 0.001$). In the group of esophageal tumors, the percentage of cells within a section showing definite immunoreactivity varied from 0%-90%. Positive expression of p21 was detected with the average of 42.5% of cells in cases, and 17.5% of cells in controls. The corresponding median values of positive cells were 50% and 15% in the case and control groups, respectively. Twenty-one cases (26.2%) were detected as p21-negative, 29 of 80 (36.3%) cases had intermediate staining (low-expression) of p21 and in 30 of 80 (37.5%) cases, we detected p21 over-expression (high staining). Conversely, the corresponding values were 45 of 80 (56.2%), 35 of 80 (43.8%), and none (0%), for the control group, respectively ($P < 0.001$).

p21-positive nuclei were detected in 45% (27/60) of normal adjacent tissue in ESCC cases. This was significantly lower than in the tumor tissues in the case group ($P = 0.001$).

Comparison of p21 and p53 protein expression in ESCC: Immunohistochemical expression of p53 and p21 varied in the proportion of stained cells and the distribution of positive cells was heterogeneous between cancer nests. Overall, there was no significant correlation between p21 and p53 expression, at all cut off values, among ESCC cases (neither in tumors, nor in the normal adjacent tissues). Combined analysis of p21 and p53 expression has been summarized in Table 2.

Relationship between the expression of p53 and p21 proteins and clinicopathological parameters, including cigarette smoking

The relationship between p53 and p21 protein expression (at any cut off value) and different demographic and clinicopathologic parameters has been analyzed in the whole series of patients, in the p53-negative and p53-positive subgroups, and in the subgroups of patients who did or did not undergo esophagectomy, separately.

In the whole series of ESCC patients, our results showed that p53 or p21 expression was not related to age category, opium use, tumor location, histology of the tumor, depth of tumor invasion, lymph node involvement, or disease stage, when they were simply dichotomized to positive and negative groups, whereas over-expression of the p53 protein was observed in 46.4% (13/28) of ever-smokers but in only 19.2% (10/52) of never-smokers; the difference was statistically significant ($P = 0.01$, OR = 3.64; 95% CI: 1.32-10.02). After controlling for the potential confounding effects of age, sex, opium use, tumor size, tumor location, depth of tumor, lymph node involvement, disease stage, histology of the tumor, and p21 expression, multiple logistic regression analysis showed similar results ($P = 0.03$, OR = 3.89; 95% CI: 1.09-13.89). We did not find any statistically significant association between cigarette smoking and p21 protein expression at any cut off value of 10% or 50%.

In addition, combined analysis of p53 and p21 expression showed that there was no significant correlation between p21/p53 expression and pathological stages or other parameters when we used different cut off values; however, the esophageal tumors only expressing high levels of p21 protein (≥ 50%) (without p53 over-expression), were significantly associated with deep invasion ($P = 0.01$).

The relationship between clinicopathological findings and p53/p21 over-expression is shown in Table 3. We did not detect any significant association between p53 or p21 over-expression with different parameters among ESCC patients, when we compared all the cut off values.

Clinical outcome

All patients were followed up, and survival analysis was performed at the end of the study period in September 2009 (Figure 3). Among the entire patient population, mean survival was 8.21 ± 4.92 mo, with a median of 7.5 mo; ranging from 4 to 24 mo. Of the 80 ESCC patients, 56.2% (45/80) underwent curative esophagectomy, including 73.3% and 26.7% with stage Ⅱ and ⅢA of ESCC, respectively (mean survival, 9.49 ± 5.02 mo; median, 8 mo; ranging from 4 to 24 mo). On the other hand, 43.8% (35/80) of the cases were categorized as inoperable ESCC patients (mean survival, 6.57 ± 4.33 mo; median, 5 mo; ranging from 4 to 17 mo).

Prognosis of ESCC patients according to clinicopathological parameters and p21 and/or p53 protein expression

The overall 6-mo, 1- and 2-year survival rates of the entire group (80 ESCC patients) were 56.7%, 26.7% and 18.6%, respectively.

Table 3 Correlation between clinicopathological parameters and p53 and p21 over-expression in esophageal squamous cell carcinoma patients *n* (%)

	Number	p53 over-expression			p21 over-expression		
		Yes	No	*P* value	Yes	No	*P* value[1]
Age (yr)							
< 60	36	10 (27.8)	26 (72.2)	NS	14 (38.9)	22 (61.1)	NS
⩾ 60	44	13 (29.5)	31 (70.5)		16 (36.4)	28 (63.6)	
Gender							
Male	45	15 (33.3)	30 (66.7)	NS	20 (44.4)	25 (55.6)	NS
Female	35	8 (22.9)	27 (77.1)		10 (28.6)	25 (71.4)	
Smoking							
Ever-user	28	13 (46.4)	15 (53.6)	0.01	14 (50)	14 (50)	0.09
Never-user	52	10 (19.2)	42 (80.8)		16 (30.8)	36 (69.2)	
Differentiation							
Well	46	12 (26.1)	34 (73.9)		20 (43.5)	26 (56.5)	
Moderate	23	8 (34.8)	15 (65.2)		6 (26.1)	17 (73.9)	NS
Poor	11	3 (27.3)	8 (72.7)	NS	4 (36.4)	7 (63.6)	
Tumor site							
Middle	59	19 (32.2)	40 (67.8)	NS	26 (44.1)	33 (55.9)	NS
Lower	20	4 (20)	16 (80)		4 (20)	16 (80)	
Size of tumor (cm)							
< 3	23	8 (34.8)	15 (65.2)		5 (21.7)	18 (78.3)	0.07
⩾ 3	53	15 (28.3)	38 (71.7)	NS	23 (43.4)	30 (56.6)	
Operability							
Operable	45	12 (26.7)	33 (73.3)	NS	17 (37.8)	28 (62.2)	NS
Inoperable	35	11 (31.4)	24 (68.6)		13 (37.1)	22 (62.9)	

[1]Not statistically significant by χ^2 test. NS: Not significant.

Results of the univariate analysis for the whole series of patients showed no influence of p53 protein expression on survival duration, even if different cut off values were considered (5% and 50%). Similarly, no significant association was found between p21 expression and survival duration, using the cut off value of 10%, whereas the 50% cut off value revealed a significant association between p21 over-expression and poor clinical outcome ($P = 0.009$). In a univariate survival analysis for the entire group of cancer patients, there was no significant survival effect for all available clinicopathologic factors for ESCC patients, except for the patients who were aged above 60 years ($P = 0.006$), or those who underwent surgical operation ($P = 0.001$); factors which were significantly associated with poorer prognosis. Our findings also revealed a significantly reduced survival period among the cases with both p21 and p53 over-expressing tumors compared to patients with p21 over-expressing tumors alone (without p53 over-expression), or in those without over-expression of both p21 and p53 proteins ($P < 0.001$).

Furthermore, to analyze the factors related to prognosis according to p53 protein expression (for both 5% and 50% cut off values), univariate and multivariate analysis were performed separately. Among the p53-positive cases, the factors related to poorer clinical outcome consisted of patients with p21 over-expressing tumors ($P = 0.009$) and those who were aged above 60 years ($P = 0.03$).

Additionally, when analyzing clinical outcome according to p53 and p21 expression in 45 patients who underwent surgery, patients with p21 over-expressing tumors showed poorer clinical outcome ($P = 0.01$). This adverse effect was still significant when the study population was restricted to the operable patients with p53 over-expression ($P = 0.004$).

The Cox proportional hazards regression model showed that age categories, surgical operation status (operable or inoperable), and p21 over-expression were independent prognostic factors (Table 4).

DISCUSSION

The significant positive expression of p53 and p21 in the ESCC patients of this studied population, compared with the healthy subjects, revealed that these proteins play an important role in ESCC development in northeastern Iran. Furthermore, we found that p53 over-expression, but not p21, was associated with cigarette smoking habit in the ESCC patients. Contradictory results have been reported regarding the association of p53 protein expression and cigarette smoking. Our finding is consistent with the studies published by Mizobuchi *et al*[7], Montesano *et al*[41] and Cruz *et al*[39], but discordant with the observations of Lam *et al*[42]. Recent studies have shown that various kinds of carcinogens produced by smoked cigarettes might be responsible for different *p53* gene mutations and p53 over-expression; thus, they may play a role in carcinogenesis, including esophageal cancer development[7,43-45]. In this regard, recent evidence from Golestan province (in northeastern Iran) inhabitants showed that moderate to high exposure to polycyclic aromatic hydrocarbon (PAH) components, one of the substances related to cigarette smoke, may be associated with esophageal carcinogenesis[46]. Therefore, it has been hypothesized that continuous exposure to specific carcinogenic components

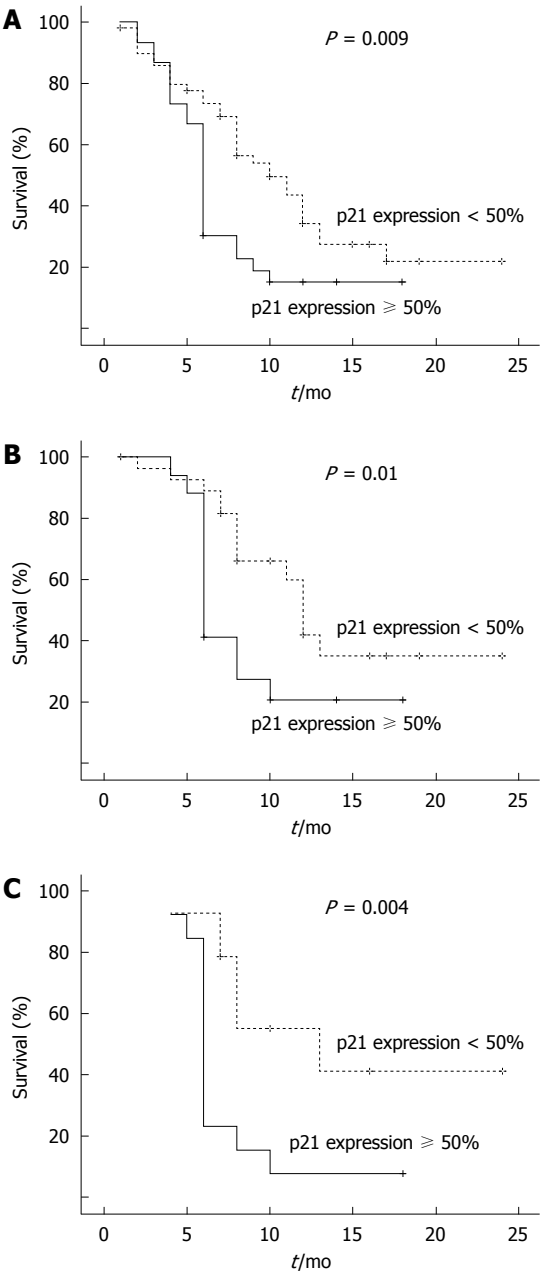

Figure 3 Kaplan-Meier survival curves for esophageal squamous cell carcinoma patients with or without p21 over-expression. A: Overall survival curves were classified by p21 over-expression in the whole series of ESCC patients; B: Survival curves in the operable group of ESCC patients, stratified according to p21 over-expression; C: Survival curves in the operable group of patients with positive expression of p53, stratified according to p21 over-expression.

of tobacco smoke in the studied area, such as PAH, may cause mutations in some important cell cycle genes, such as *p53*, leading to over-expression and abnormal accumulation of the translated proteins. This specific mutation may result in the formation of a dysfunctional protein, which is sequestered and accumulated in the cell, leading to cancer development. These observations provide support for further studies to evaluate the effect of possible carcinogen components of tobacco smoke, such as PAH, in ESCC patients residing in this region.

In the present study, positive expression of p53 protein in the peripheral layers of cancer nests, which are representative of the most proliferative and invasive cells in the esophageal SCC (due to the inactivation by mutation or deregulation of other cancer-related genes in the cell cycle), may suggest that the p53-positive expression is a frequent genetic alteration and plays an important role in the carcinogenesis of esophageal carcinoma in the studied population.

Recent studies have indicated that alteration in the *p53* gene, as well as p53 protein accumulation, is frequently detected in dysplastic or precancerous lesions adjacent to ESCC tumors[47]. In the present study, 10% (6/60) of morphologically normal esophageal specimens adjacent to tumors showed p53 positive staining, including 3 samples with dysplastic lesions, one sample with moderate to severe esophagitis, and another two normal samples with no pathologic change. Although all of these specimens were positive for p21 immunostaining, the frequency and intensity of p21 expression were greater in dysplastic lesions than in the others. The observation of p53-positive expression in the adjacent dysplastic lesions (with or without p53 positivity in corresponding tumor) in this study supports the concept that potentially multiple origins, through similar or independent genetic alterations, may result in the development or recurrence of esophageal tumor in this site (either from the same clone or a different one as a consequence of other involved molecular alterations and pathways). Therefore, it is important to note that in patients, who have had the primary tumor removed, p53 accumulation may be a risk factor for tumor recurrence. This finding may be an important factor for the screening of the ESCC patients in a high-risk population. Therefore, immunohistochemical staining of p53 protein in the remaining unresected normal-appearing esophagus, beyond the normal margin, may be a valuable tool in these patients, to evaluate the risk of developing a secondary ESCC after an esophagectomy. Further prospective, large-scale studies are required as a validation set to support this concept.

Regarding the association between p21 and p53 protein expression in cancer patients, several studies have shown that one of the important ways to investigate the functional status of p53 is to evaluate some of its downstream effectors such as $p21^{Waf1/Cip1}$[48]. Unlike p53, the positive expression of p21 is most often representative of the wild-type protein since no mutations in this gene have been detected in a large number of human tumors[38,49]. p21 protein may be regulated either in a p53-dependent or -independent manner. In our study we found no significant correlation between p53 and p21 proteins, and co-expression of p21 and p53 proteins in a proportion of ESCC cases supports the hypothesis that activation of p21 was regulated through a p53-independent pathway in this series of esophageal tumor samples, in agreement with a previous report by Seta *et al*[50], who showed there was no correlation between the expression of these proteins in esophageal or gastric cancer. Similar results were also reported by Yasui *et al*[51] and Gomyo *et al*[52] for gastric cancer.

Table 4 Log-rank and proportional hazard regression analysis (Cox method) for clinicopathological parameters in esophageal squamous cell carcinoma patients

	Mean survival time (mo)	Log rank *P* value	Cox-regression		
			HR	95% CI	*P* value[1]
Operability					
Operable	12.68 ± 2.56	0.001	2.27	1.30-3.97	0.004
Inoperable	7.08 ± 1.72				
p21 over-expression					
Yes	7.41 ± 1.78	0.009	1.82	1.02-3.25	0.04
No	11.71 ± 2.36				
p53 over-expression					
Yes	8.00 ± 2.06	0.30	1.23	0.67-2.26	NS
No	10.69 ± 2.08				
Tumor size (cm)					
< 3	12.58 ± 3.28	0.06	1.75	0.92-3.32	0.08
⩾ 3	9.43 ± 2.18				
Age (yr)					
< 60	12.61 ± 2.46	0.006	2.30	1.29-4.09	0.005
⩾ 60	8.40 ± 2.14				

[1]Not statistically significant by χ^2 test. NS: Not significant; HR: Hazard ratio; CI: Confidence interval.

Several studies have investigated the significant prognostic impact of p21 over-expression in different cancers, including esophageal carcinoma[35,36,52,53]. However, the results are contradictory[54,55]. The discrepancy in the findings might be due to lack of a standard classification for p21 immunostaining interpretation, or it may depend on different characteristics of malignant cells or different molecular markers regulating p21 expression in a specific tissue or tumor type. In the present study, we adopted the cut off value of 50% nuclear staining to indicate p21 over-expression, as it was applied in some of the previous studies[53]. Using these criteria, our results showed that the prognosis of esophageal cancer patients deteriorates with p21 over-expression (in both univariate and multivariate survival analysis). This is consistent with the study by Sarbia *et al*[53] who showed an adverse survival effect of p21 over-expressing esophageal tumors when they considered the cut off value of 50% as p21 over-expression. Goan *et al*[36] also indicated that p21 over-expression was associated with adverse prognosis in ESCC patients. However, this result contradicts the result of Shimada *et al*[56]. In addition, the adverse survival effect of p21 over-expression in the present study was still significant when the study population was restricted to the patients with p53-positive expression who underwent surgical operation. Some studies have shown that combined analysis of p53 and p21 expression may provide more prognostic information than evaluation of either variable alone[57,58]. In this regard, our findings also revealed a significantly reduced survival period among the cases with both p21 and p53 over-expressing tumors than in patients with p21 over-expressed tumors alone (without p53 over-expression), or than in those without over-expression of both p21 and p53 proteins. This may suggest a possibly more malignant behavior of the tumors when they over-express both p53 and p21 proteins. In other words, patients who harbor p21 over-expressing tumor have a compromised survival that could be superimposed by the adverse effect of non-functional accumulated p53, leading to poorer prognosis.

Concerning the adverse prognostic effect of p21 over-expression, recent studies have shown that despite the role of p21 in cell cycle arrest, this protein could contribute to the inhibition of DNA repair and mitotic control. In the presence of p53 mutation, the adverse survival effect of p21 over-expression could be increased, leading to uncontrolled high expression of p21, as well as sustained genomic instability, leading to facilitation of the progression of the tumor[36,59]. Therefore, this phenomenon may also be responsible for the adverse survival effect of p21 over-expression among the studied population in the present study.

In conclusion, this is the first study focused on evaluating the prognostic effect of p21 and p53 protein expression, as well as their role as a target for cigarette smoking, in ESCC patients in northeastern Iran which is a high-incidence area for this type of cancer. Our results showed that (1) p53 and p21 expression play an important role in ESCC development in northeastern Iran; (2) p53 as a target of cigarette smoking plays a critical role in ESCC development among this high-risk population; (3) the presence of abnormally accumulated p53 in the morphologically normal tissue adjacent to the resected tumor may be a predictor of future recurrence of tumor, thus evaluating the remaining normal esophageal tissue after resection of tumor could help to indicate a population who are at higher risk for tumor recurrence at this site; and finally (4) we indicated the adverse survival effect of p21 over-expression in the ESCC patients of northeastern Iran. Therefore, our data suggest that the immunohistochemical assessment of p21 over-expression, in relation to p53 over-expression, in esophageal cancer patients may provide useful prognostic markers for identifying the subgroup of high risk patients with poor clinical outcome who need closer postoperative follow up, and probably a more intensive therapeutic protocol.

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COMMENTS

Background

The incidence of esophageal squamous cell carcinoma (ESCC) is high in northeastern Iran. This is due to environmental and genetic risk factors. *p53* gene mutation appears to have a central role in the progression of esophageal cancer in patients who have a positive history of cigarette smoking. The current study was conducted to investigate the immunohistochemical expression of p53 and p21 among ESCC patients from northeastern Iran in relation to possible risk factors, such as cigarette smoking, and to evaluate whether their expression is a prognostic factor according to p53-dependent and -independent pathways.

Research frontiers

This study showed that the presence of abnormally accumulated p53 in the morphologically normal tissue adjacent to a resected tumor may be a predictor of future recurrence of the tumor. Also, results show the adverse survival effect of p21 over-expression on the ESCC patients of northeastern Iran.

Innovations and breakthroughs

This is the first study describing progression of ESCC in patients who are resident in northeastern Iran with a positive history of cigarette smoking.

Applications

The results of this study may provide useful prognostic markers, such as p53 and p21 over-expression, for identifying the subgroup of high risk patients with poor clinical outcome who need closer follow up, and probably more intensive therapeutic protocol, during postoperative management.

Peer review

As this author mentioned, over-expression of p53 in immunohistochemistry is generally thought to be mutated p53. Sometimes it is difficult to say whether these all originate from the mutation, because there are other ways of inactivation of p53. The author should try to clarify how the p53 overexpression happened or give more scientific evidence or reference about this point.

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BRIEF ARTICLE

Etiology and long-term outcome of extrahepatic portal vein obstruction in children

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Abstract

AIM: To study the management and outcome of children with extrahepatic portal vein obstruction (EHPVO) in a whole country population.

METHODS: A nationwide multicenter retrospective case series of children with EHPVO was conducted. Data on demographics, radiographic studies, laboratory workup, endoscopic and surgical procedures, growth and development, were extracted from the patients' charts. Characteristics of clinical presentation, etiology of EHPVO, management and outcome were analyzed.

RESULTS: Thirty patients, 13 males and 17 females, 19 (63.3%) Israeli and 11 (36.7%) Palestinians, were included in the analysis. Age at presentation was 4.8 ± 4.6 years, and mean follow-up was 4.9 ± 4.3 years. Associated anomalies were found in 4 patients. The incidence of EHPVO in Israeli children aged 0-14 years was 0.72/million. Risk factors for EHPVO were detected in 13 (43.3%) patients, including 9 patients (30%) with perinatal risk factors, and 4 patients (13.3%) with prothrombotic states: two had low levels of protein S and C, one had lupus anticoagulant, and one was homozygous for methyltetrahydrofolate reductase mutations. In 56.6% of patients, no predisposing factors were found. The most common presenting symptoms were an incidental finding of splenomegaly (43.3%), and upper gastrointestinal bleeding (40%). No differences were found between Israeli and Palestinian children with regard to age at presentation, etiology and clinical symptoms. Bleeding occurred in 18 patients (60%), at a median age of 3 years. Sclerotherapy or esophageal banding was performed in 20 patients. No sclerotherapy complications were reported. Portosystemic shunts were performed in 11 patients (36.6%), at a median age of 11 (range 3-17) years: splenorenal in 9, mesocaval in 1, and a meso-Rex shunt in 1 patient. One patient underwent splenectomy due to severe pancytopenia. Patients were followed up for a median of 3 (range 0.5-15) years. One patient died aged 3 years due to mucopolysaccharidase deficiency type Ⅲ. None of the patients died due to gastrointestinal bleeding.

CONCLUSION: EHPVO is a rare disorder. The etiological factors are still mostly unknown, and the endoscopic and surgical treatment options ensure a good long-term prognosis.

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Key words: Children; Extrahepatic; Obstruction; Outcome; Portal; Vein

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INTRODUCTION

Extrahepatic portal vein obstruction (EHPVO), although rare in children, is an important cause of portal hypertension and upper gastrointestinal (UGI) bleeding in the pediatric age group[1,2]. The etiology of EHPVO is diverse and risk factors are usually detected in less than half of patients, and include perinatal events such as umbilical catheterization and sepsis, and prothrombotic disorders[2-4].

We aimed to study the evaluation, management and outcome of children with EHPVO in a whole country population.

MATERIALS AND METHODS

This study is a multicenter retrospective case series of children with EHPOV diagnosed and followed in all pediatric gastroenterology and hepatology divisions in Israel during the period January 1, 1993 to December 31, 2008. All pediatric gastroenterologists and hepatologists registered in the country were approached by mail and telephone and asked to participate in the study. The patients were allocated *via* the hospital or outpatient clinic archive registrations, including searches for hospitalizations, outpatient visits, radiological, endoscopic and surgical procedures with one or more of the following diagnoses: portal vein, thrombosis, splenomegaly, hepatomegaly, gastrointestinal bleeding, varices, sclerotherapy, ligation, liver biopsy, elevated liver enzymes, and shunt. Patients with portal vein obstruction and portal hypertension due to chronic liver disease were excluded after chart reviews. The referral population included both Israeli and Palestinian children referred to a medical center in Israel from the Gaza Strip and West Bank.

Data on demographics, radiographic studies, laboratory workup, endoscopic and surgical procedures, growth and development, were extracted from the patients' charts.

Table 1 Demographic data, clinical presentation and risk factors for extrahepatic portal vein obstruction in the study group

	n (%)
Gender	
Male	13 (43.3)
Female	17 (56.7)
Ethnicity	
Jewish	15 (50)
Arab	14 (46.6)
Russian	1 (3.3)
Referral	
Israeli	19 (63.3)
Palestinian	11 (36.7)
Gestational age	
Term	23 (76.7)
Preterm	7 (23.3)
Clinical presentation	
Splenomegaly	13 (43.3)
UGI bleeding	12 (40.0)
Cytopenia	5 (16.6)
Elevated liver enzymes	2 (6.6)
Risk factor for EHPVO	
Perinatal events	
Umbilical catheterization	6 (20)
Omphalitis	2 (6.6)
Sepsis, NEC	1 (3.3)
Hypercoagulable state	
APLA syndrome	2 (6.6)
Protein S and C deficiency	1 (3.3)
MTHFR mutation homozygosity	1 (3.3)
Unknown	17 (56.6)

EHPVO: Extrahepatic portal vein obstruction; UGI: Upper gastrointestinal; MTHFR: Methyltetrahydrofolate reductase; APLA: Anti phospholipid antibodies.

Characteristics of clinical presentation, etiology of EHPVO, management and outcome were analyzed.

Statistical analysis

The data were analyzed using Bio-medical P-series[5]. Discrete variables were compared using Fisher's exact test. Continuous variables were compared using the Mann-Whitney *U*-test, since the sample sizes were relatively small. A P-value $\leqslant 0.05$ was considered significant.

RESULTS

Thirty-two children were identified. Two patients were excluded due to missing clinical data, and 30 patients, 13 males and 17 females, were included in the analysis. The demographic data of the patients are presented in Table 1. Fourteen patients (46.6%) were Jewish, 15 (50%) were Arab, and 1 (3.3%) was of Russian descent. Nineteen (63.3%) patients were Israeli and 11 (36.7%) were Palestinians. The age at presentation was 4.8 ± 4.6 years (median 3.5 years, range 1 mo-14 years), and the mean follow-up was 4.9 ± 4.3 years (median 3 years, range 1-15 years). Associated congenital anomalies were found in 4 patients, including: cardiac anomalies (3) and mucopolysaccharidase deficiency type Ⅲ (1).

The diagnosis of EHPVO was based on clinical signs and symptoms of portal hypertension, as well as ultrasonographic findings of portal vein cavernous transformation, in the absence of any chronic liver disease. Additional radiographic evaluations were performed in 8 patients at diagnosis and during follow-up, including computed tomography (CT) with angiography (5), magnetic resonance imaging (MRI) (4), and angiography + venography (2).

Liver biopsy was performed in 20 (66.6%) patients. The histology of 10 biopsies was within normal limits. In 6 biopsies, minor changes including hepatocyte ballooning with mild sinusoidal dilatation were reported, and in 4 biopsies regenerative nodular hyperplasia was found. In these 4 patients EHPVO obstruction was diagnosed by US and CT-angiography or MRI. The mechanism of regenerative nodular hyperplasia may be similar to other hepatic findings resulting from deprivation of portal flow to the liver[4].

Incidence of EHPVO

The calculated incidence in Israeli children aged 0-14 years was 0.72/million. This calculation was based on an average number of children at this age in Israel during the years 1993-2008 (1.85 million, range 1.7-2.0 million). However, it cannot be ruled out that children with no symptoms and no complications of EHPVO were not diagnosed, and that the incidence may be higher.

The incidence in Palestinian children could not be calculated, due to referral bias. Some children may have been referred to other countries for evaluation, or followed-up in local hospitals if no bleeding or other complications occurred.

Etiology of EHPVO

Risk factors for EHPVO were detected in 13 (43.3%) patients (Table 1). Nine patients (30%) had perinatal risk factors including umbilical catheterization (6), omphalitis (2), and neonatal sepsis with necrotizing enterocolitis (1). Detailed prothrombotic profiles were available in 28 patients, including: protein S, protein C, antithrombin Ⅲ, lupus anticoagulant, factor V Leiden and factor Ⅱ mutations and methyltetrahydrofolate reductase (MTHFR) mutations. Janus kinase 2 (JAK2) V617F mutation of the prothrombin gene was assessed in 5 patients. Prothrombotic states were found in 4 patients (13.3%): two had low levels of protein S and C, lupus anticoagulant was positive in one, and one was homozygous for MTHFR mutations. In addition, 2 patients had mildly reduced activity of protein C levels, however, such levels were thought to be secondary.

In the majority of patients, 17 (56.6%), no predisposing factors for EHPVO were found.

Clinical course

The most common presenting symptoms were an incidental finding of splenomegaly on physical examination (43.3%), and UGI bleeding manifested as hematemesis and/or melena (40%). In two patients, failure to thrive with a weight under the 3rd percentile was noted at presentation in addition to the presenting symptoms.

Table 2 Clinical characteristics and course of Israeli and Palestinian children with extrahepatic portal vein obstruction *n* (%)

	Israeli	Palestinian	*P*
Gender (M/F)	11/8	2/9	0.06
Age at diagnosis (yr)			
mean ± SD	6 ± 5.1	3.5 ± 3.8	
Median (range)	7 (0.1-16)	2 (0.75-12)	NS
Etiology			
Perinatal events	4 (21)	4 (36.3)	NS
Hypercoagulability	2 (10.5)	2 (18.2)	
Variceal bleeding	9 (47.3)	9 (81.8)	NS
Follow-up (yr, mean ± SD)	6.5 ± 5.3	3.0 ± 2.2	NS
Surgery	7 (36.8)	4 (36.3)	NS

NS: Not significant.

Table 3 Endoscopic and surgical treatment of children with extrahepatic portal vein obstruction

Procedure	*n* (%)
Sclerotherapy	13 (43.3)
Variceal banding	7 (23.3)
Surgery	
Splenorenal shunt	9 (30.0)
Mesocaval shunt	1 (3.3)
Meso-Rex shunt	1 (3.3)
Splenectomy	1 (3.3)

A comparison between Israeli and Palestinian children did not reveal any significant differences with regard to the age at presentation, etiology and clinical symptoms (Table 2).

Outcome

Overall, 18 patients (60%) had bleeding: 12 (40%) at presentation and an additional 6 patients (20%) during follow-up. The median age at the first bleeding episode was 3 (range 0.75-13) years. Twenty-two patients, who had esophageal varices on upper endoscopy, received propranolol for secondary or primary bleeding prevention. Sclerotherapy or esophageal banding was performed in 20 patients. In 18 of these patients, the procedures were performed during and after bleeding, and in 2 patients banding was performed as primary prevention (Table 3). No complications of sclerotherapy were reported.

Shunt operation was performed in 11 patients (36.6%), at a median age of 11 (range 3-17) years. The indication was uncontrolled bleeding despite variceal banding in 10 patients, and emergency shunt for failure of bleeding control in one. The types of shunt were splenorenal in 9, mesocaval in 1, and meso-Rex in 1 patient. One patient underwent splenectomy due to severe pancytopenia (Table 3).

Patients were followed up for a median of 3 (range 0.5-15) years. One patient died aged 3 years due to mucopolysaccharidase deficiency type Ⅲ. None of the patients died due to gastrointestinal bleeding.

DISCUSSION

The present study summarizes a national experience of EHPVO in children with over 15 years of follow-up. The average incidence of EHPVO in children age 0-14 years in the current study was very low at 0.72 per million. Although EHPVO is an important cause of portal hypertension and gastrointestinal bleeding in children, the exact incidence of the disorder is unknown[6]. The available case series are mostly retrospective, summarizing the experience of one or multiple referral centers[2,7]. The current study is the only nationwide study including all patients diagnosed over a 15-year period, enabling the calculation of EHPVO incidence.

The results are in agreement with previous studies reporting an unknown etiology for EHPVT in over 50% of children[2-4]. Neonatal events, including umbilical catheterization and sepsis, were possible causes of EHPVO in a small number of patients in the current study, as reported by others[2]. In a retrospective study of 133 infants diagnosed with portal vein thrombosis (PVT) within the first month of life, an umbilical catheter was inserted in 73%[8]. Of 29 infants with grade Ⅲ PVT, 62% progressed to portal hypertension. It is, therefore, surprising that umbilical catheterization accounts for a minority of cases of EHPVO in children in different studies[2,7]. Since the mean follow-up period of the infants in the study by Morag *et al*[8] was only 79 d, this may indicate that most of the PVT seen post-umbilical catheterization either resolve or remain without clinical significance.

Venous thrombosis has been associated with thrombophilia[3]. Factor V Leiden mutation, which is the most common inherited cause of thrombophilia, has been described in association with hepatic vein thrombosis, but its association with PVT is questionable[3,9]. A lack of association between factor V Leiden mutation and PVT was reported in 3 studies[7,10,11]. In contrast, a high prevalence of this mutation in children with PVT (6/23 and 12/40 patients) was found in 2 other studies[12,13]. In the current study, one child had factor V Leiden mutation and an umbilical catheter, thus, the exact cause of EHPVO cannot be determined. Inherited deficiencies in protein C, protein S, antithrombin Ⅲ and prothrombin, increase the risk of venous thrombosis and are associated with EHPVO in adults[10,14,15]. Gurakan reported 5 of 12 pediatric patients with EHPVO to have protein C, S, anti thrombin Ⅲ or combined deficiencies[7]. Higher rates were found in Egyptian children - 27.5% had protein C and 2.5% had antithrombin Ⅲ deficiency[13]. However, in a study of 14 patients and their parents, the frequency of protein C, S and antithrombin Ⅲ deficiency was 43% in the PVT patients but none were inherited[16]. Mack *et al*[17] showed, that coagulation defects are pathophysiologic consequences of EHPVO, the consequence of depriving the liver of portal venous flow, and that surgical restoration of intrahepatic portal venous flow corrects the abnormalities. In the current study, 2 patients (6.6%) had low protein C values, one of them combined with protein S deficiency. Antiphospholipid antibody syndrome, identified in one of our patients, may also manifest as arterial or venous thrombosis[18]. An acquired JAK2 mutation (JAK2V617F) was recently reported in the majority of patients with polycythemia vera and essential thrombocytosis[19], and in 36% of adults with EHPVO[20]. In children, JAK2V617F screening was negative in one study[2], and was negative in the 5 patients screened in the current study.

The outcome of children with EHPVO depends on the control of variceal bleeding. Sclerotherapy and banding are effective therapies for bleeding esophageal varices[2,21,22], and may achieve long-term variceal eradication in 50% of patients[2]. Similarly, in the current study, long-term bleeding control was achieved in 50% of bleeding children. Portosystemic shunts were performed in 36.6% of patients, a higher rate than that of 8%-17% reported by others in recent studies[2,6,7]. The rate of surgery was similar for Israeli and Palestinian patients, demonstrating that there was no selection of patients living in remote areas for shunt operation. Most of the patients were followed in large centers, in which all endoscopic techniques are available, and the high surgical rate may reflect patients with more severe disease. The prognosis of patients in the current study was good, with no bleeding or liver related mortality, in agreement with other studies reporting mortality in less than 10% of patients[2,7].

The current study has a few limitations. One is the possibility of under detection in children with no symptoms and no complications of EHPVO, resulting in a lower than actual incidence. Another limitation is the shorter patient follow-up compared with other studies. The median follow-up in the current study was 3 years with a mean of 4.9 years, compared to a median of 6 years in one study[2] and a mean of 7.4 years in another study[7]. As a result, the long-term outcome may be worse than that found in the current study.

In conclusion, although EHPVO is an important cause of portal hypertension in children, it is a rare disorder. The etiological factors are still mostly unknown, and the endoscopic and surgical treatment options ensure a good long-term prognosis.

COMMENTS

Background

Extraintestinal portal vein obstruction (EHPVO), although rare in children, is an important cause of portal hypertension and upper gastrointestinal bleeding from varices in the pediatric age group. It accounts for almost 70% of children with portal hypertension. The etiology of EHPVO is diverse and risk factors are usually detected in less than half of patients and include congenital abnormalities and perinatal events such as exchange transfusions, umbilical catheterization and sepsis, and hypercoagulable states.

Research frontiers

Improvements in the definitions and tests for hypercoagulable states allow more extensive studies on the etiology of EHPVO. Medical control of acute variceal bleeding and long-term endoscopic control of varices by sclerotherapy/ ligation may improve the long-term outcome of children with EHPVO. Surgical options for shunts in patients who fail endoscopic control of bleeding include the recent introduction of the meso-Rex bypass, which results in restoration of normal blood flow to the liver.

Innovations and breakthroughs

Calculation of the incidence of EHPVO in this first national study revealed a low incidence of 0.72/million.

Applications

The long-term outcome of EHPVO in children is good, with low mortality. Variceal bleeding can be controlled in most patients, and shunt surgery is needed in about a third of patients.

Terminology

Portal hypertension caused by extrahepatic portal vein obstruction occurs when the site of the blockage is the portal vein before the blood reaches the liver. A portal cavernoma is usually formed. This disease entity is distinct and not primarily associated with primary liver disease.

Peer review

The study, though it is a retrospective analysis, is informative, well written and gives a good overview on the incidence, underlying causes and treatment of EHPVO in children in Israel.

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BRIEF ARTICLE

Glycemic index, glycemic load and insulinemic index of Chinese starchy foods

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Abstract

AIM: To determine the glycemic index (GI), glycemic load (GL) and insulinemic index (II) of five starchy foods that are commonly used in Chinese diets.

METHODS: Ten healthy subjects aged between 20-30 years were recruited. Each subject was asked to consume 50 g of available carbohydrate portions of test foods and reference food. Finger capillary blood samples were collected at the start of eating and 15, 30, 45, 60, 90 and 120 min after consumption. The GI and II of foods were calculated from the ratio of incremental area under the glucose/insulin response curves of test and reference foods. The GL for each test food was determined from its GI value and carbohydrate content.

RESULTS: The results showed that brown rice elicited the highest postprandial glucose and insulin responses, followed by taro, adlay, yam and mung bean noodles, which produced the lowest. Among the five starchy foods, brown rice evoked the highest GI and GL at 82 ± 0.2 and 18 ± 0.2, followed by taro (69 ± 0.4, 12 ± 0.2), adlay (55 ± 0.4, 10 ± 0.2), yam (52 ± 0.3, 9 ± 0.0) and mung bean noodles (28 ± 0.5, 7 ± 0.2), respectively. The II values of the test foods corresponded with GI values. Similarly, brown rice gave the highest II at 81 ± 0.1, followed by taro (73 ± 0.3), adlay (67 ± 0.3), yam (64 ± 0.5) and mung bean noodles (38 ± 0.3). All five starchy foods had lower GI, GL and II than reference bread ($P < 0.05$).

CONCLUSION: The GI, GL and II values of starchy foods provide important information for the public to manage their diet and could be useful for the prevention of lifestyle-related diseases such as diabetes mellitus.

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Key words: Glycemic response; Glycemic index; Glycemic load; Insulinemic response; Insulinemic index

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INTRODUCTION

Insulin resistance increases the risk of type 2 diabetes[1-3]. One characteristic that can be associated with insulin resistance is hyperinsulinemia that may result in deterioration of β-cell function, which is involved in the pathogenic process of diabetes[4]. In the context of current dietary strategies to prevent hyperinsulinemia and insulin resistance, it is imperative to consider diets/foods in terms of their ability to reduce the degree of postprandial glycemia

and insulinemia[5]. These issues have important public health implications. Any diet to counteract diabetes should be evaluated for its effects on glucose response and insulin secretion. To do this, it is urgent and necessary to continuously determine the glycemic index (GI) and insulinemic index (II) values of foods in different countries, especially the GI of agricultural foods.

GI was introduced to describe the extent to which different foods elicit varying degrees of postprandial blood glucose. It is defined as the incremental area under the 2 h blood glucose response curve (IAUC) after consuming a test food compared to the corresponding area after a carbohydrate-equivalent amount of a reference food (either glucose or white bread)[6,7]. Expanding this theory to the postprandial insulin levels evoked by foods, the II of foods can also be determined from the corresponding incremental blood insulin areas[8]. Because insulin is the hormone that maintains blood glucose homeostasis, a food or diet high in II could induce a higher degree of postprandial insulin concentration and thus result in higher insulin demand in the long term[9,10]. Therefore, it is compulsory to grade foods based on their GI, along with the II, to prevent both postprandial glycemia and insulinemia in humans. Glycemic load (GL), on the other hand, is a concept that summarizes both GI and the carbohydrate content and is considered to represent the overall glycemic effects of a food[11]. Recent studies have shown that increased dietary GL resulted in predictable increases in glycemia and insulinemia in humans[12,13]. Therefore, it is important to evaluate the concept of GI value of foods together with their concurrent II and GL values.

Tubers and cereals have been considered as the main carbohydrate sources in Chinese diets since the early 1960s. They are not only rich in starch, but also contain vitamins, minerals, phytoestrogens, and trace elements. In the agricultural epoch of Taiwan, where rice and grains are considered rare and expensive, people often consume tubers, such as taro and yam, as a main meal or as a rice substitute to help them harness energy for endurance farm work. In the book, "Ben Chou Gun Mu"[14], a very famous Chinese ancient medical book, they were even described as having medical purposes. With rapid development of the economy, however, eating habits and lifestyle in Taiwan are changing. There is some concern that people think it is detrimental to consume tubers and some cereal products because they are high in starch and regular eating may cause hyperpostprandial glucose responses. Some people even avoid grains or tubers in their diets, particularly diabetic patients. Therefore, it is necessary to evaluate these foods according to their glycemic and insulinemic responses, since they are involved in diet management that helps maintain normoglycemia (possibly also maintaining insulin demand). The five most available starchy foods that are controversial regarding their glycemic effects on humans were chosen for this study. The proximate nutritional components and indigestible starch [dietary fiber (DF) + resistant starch (RS)] were also evaluated in this study.

MATERIALS AND METHODS

Ethics

The study was approved by the Institutional Review Board of Kaohsiung Medical University. Informed consent was obtained from each subject before the enrollment.

Study subjects

Ten healthy subjects were selected for the study. The subjects were six females and four males, aged between 20-30 years, with a mean body mass index (BMI) of 20.6 ± 0.6 (BMI ± SE, in kg/m^2). Subjects were recruited based on the following criteria: (1) healthy weight, stable for 6 mo prior to the study; (2) not being on a diet; (3) non-smoker; (4) not taking prescription medication; (5) normotensive; and (6) normal fasting glucose[7]. All subjects were asked to avoid alcohol, legumes and fried foods, eat a regular meal the night before each test, and refrain from unusual eating habits and activity the day before each test. Subjects were also required to complete a food questionnaire before each test to ensure that they had no irregular eating habits. The procedures of the study were orally explained to the subjects, and by written notification.

Test foods

Five starchy foods and one reference food were tested in 50 g available carbohydrate portions. The test foods examined included adlay (*Coix lachryma-jobi L.*), brown rice (variety, Tai Ken #9) (*Oryza sativa L. japonica*), mung bean noodles (glass or cellophane noodles), taro (*Colocasia esculenta L. Schott*) and yam (Chinese sweet potato) (*Ipomoea batatas L. Lam*). Brown rice was manufactured by the Union Rice Company (Taipei, Taiwan). Mung bean noodles were produced by the Longkow Company (Taipei, Taiwan). Taro and yam were purchased from a local farm (Kaohsiung County, Taiwan). Regarding food preparation, brown rice was prepared by a preliminary soaking (the ratio of rice to water was 1:1.5) overnight, and cooked by a rice cooker (Tatung Co., Ltd. Taiwan) right before the tests. Mung bean noodles were boiled. Taro and yam were skin peeled, cut into 5 cm cubes and steamed by the rice cooker (Tatung Co., Ltd. Taiwan). The reference food, white bread, was laboratory made the day prior to the tests.

Experimental procedures

This study was conducted using internationally recognized GI methodology[6,7,15,16]. All subjects were blind to the name of the food being tested. White bread was the reference food (GI = 100%) against which all test foods were compared. Subjects arrived at the laboratory at eight to nine o'clock in the morning after 10-12 h overnight fast. Each subject was fed equivalent 50 g available carbohydrate of test foods or reference food in random order. To minimize day to day variation of glucose tolerance, the reference food was tested in triplicate in each subject. All test and reference foods were served with 220 mL of water. An automatic lancet device (Safe-T-Pro; Roche Diag-

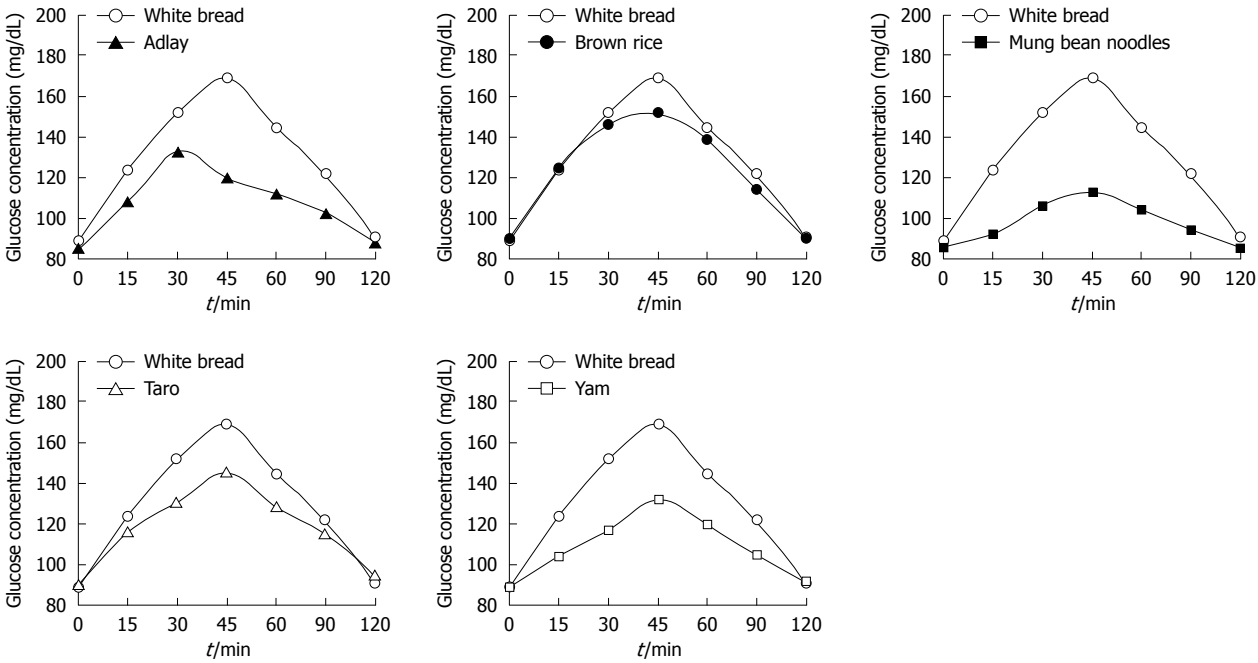

Figure 1 Mean glucose concentrations elicited by five different starchy foods in healthy subjects. Data are expressed as the change in plasma glucose concentration from the fasting baseline concentration.

nostics GmbH Mannheim, Germany) was used to collect finger capillary blood samples (1.5 mL). Blood samples were taken immediately before the start of the study (0 min) and 15, 30, 45, 60, 90 and 120 min after the start of eating. The blood samples were collected in heparinized tubes and centrifuged at 10 500 × *g* for 3 min at 4℃ to obtain plasma. Plasma was spotted onto a slide which contained a reagent layer (glucose oxidase and peroxidase) (Fuji Dri-Chem 3000; Fuji Film, Kanagawa, Japan) and analyzed with an automatic biochemistry analyzer (Fuji Dri-Chem 3000s, Fuji Film, Kanagawa, Japan) for glucose concentrations on each test day. Plasma insulin concentrations were analyzed in duplicate using an enzyme-linked immunosorbent assay (ELISA) with immunoassay kit (Insulin ELISA, Mercodia AB, Uppsala, Sweden) and microplate spectrophotometer (PowerWave XS, Bio Tek, Winooski, VT, USA).

Glycemic and insulin index determinations

The GI/II was calculated from the ratio of the IAUC of the blood glucose/insulin response curve of test food containing 50 g of available carbohydrate and the same amount of reference food (mean IAUC of three reference white bread samples) expressed as a percentage. Because the GI value of white bread is 71 (measured in advance), therefore, the resulting values need to be multiplied by 0.71 in order to convert them to GI values based on glucose[17-19].

Proximate composition analysis

The fat, protein and carbohydrate contents of test foods were analyzed according to AOAC methods[20]. Crude fat was estimated by solvent extraction in a soxhlet apparatus for 14-16 h with petroleum ether. Crude protein was analyzed by determining the total nitrogen in dried food samples using micro-kjeldahl procedures. A factor of 6.25 was used to convert 'N' (nitrogen) value into protein[20]. The analyses of RS + DF were carried out by the method of Onyango and others[21,22] with a slight modification. All measurements were in triplicate.

Statistical analysis

Results are presented as mean ± SE. Insulin concentrations were multiplied by a factor of 6.0 to convert the concentration from mU/L to pmol/L (scientific units). Analysis of variance was performed by using SPSS Windows Release 13.00 (Standard Version, Germany) to determine significant differences. A value of $P < 0.05$ was considered significant.

RESULTS

Postprandial glucose and insulin responses

The study protocol was well tolerated. All 10 subjects completed the study. The mean plasma glucose responses curves for the reference and five test foods are displayed in Figure 1. The reference food produced a large rise in blood glucose during the first 45 min and the greatest overall glycemic response. All test foods had similarity in their peak blood glucose concentrations, except adlay which reached a glycemic peak at 30 min. All test foods, however, varied in their overall glycemic responses. Among the test foods, the brown rice elicited the highest glycemic responses followed by the taro, adlay and yam, and the mung bean noodles produced the lowest. Figure 2 shows the mean plasma insulin response curves for the reference and five test foods. The reference food

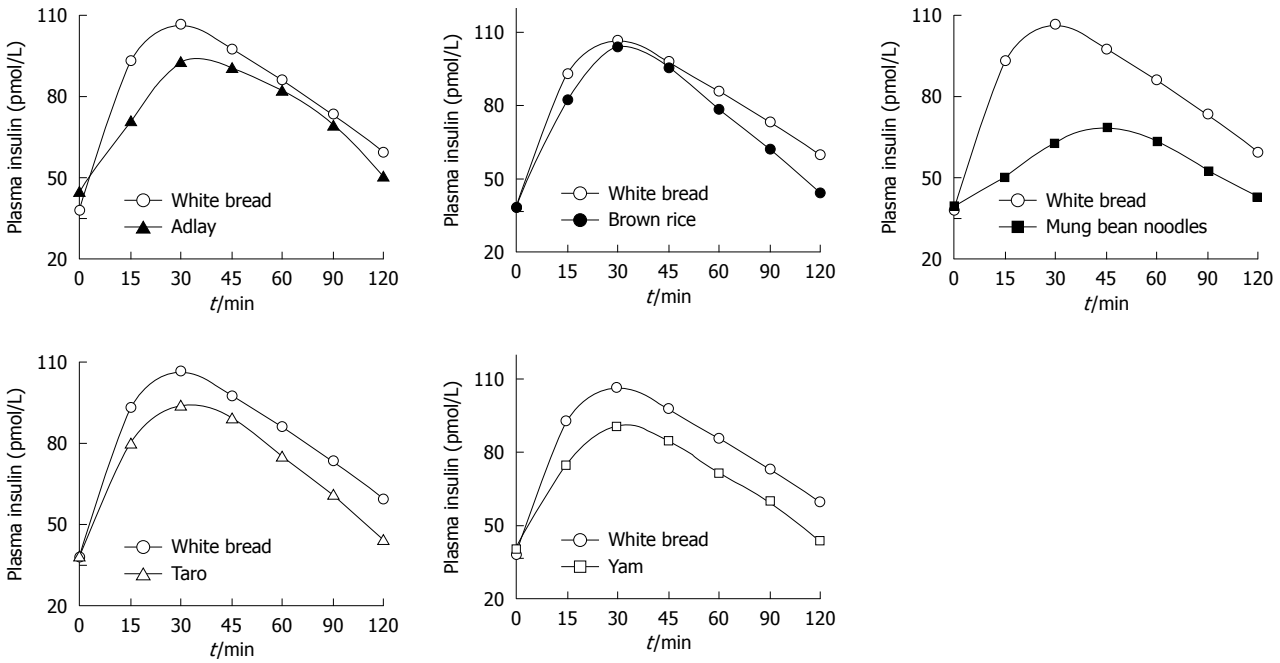

Figure 2 Mean insulin concentrations elicited by five different starchy foods in healthy subjects. Data are expressed as the change in plasma insulin concentration from the fasting baseline concentration.

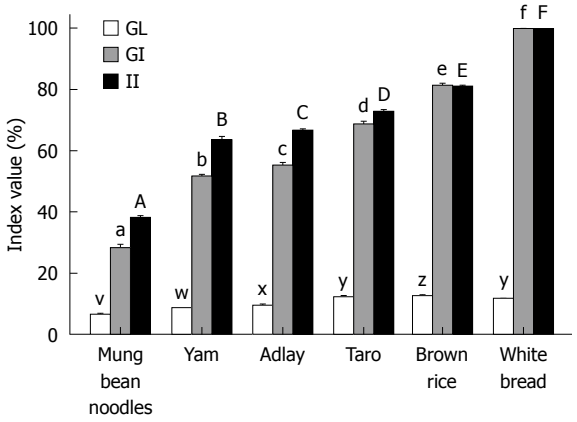

Figure 3 Glycemic index, glycemic load and insulinemic index values of the starchy foods. The mean glycemic index (GI), glycemic load (GL) and insulinemic index (II) for the reference food (white bread) and the five tested starchy foods. For the GI values, columns with different superscripts (a, b, c, d, e, f) are significantly ($P < 0.05$) different. Columns representing the GL values with different superscripts (v, w, x, y, z) are significantly different ($P < 0.05$). Columns representing the II values with different superscripts (A, B, C, D, E, F) are significantly different ($P < 0.05$).

produced the highest peak plasma insulin concentration and the largest overall plasma insulin responses, followed by the brown rice, and the mung bean noodles elicited the lowest plasma insulin responses. All five test foods and the reference food reached their highest response peak at 30 min, except mung bean noodles which reached a peak at 45 min. The plasma insulin responses observed for the five test foods showed a similar profile to their concurrent glycemic responses.

GI, GL and II

The GIs, GLs and IIs of all the test foods are presented in Figure 3 and the classifications of GIs and GLs are showed in Table 1. The mean GI and GL values of the white bread reference were significantly greater ($P < 0.001$) than the mean GI and GL values of each of the test foods. The II values of the test foods corresponded with the GIs. The mean II value of the white bread was significantly higher ($P < 0.05$) than the mean II values of each of the five test foods.

Proximate nutrition components

The nutrient levels and RS + DF are listed in Table 2. The RS + DF content of the yam, mung bean noodles, and adlay was intermediate (15-20 g), whereas the taro and reference white bread was low (9-10 g). We further estimated the caloric values of the test foods from their carbohydrate, fat and protein contents. All five test foods had caloric values ranging from 330 to 384 kcal (= 1379-1605 kJ) per 100 g.

DISCUSSION

The present study evaluates the GI, GL and II of five starchy foods that are traditionally used in the Chinese diet. The results suggest that brown rice produces the highest glycemic and insulinemic responses and has a GI lower than white rice cooked in a rice cooker (i.e. GI = 99-156)[11]. This result is surprising as a characteristic of brown rice is that the thick bran layer retained in brown rice is often composed of higher fiber content than white counterparts. As judged by several reports[23,24], the rate of gastric emptying of starch and digestibility of starch influence the glucose responses and GI values. The effects of fiber and RS on gastric emptying and digestibility have been evaluated in previous studies, showing that fiber and

Table 1 Glycemic index, glycemic load and insulinemic index of the test foods

	Glycemic index[1,3] (%)		Glycemic load[2,3] (g)		Insulinemic index (%)
	mean ± SE	Classification	mean ± SE	Classification	mean ± SE
Adlay	55 ± 0.40	Low	9 ± 0.15	Low	67 ± 0.27
Brown rice	82 ± 0.22	High	18 ± 0.15	Medium	81 ± 0.13
Mung bean noodles	28 ± 0.50	Low	7 ± 0.15	Low	38 ± 0.26
Taro	69 ± 0.35	Medium	12 ± 0.16	Medium	73 ± 0.30
Yam	52 ± 0.25	Low	9 ± 0.00	Low	64 ± 0.45
White bread	100[3]	High	12	Medium	100[3]

[1]Level of glycemic indexs (GIs) were classified according to high (> 69), medium (56-69) and low (< 56) GI; [2]Level of glycemic loads (GLs) were classified as high (> 20), medium (11-19), and low (< 10) GL; [3]White bread was used as reference food and was defined as 100.

Table 2 Major nutrient components and resistant starch content of the test foods (mean ± SE)

	Carbohydrate[1] (g/100 g)	Protein[1] (g/100 g)	Fat[1] (g/100 g)	RS + DF[1] (g/100 g)	Calories (kcal/100 g)
Adlay	85.9 ± 0.5	6.7 ± 0.1	2.5 ± 0.0	15.1 ± 0.5	329.9
Brown rice	86.2 ± 0.1	5 ± 0.1	1.7 ± 0.1	30.8 ± 0.6	380.1
Mung bean noodles	93.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	15.7 ± 0.5	374.0
Taro	89.9 ± 0.1	3.25 ± 0.0	0.1 ± 0.0	9.8 ± 0.3	373.5
Yam	89.1 ± 0.1	2.12 ± 0.2	2.1 ± 0.2	20.2 ± 0.5	383.8
White bread	49.9 ± 0.5	9.4 ± 0.1	7.5 ± 0.0	8.8 ± 0.4	304.7

[1]Analyzed by dry weight. DF: Dietary fiber; RS: Resistant starch.

RS are indigestible and could delay gastric emptying[25-27]. Therefore, lower blood glucose responses and GIs are expected in brown rice. The present results, however, indicated that the brown rice we tested is considered as high GI and medium GL food. This information appears to coincide with clinical observations of a significant rise in postprandial blood glucose after consuming brown rice in both diabetic patients and healthy consumers. Traditionally, when cooking brown rice, it has often been soaked in cold water before cooking to reduce the hardness and chewy mouthfeel after cooking. A possible explanation for the high GI is that the process of soaking allows starch granule expansion and performance of better gelatinization, leading to improved digestibility and consequently a higher GI level is observed.

The result regarding mung bean noodles showed lower glucose and insulin responses than bread and produced the lowest GI and GL among the five starchy foods, although higher carbohydrate content was observed. Generally, mung bean noodles are made of mung bean or pea starch, high in amylose, which has been reported to have the effect of lowering GI[28]. Taro and yam both have long been used in the Chinese diet. There have been times when rice was considered rare and expensive, so yam has often been eaten as a sweet dessert or used as a rice substitute in traditional diets. The postprandial glucose and insulin responses elicited by yam are slightly lower than taro, and thus gave lower GI values. These properties of yam and taro can be encouraging for people who are concerned about their postprandial blood glucose levels. It is interesting to note that taro and yam both have similar carbohydrate contents, however they produced variable GI and GL values. They also had lower GI and GL than bread (the reference food), despite the fact that carbohydrate level in bread was much lower than in taro and yam. An unexpected observation was the relationship between GI and II (i.e. II has usually been described as lower than the relative GI values). In our results, the IIs observed from the five starchy foods were higher than their relative GIs. For example, the II of adlay was 67 ± 0.3; its GI, however, was 55 ± 0.4**.** Previous studies indicated co-ingestion of fat and/or protein could increase insulin responses and potentially elicit higher insulinemic responses than relative glycemic responses[29]. In the present study, fat and protein contents were observed among the five test foods and insulin responses are higher than their relative glycemic responses, consequently higher II than the corresponding GI values were found. This result implies that co-ingestion of fat and protein in real foods may influence insulin secretion, despite similar amounts of carbohydrate in their contents. The effect may be viewed as increasing glycemic and insulin responses as higher protein and/or fat contents in the starchy foods are measured. With regard to calorie content, all five starchy foods contained calories of about 368 kcal (per 100 g), which did not reach statistical significance ($P < 0.05$) when compared with white bread (305 kcal). Accordingly, food with lower GI has better satiety than high GI items. Therefore, the actual calorie input may be much lower in mung bean noodles, adlay, taro and yam than in brown rice and white bread.

Based on the correlation analysis, our results suggested that the RS + DF were negatively correlated with the GI and II values (r^2 = -0.66 and -0.10, respectively), and positively correlated with GL (r^2 = 0.49). Although this result is in line with previous findings[30,31], showing

that indigestible starch reduces postprandial glucose and insulin responses, the study may overestimate the amount of RS and DF in the test foods. All the test foods were served hot (approximately 60℃) to the subjects for GI determination. In the RS + DF analysis, however, all the test foods needed to be cooled and dried before proceeding to analytical procedures. The performance of cooling and drying allows retrogradation to occur in amylose chains and may increase the production of RS (retrograded amylose)[32]; consequently higher RS was observed. In particular, this applied to brown rice and yam.

Comparing GI data from other nations, the GI values of starchy foods produced in Taiwan are slightly different to that of counterpart foods produced overseas[9,11,19]. Findings such as this reveal that GI and II values of foods from different countries need to be determined strictly following their own recipes. The GI values of a food could vary when food preparation, cooking methods, food processing, GI testing methods[19] and even geographical location are different. This is more applicable for raw agricultural products. Hence, food with equivalent carbohydrate does not induce similar glycemic and insulinemic responses. This means that GI and GL, along with II values of foods, need to be determined at the same time, in order to provide better understanding as to their postprandial glycemic and insulinemic effects. The present study emphasizes that mung bean noodles, adlay and yam are low GI and GL foods but have variable degrees of II values. The results of this study may provide important information for the public to manage their diet and may prove useful for the prevention of lifestyle-related diseases, such as diabetes mellitus. Continuously evaluating GI values of foods, along with their relative GL and II values, is necessary for individual countries.

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COMMENTS

Background

The glycemic and insulinemic effects of foods are relevant to the development of some lifestyle-related diseases and are involved in the therapeutic dietary plan of chronic diseases. However, the glycemic index (GI) and insulinemic index (II) of Chinese traditional starchy foods have not yet been adequately examined.

Research frontiers

GI, one of the most talked about topics in nutrition today, has recently been recommended as a potential tool for both diabetic and individual use. Research has shown that food GI and II values are relevant to the degree of postprandial glycemia and insulinemia and are involved in the therapeutic dietary plan for some lifestyle-related diseases. The need to continuously determine the postprandial glycemic and insulinemic effects of foods is still valuable for health professionals and researchers, and in particular, the GI and II data of agricultural products carried out in individual countries. Although some studies have evaluated the GI of Chinese foods, the glycemic and insulinemic effects of Chinese starchy foods have not yet been examined in parallel. Therefore, it was informative to evaluate the GI, glycemic load (GL) and II of Chinese starchy foods, since they are beneficial for dietary therapy and meal planning.

Innovations and breakthroughs

The present study evaluated the GI and II of five starchy foods that are commonly used in the Chinese traditional diet. The results will provide some preliminary information on both postprandial insulinemic and glycemic effects of Chinese starchy foods and prove useful for consumers to manage their diets, particular for diabetic patients.

Applications

Since a dietary approach is involved in the prevention and management of some chronic diseases, the results of this study will assist the public and health professionals in their meal planning and dietary management.

Terminology

Glycemic effect is expressed as the incremental area under the curve (AUC) of blood glucose response (120 min). Insulinemic effect of food is referring to as the AUC of the blood insulin response. GI is defined as the incremental blood glucose area (120 min) after ingestion of 50 g of available carbohydrates in the test food as a percentage of the corresponding area after an equivalent amount of carbohydrate from a reference food (either white bread or glucose). II is defined as the incremental blood insulin area after eating of 50 g of available carbohydrates in the test food as a percentage of the corresponding area after an equivalent amount of carbohydrate from a reference food. GL is calculated from the GI value of a food multiplied by the amount of carbohydrate in a usual portion size, divided by 100.

Peer review

The authors provided clinically meaningful data for glycemic control of diabetic patients and this reviewer agrees that preventing hyperinsulinemia after feeding would also be important for that.

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BRIEF ARTICLE

CABYR RNAi plasmid construction and NF-κB signal transduction pathway

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Abstract

AIM: To construct the CABYR RNAi plasmid and study its relation with the nuclear factor (NF)-κB signal transduction pathway.

METHODS: Human CABYR mRNA sequence was obtained from GenBank. The structure of cDNA sequence for the short hairpin RNA was *Bbs* I + sense + loop + antisense + transcription terminator + *Kpn* I + *Bam*H I. A CABYR silencing plasmid was constructed and transfected into the human embryo cell line 293T. Quantitative real-time polymerase chain reaction was used to analyze CABYR and NF-κB gene expression.

RESULTS: The CABYR and NF-κB expressions were detected in 293T cells. The oligonucleotide (5'-GCTCAGATGTTAGGTAAAG-3') efficiently silenced the expression of CABYR. The expression of NF-κB was not significantly affected by silencing CABYR (P = 0.743).

CONCLUSION: CABYR can be found in the human embryo cell line 293T. Cabyrmid 2 can efficiently silence its target, CABYR, indicating that CABYR is not related with the NF-κB signal transduction pathway.

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Key words: CABYR; Plasmid; Nuclear factor-κB; Signal transduction; RNAi; Cabyrmid 2

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INTRODUCTION

Tumors are the result of multiple genetic mutations in cells. The mutated genes generally affect the signal transduction pathways, inducing changes in the bionomic and hereditary characteristics of tumor cells (TC)[1]. Many abnormalities in various signal transduction pathways of TC have been reported, including the ILK, AP-1, Wnt and nuclear factor (NF)-κB pathways[2,3]. The NF-κB signal transduction pathway is known to enhance the transcription of target gene related to apoptosis, proliferation and differentiation of lymphocytes. Abnormalities in this pathway have been found in many TC. Inhibition of the NF-κB pathway can suppress the growth and metastasis of pancreatic carcinoma, and decrease chemo-drug resistance[2-6]. The NF-κB signaling pathway contains a positive feedback mechanism[7,8] and has crosstalk with other signaling pathways, such as PI3K/AKt[4,9,10], NOTCH1[11], K-ras[12] and Hedgehog[13]. Dysfunction of the NF-κB pathway can contribute to the development of tumors. Some signal transduction pathways related to the phos-

phorylation of proteins are of the most important regulation mechanism present in cells. CABYR is a calcium-binding tyrosine phosphorylation-regulated protein that has been detected in testis and also in lung cancer[14], and its CR-A and CR-B contain 5 PXXP consensus motifs[15], the cognate sites for SH3 which is one of the signal transduction protein modular binding domains. IκBα molecule, a key regulatory subunit of the NF-κB signal transduction pathway, can be regulated by the PI3K/Akt pathway. CABYR spliceosome Ⅲ/Ⅴ can act as an ideal substrate for glycogen synthase kinase-3 (GSK3)β within the extensin-like domain. GSK3β is one of the most important transduction proteins involved in many signal transduction pathways, and plays a vital role in tumorigenesis. We hypothesize that CABYR may be related with the NF-κB signal transduction pathway, affecting basal expression of NF-κB subunit, phosphorylation of IκBα, and DNA binding ability.

MATERIALS AND METHODS

Cell culture

Human embryo cell line 293T, obtained from Department of Immunology at Shanghai Tongji University (Shanghai, China), was maintained by passing twice a week in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Gaithesburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 10 mg/mL streptomycin at 37℃ in an atmosphere containing 5% CO_2. Each cell line was passed at 70%-80% confluence. The cells were subjected to 6 treatment regimens based on the following groups: CABYR1, CABYR2, CABYR3, empty vector, blank and transfection efficiency reference.

Plasmids and transfection

Human CABYR mRNA sequence was obtained from GenBank (Accession number NM_153768). Three possible target sites to this sequence were chosen with the GenScript SiRNA target finder. BLAST was used to identify whether they are exclusive to CABYR. The target sequences are 5'-CCATCAAACATCAACCAGT-3' (nt 240-258), 5'-GCTCAGATGTTAGGTAAAG-3' (nt 627-645) and 5'-GCTCTCTGACACATCTTT-3' (nt 1256-1273). A short hairpin RNA (shRNA) was designed for use in RNA interference (RNAi) according to the three targets. The TTCAAGAGA sequence is part of the "loop" motif in the shRNA, and the transcription terminator is TTTTT. Restriction endonuclease sites for *Bbs*I, *Kpn* I + *Bam*H I were incorporated into the shRNA sequence, along with a sense and antisense sequence for each cDNA. Single-stranded oligonucleotide DNAs were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China). Relative cDNAs are composed of a DNA double strand and a conjunct with pSilence1.0 plasmid. The plasmids were transformed into competent cells and possible transformants were identified, which are resistant to ampicillin. The plasmids were purified using the plasmid minipreps purification system B (BioDev, Beijing, China), then incubated with *Kpn* I to linearise and sequenced to confirm their identity. The 293T cells were transfected with CABYR-shRNA, shRNA control and pEGFP (reference of transfection efficiency) using the Effectene transfection kit (Qiagen, Hilden, Germany). CABYR-shRNA 1, CABYR-shRNA 2 and CABYR-shRNA 3 were designated as Cabyrmid 1, Cabyrmid 2 and Cabyrmid 3, respectively.

Semi-quantitative polymerase chain reaction

The β-actin gene was used as the reference gene when the results were quantified. The primers employed were β-actin (forward: 5'-ACAGAGCCTCGCCTTTGCC-3' and reverse: 5'-CATGTCGTCCCAGTTGGTG-3'), CABYR exon2 (forward: 5'-CAACCCATCAAACATCAACC-3' and reverse: 5'-TGCCATTGCTAACATCTGAG-3'), CABYR exon 4 (forward: 5'-CAGACACAGACGAGGACAATG-3' and reverse: 5'-TCC GTT TGC TCA GTG CCT-3'), NF-κB (forward: 5'-GAGACATCCTTCCGCAAACT-3' and reverse: 5'-TCCTTCCTGCCCATAATCA-3'). Total RNA was extracted from 293T cells using Trizol (Invitrogen, Shanghai, China) following its manufacturer's instructions, with quality and quantity determined by measuring the optical density at 260 nm and 280 nm. An $A_{260/280}$ ratio of approximately 1.8 indicated that the RNA sample was of sufficient purity. The RNA integrity was also checked by electrophoresis. Total RNA was reverse transcribed into cDNA using a RevertAid™ cDNA first strand synthesis kit (Fermentas, Ontario, Canada). Thirty cycles of semi-quantitative polymerase chain reaction (PCR) were conducted in a 25 μL volume, with an annealing temperature of 56℃. The PCR products were visualized by agarose gel electrophoresis.

Real-time analysis of gene expression

Changes in NF-κB expression were detected before and after CABYR RNAi treatment by quantitative real-time PCR (qPCR). Total RNA was extracted and reverse transcribed into cDNA using a RevertAid™ cDNA first strand synthesis kit (Fermentas, Ontario, Canada). The primers used in the qPCR are CABYR exon 4 (forward: 5'-CAGACACAGACGAGGACAATG-3' and reverse: 5'-TCCGTTTGCTCAGTGCCT-3'), β-actin (forward: 5'-GCACTCTTCCAGCCTTCCTT-3' and reverse: 5'-GGTCTTTGCGGATGTCCA-3'), NF-κB (forward: 5'-GAGACATCCTTCCGCAAACT-3' and reverse: 5'-TCCTTCCTGCCCATAATCA-3'). cDNA for the blank group was diluted at 1:1, 1:10, 1:100, 1:1000 and 1:10000 and used in over 40 cycles of two-step qPCR in a 25 μL volume, with an annealing temperature of 62℃. A SYBR Premix Taq kit from Takara Bio (Shiga, Japan) was used. The results were analyzed using the Rotor-gene real-time analysis software.

Statistical analysis

All the experiments were repeated three times and the results were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The statistical analytical method was one-way ANOVAD. $P < 0.05$ was considered statistically significant.

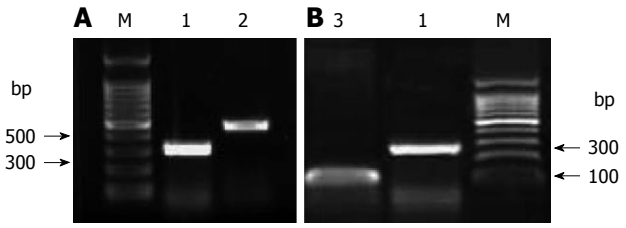

Figure 1 Basic expression of CABYR (A) and nuclear factor-κB (B) at mRNA level in 293T cells. Total RNA was extracted from 293T cells with Trizol and reverse transcribed to 293T cDNA with the primer Oligo(dt). The target fragment was amplified by semi-quantitative polymerase chain reaction and analyzed by agarose electrophoresis (1%). CABYR and nuclear factor (NF)-κB were detectable in 293T cells, indicating that 293T cells can be used to identify the efficient silence fragment for CABYR and study the relation between CABYR and NF-κB. 1: β-actin; 2: CABYR; 3: NF-κB; M: Marker.

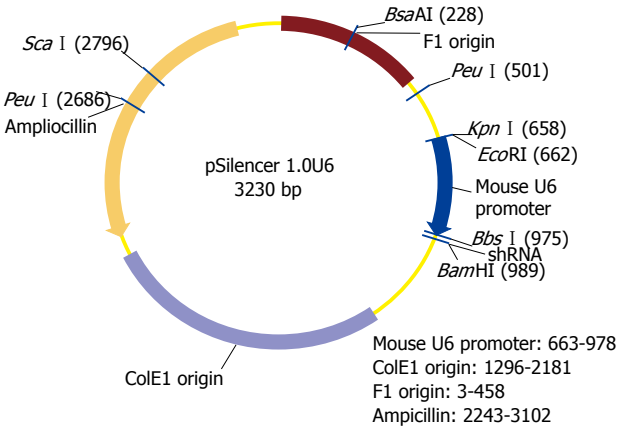

Figure 2 Construction of CABYR shRNA eucaryon expression vector by inserting the target gene fragment into pSlience1.0 (3.23 kb) between *Bbs* I and *Bam*HI.

RESULTS

Basal expression of CABYR and NF-κB mRNA in 293T cells

The transcripts of CABYR and NF-κB were detected in 293T cells (Figure 1).

Construction of CABYR shRNA eukaryotic expression vector

The gene fragment was inserted between the restriction sites of *Bbs* I and *Bam*H I in pSlience1.0 (Figure 2). The plasmid also contained a restriction site of *Kpn* I, and a *Kpn* I recognition sequence was incorporated into the ends of our gene fragment. Possible recombinant plasmids were identified by digesting with the appropriate restriction endonuclease and a 396-397 bp product would be liberated if the target fragment was correctly incorporated (Figure 3). The identified recombinant plasmids were sequenced by Shanghai Sangon Company for further verification (Figure 4).

CABYR shRNA expression in different groups

GFP was highly expressed in the reference group, indicating that a high efficiency of transfection can be achieved in the other groups under the same conditions (Figure 5). CABYR mRNA was also expressed in the blank control, vacant vector control and CABYR RNAi groups. The CABYR mRNA expression was decreased in CABYR RNAi group, indicating that 5'-GCTCAGATGTTAGGTAAAG-3' is an efficient silencing target for CABYR (Figure 6).

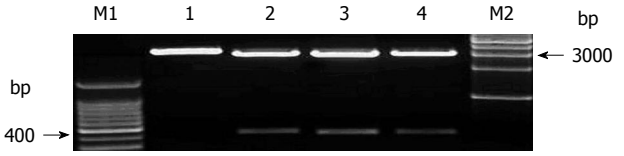

Figure 3 Construction of CABYR shRNA eucaryon expression vector by inserting the target fragment we constructed into the RNAi plasmid. 1:vacant vector; 2: Cabyrmid 1 vector; 3: Cabyrmid 2 vector; 4: Cabyrmid 3 vector; M1: Marker 1; M2: Marker 2.

Table 1 Relation between CABYR and nuclear factor-κB signal pathway (mean ± SD)

Group	CABYR [Δ(ΔCT) value]	NF-κB [Δ(ΔCT) value]
Control group	-0.06 ± 0.18	-0.38 ± 0.51
Cabyrmid 1	0.19 ± 0.23	-0.10 ± 0.25
Cabyrmid 2	2.11 ± 0.15[a]	-0.05 ± 0.79
Cabyrmid 3	0.76 ± 0.33	-0.15 ± 0.20
F value	51.928	0.381

[a]$P < 0.05$ *vs* control, cabyrmid 1 and cabyrmid 3 groups. NF: Nuclear factor; CT: Computed tomography.

Relation between CABYR and NF-κB signal pathway

According to the standard curve generated, CT exhibited a strong linear correlation between CABYR and NF-κB at different diluted concentrations, thus the precise results could be obtained using qPCR. The M value for β-actin, CABYR and NF-κB was approximately uniform, indicating that their amplification efficiency is similar. The concentration of target fragment was analyzed using the Δ(ΔCT) method. The results showed that the mRNA expression was obviously decreased in the siRNA2 group, indicating that 5'-GCTCAGATGTTAGGTAAAG-3' can silence the expression of CABYR mRNA transcript, while the expression of NF-κB was not affected by silencing CABYR ($P = 0.743$), displaying that CABYR has no significant effect on the expression of NF-κB (Table 1).

DISCUSSION

In this study, a CABYR silencing plasmid was constructed with its function observed. CABYR, first identified in the testis by Naaby-Hansen *et al*[16], plays a key role in protein tyrosine phosphorylation and increases the concentration of intracellular calcium. Its transcript variants encode multiple protein isoforms, but spliceosome Ⅲ/Ⅴ is not specific for testis[15]. CABYR can be found in pancreas, fetal brain, liver, motile cilia of human bronchus

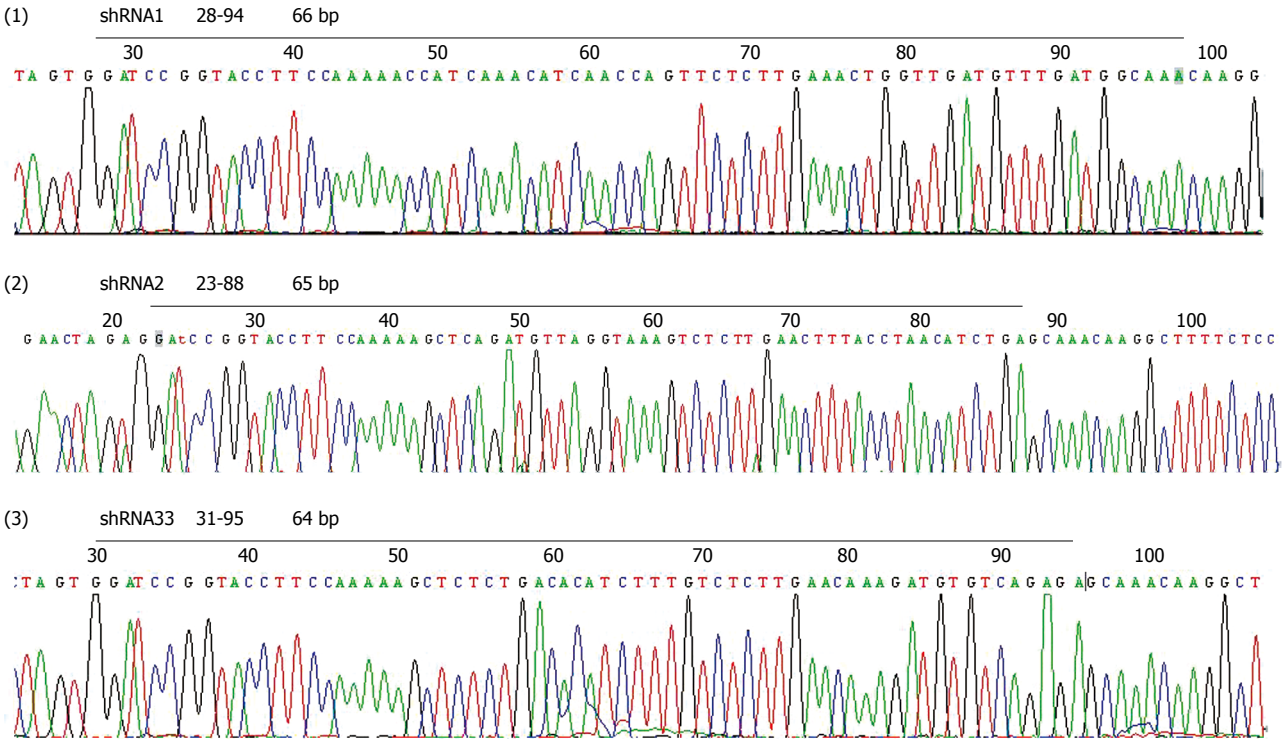

Figure 4 Construction of CABYR shRNA eucaryon expression vector by locating shRNA in plasmid.

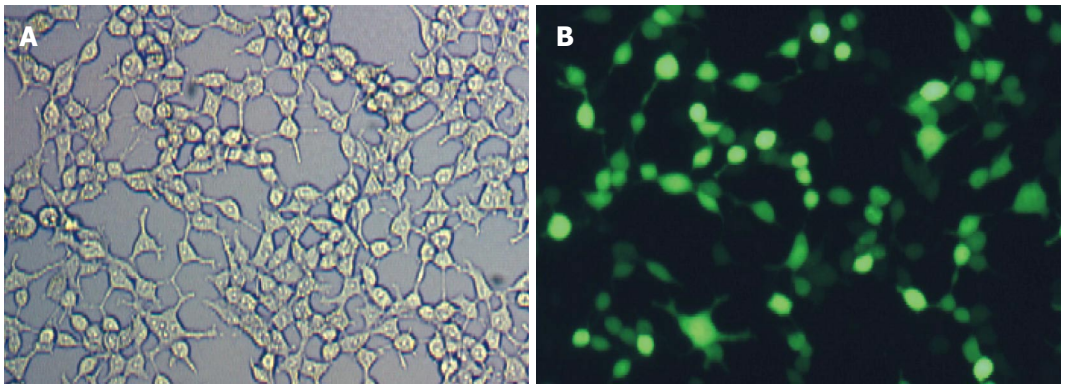

Figure 5 CABYR shRNA expression in reference group (A) and CABYR mRNA expression in other groups (B). A: HE stain, × 100; B: Blue fluorescent, ×100.

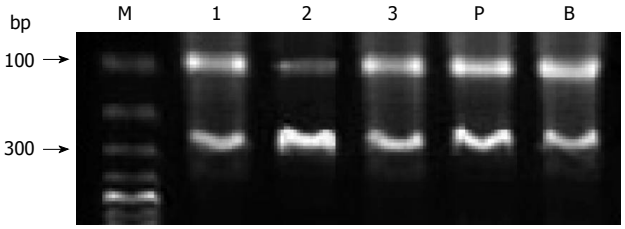

Figure 6 CABYR mRNA expressions in different groups after transfection. M: Marker; 1: Cabyrmid 1; 2: Cabyrmid 2; 3: Cabyrmid 3; P: Vacant vector; B: Blank.

and fallopian tubes[17]. In this study, CABYR was identified in the human embryo cell line 293T.

Cabyrmid 2 is an effective CABYR silencing plasmid. In this study, 3 target fragments of CABYR were designed using the GenScript SiRNA target finder and compared with the corresponding sequences in GenBank to determine its specificity[18]. The CABYR shRNA we constructed, inserted downstream from the strong U6 promoter in pSilence 1.0 and transcribed, which became a functional siRNA with an ability to degrade CABYR mRNA exclusively. The effective CABYR shRNA was screened by transfecting 293T cells *via* lipofection as previously described[19,20]. In this study, a highly effective CABYR silencing site, 5'-GCTCAGATGTTAGGTAAAG-3', was found, and a short hairpin plasmid that could effectively silence CABYR expression was constructed, which was designated as Cabyrmid 2.

The expression or repression of CABYR had no effect on NF-κB signaling pathways in our study. It has been shown that CABYR spliceosome Ⅲ/Ⅴ can act as an ideal substrate for GSK3β in the extensin-like domain[21]. GSK3β is known to play a key role in tumorigenesis[22-26]

in conjunction with PI3K/Akt which plays a role in the regulation of the NF-κB transduction pathway. NF-κB plays an important role in embryo growth, differentiation and apoptosis of lymphocytes, immunological and inflammatory reactions[27-31]. Abnormal CABYR and NF-κB have been detected in the same cancers, and CABYR possesses a tyrosine kinase activity which is an important kinase in various signaling pathways, suggesting that CABYR may be related with NF-κB. However, no significant effect of CABYR was observed on the expression of NF-κB in this study.

Two reasons can explain why CABYR had no significant effect on the expression of NF-κB in this study. One is that the 293T cells were used while the NF-κB signaling pathway was normal. If their relation was detected in Bxpc3 (NF-κB dysfunction), other results may be observed. The other is that NF-κB exists as an inactive precursor (p50, p60, IκBα) in cytoplasm. After NF-κB is activated, IκBα is phosphorylated and detached from the conglomeration. The remaining molecules enter the nuclei and adhere to target DNA, thereby enhancing transcription. Phosphorylation of IκBα is a key step in the NF-κB pathway. Though no significant effect of CABYR was observed on the expression of NF-κB in this study, CABYR possesses a tyrosine kinase activity possibly affecting the NF-κB pathway by phosphorylating IκBα. It has been reported that G3BP2 (RasGAP SH3-binding protein 2) is able to discriminate between amino terminals of IκBα[32] related with the retention of IκBα/NF-κB conglomeration in cytoplasm. CABYR also contains a PXXP motif, similar to G3BP2 which is a core part of the SH3 aglucone. A study involving the influenza A virus demonstrated that the structure of SH3 plays a key role in determining the activity of PI3K/Akt[33]. The PI3K/Akt signaling pathway can also regulate the phosphorylation of IκBα, indicating that CABYR may take part in the regulation of the NF-κB signaling pathway.

In summary, CABYR is not exclusive to the testis and codes for a calcium-binding tyrosine-phosphorylation regulated protein that is intimately involved in calcium signaling. Cabyrmid 2 can efficiently silence CABYR expression rather than the expression of NF-κB in 293T cells.

COMMENTS

Background

Tumor is a polygene mutation disease. The mutation gene effects on the signal transduction pathway inducing tumor cell's (TC). Many abnormalities of signal transduction pathway in TC have been reported, such as ILK, AP-1, Wnt, and nuclear factor (NF)-κB. Recently NF-κB signal transduction pathway was hotly researched. It can enhance the transcription of the target gene relating to the apoptosis, proliferation and differentiation of lymphocyte. And its abnormality was also been found in many TC. CABYR is an capacitation related calcium binding tyrosine-(Y)-phosphorylation regulated gene, it has no absolute testis specificity, it was also reported that CABYR antigen was detected in many cancers such as lung cancer.

Research frontiers

NF-κB signal transduction pathway can enhance the transcription of the target gene relating to the apoptosis, proliferation, and differentiation of lymphocyte *etc*. The inhibition of NF-κB can depress growth and metastasis of pancreatic carcinoma, and decrease the chemo-drug resistance. NF-κB signal transduction pathway has many crosstalk with other signal pathway. This indicated that the dysfunction of NF-κB signal pathway contribute an important part of tumors development. CABYR is an capacitation related calcium binding tyrosine-(Y)-phosphorylation regulated gene. its CR-A and CR-B contain five PXXP consensus motifs, the cognate sites for SH3 ,one of the signal transduction protein Modular Binding Domains, interaction. On another side IκBα, the key regulated subunit of NF-κB signal transduction pathway, can been regulated by the PI3K/Akt signal pathway. CABYR splicesome Ⅲ/Ⅴ act as an ideal substrate for GSK3beta (glycogen synthase kinase-3) within the extensin-like domain. The GSK3beta is one of the most important transduction proteins involving in many signal transduction pathway which play a key role in tumorous genesis and development including PI3K/Akt signal pathway. So the authors hypothesised that CABYR may have some relationship with NF-κB signal transduction pathway.

Innovations and breakthroughs

The authors constructed CABYR silence plasmid (Cabyrmid 2) and proved that CABYR RNAi plasmid 2 is the efficient silence target to CABYR. They also found that CABYR may have no relationship with NF-κB signal transduction pathway.

Applications

CABYR silence plasmid (Cabyrmid 2) may help the authors in the future research of CABYR.

Terminology

CABYR is a calcium binding tyrosine phosphorylation regulator gene. It was first found in testis by Naaby-Hansen in 2002. CABYR plays a key role in capacitation involving protein tyrosine phosphorylation and increased intracellular calcium. Transcript variants of this gene encode multiple protein isoforms. And its spliceosome Ⅲ/Ⅴ wasn't seem to be absolute testis specificity. It also be found in the pancreatic tissue, fetal brain, sclerosis liver as well.

Peer review

The authors have presented a basic study with convincing data. It will be suitable for publication after revision.

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BRIEF ARTICLE

Up-regulation of PIK3CA promotes metastasis in gastric carcinoma

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Abstract

AIM: To explore expressions of PIK3CA in the progression of gastric cancer from primary to metastasis and its effects on activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

METHODS: mRNA and protein levels of PIK3CA were assessed, respectively, by real-time quantitative polymerase chain reaction and immunohistochemistry in specimens of normal gastric mucosa, primary foci and lymph node and distant metastasis of gastric cancer. Akt and phosphorylated Akt protein were also examined by Western blotting in these tissues, in order to analyze the effect of PIK3CA expression level changes on the activation of PI3K/Akt signaling pathway.

RESULTS: PIK3CA mRNA in lymph node metastasis were approximately 5 and 2 folds higher, respectively, than that in the corresponding normal gastric mucosa and primary gastric cancer tissues ($P < 0.05$), while no statistical significance was found compared with distant metastasis. Immunohistochemically, PIK3CA protein expression was discovered in 7 (35%) specimens of 20 primary foci *vs* 10 (67%) of 15 of lymph node metastasis or 11 (61%) of 18 of distant metastasis (35% *vs* 67%, $P = 0.015$; 35% *vs* 61%, $P = 0.044$). With the increased level of PIK3CA expression, the total Akt protein expression remained almost unchanged, but p-Akt protein was upregulated markedly.

CONCLUSION: Increased expression of PIK3CA is expected to be a promising indicator of metastasis in gastric cancer. Up-regulation of PIK3CA may promote the metastasis of gastric cancer through aberrant activation of PI3K/Akt signaling.

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Key words: PIK3CA; Phosphatidylinositol 3-kinase/Akt pathway; Metastasis; Gastric cancer; Akt

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INTRODUCTION

Gastric cancer, the most common gastrointestinal cancer, is the leading cause of cancer death. Invasion and metastasis are the main biological characteristics of malignant tumors, and also the important factors contributing to the death of gastric cancer patients and affecting their therapeutic efficacy. Currently, no method has been available to predict metastasis of gastric cancer. Therefore, studies on the molecular mechanism of metastasis are crucial for diagnosis, treatment and prognosis of gastric cancer.

Several molecular pathways are known to play a role in gastric cancer development and progression[1-3]. The most important pathway may be the recently discovered phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (Akt) signaling pathway.

The PIK3CA gene, which is located on chromosome 3q26.3, encodes the key enzymatic subunit p110α of PI3K[4]. It has been shown that mutations of the PIK3CA gene are highly prevalent in a variety of human solid tumors including colon, gastric, breast and pituitary cancer[5-9], which can lead to dysregulation of PI3K/Akt signaling pathway at several levels[10]. Guo *et al*[11] reported that mutant PIK3CA-bearing colon cancer cells displayed increased enzymatic activity of PI3K compared with wild-type PIK3CA-bearing colon cancer cells. In addition, the former showed a significantly enhanced level of phosphorylation of Akt as well as cell invasion and metastasis, which is consistent with the findings from Samuels *et al*[12]. Although many studies have implicated PIK3CA mutations with features of transformation[13,14], relationship between PIK3CA expression and metastasis of gastric cancer and aberrant activation of PI3K/Akt signaling pathway has not been elucidated to date.

Our previous work showed that higher expression levels of PIK3CA were associated with lower differentiation of gastric cancer cells and stronger ability of invasion and metastasis, suggesting that PIK3CA gene may contribute to differentiation, invasion and metastasis in gastric cancer cells[15]. To further explore the correlation between PIK3CA expression and metastasis of gastric cancer, expression levels of PIK3CA were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry in different gastric cancer tissues. In addition, we investigated the effects of changes of PIK3CA expression levels on activation of PI3K/Akt signaling in order to provide important experimental evidences for the molecular mechanism of invasion and metastasis in gastric cancer.

Table 1 Primers used in real-time quantitative polymerase chain reaction

Primer	Nucleotide sequence (5'→3')
PIK3CA (+)	TGCTAAAGAGGAACACTGTCCA
PIK3CA (-)	GGTACTGGCCAAAGATTCAAAG
β-actin (+)	CTGAGCAGATCATGAAGAC
β-actin (-)	CTTGGTGGACGCATCCTGAG

"+" and "-" mean sense and antisense oligos, respectively.

MATERIALS AND METHODS

Gastric cancer specimens

From March 2008 to April 2010, 53 gastric carcinoma patients (45 with intestinal and 8 with diffuse gastric carcinoma) consisting of 29 males and 24 females, with a median age of 48 years (range from 20-72 years) admitted to the Department of Gastroenterology of our hospital and Guangdong Armed Police Hospital were assessed. Informed consent was obtained before operation from each patient for research use of the resected cancer lesions. Gastric cancer tissue samples including 20 primary (stage Ⅰ-Ⅱ), 15 lymph node metastasis (stage Ⅲ) and 18 distant metastasis (stage Ⅳ) in gastric cancer were verified by pathological diagnosis. Ten normal tissue samples were obtained from around tumor tissues. All samples were immediately frozen in liquid nitrogen or fixed in 5% formaldehyde solution for subsequent analysis. None of the gastric cancer patients had received preoperative radiotherapy, chemotherapy or biotherapy. This study was approved by the Ethics Committee of the Guangzhou Medical College.

RT-qPCR assays

Transcript abundance of PIK3CA and β-actin (internal control) was quantified by RT-qPCR on total RNA isolated from normal gastric mucosa, and gastric cancer tissues of primary foci, lymph node and distant metastasis. Briefly, 1 μg of total RNA was reversely transcribed in a reaction volume of 25 μL using oligodT(15) primers and M-MLV reverse transcriptase (Promega). The primers used for amplification are shown on Table 1. The PCR amplification and fluorescence detection were carried out in 20 μL solution, with 200 nmol/L of each primer and 5 μL of cDNA serving as templates, and SYBR Green PCR master Mix (ABI) using the ABI Prism 7500 Sequence Detection System was conducted following the manufacturer's instructions. Each cDNA was analyzed in triplicate for both target genes and β-actin housekeeping genes. The cycling conditions were 50℃ for 2 min, 95℃ for 10 min followed by 40 cycles with each cycle consisting of 30 s at 95℃, and 1 min at 60℃. For each sample, a standard quantity was calculated using the $2^{-\Delta\Delta C(T)}$ method according to previously described protocol[16].

Immunohistochemistry

Immunohistochemical analysis was performed using

DAKO Envision kits according to the manufacturer's protocol. Briefly, 4-μm sections were cut into coated slides and were deparaffined using routine techniques. After treatment with 3% hydrogen peroxidase for 10 min to block endogenous peroxidases, the sections were subsequently incubated with monoclonal antibodies (rabbit anti-human PIK3CA, Cell Signaling Technology) for 30 min at room temperature, washed with Tris Buffered Saline for 10 min and reacted with Envision TM (Dako) for 30 min. Labelling was then detected as above using 3,3'-diaminobenzidine. Negative control was obtained by omitting the primary antibody. Samples mixed with only Phosphate Buffered Saline (PBS) buffer were treated as negative controls. For the evaluation of immunostaining, at least 1000 cells were counted from randomly selected 10 fields of vision and staining intensity as well as number of positive cells were assessed according to Hara's method with minor modifications[17]. The staining was scored as follows: 0-1, < 20% cells with no or faint staining (negative); 2-4, 20%-50% cells with moderate staining (positive); and 5-6, ⩾ 50% with marked staining (strong positive).

Western blotting

The normal gastric mucosa, and gastric cancer tissues of primary foci, lymph node and distant metastasis were polished to powder in liquid nitrogen and then were lysed in protein extraction buffer [0.5 mmol/L Tris.Cl (pH 7.0), 0.1% β-mercaptoethanol, 0.5 mmol/L ethylenediaminetetraaceticacid (EDTA) (pH 7.0), 0.5 mmol/L ethyleneglycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (pH 7.0), and 2 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2.5 mg/mL Aprotinin, 1 mmol/L dithiothreitol (DTT), 0.5% Triton X-100]. The lysates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Life Sciences). The membranes were blocked for 1 h, probed with the anti-phosphorylated Akt (Ser473), anti-Akt and anti-β-actin (Cell Signaling Technology) antibodies, respectively, and then reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (BestBio).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 17.0). Differences in the results between groups were analyzed by the Mann-Whitney *U*-test, and a *P* value of less than 0.05 was taken as significant.

RESULTS

Analyses of PIK3CA mRNA and protein expression

PIK3CA mRNA expression was detected by RT-qPCR in normal gastric mucosa, and gastric cancer tissues of primary foci, lymph node and distant metastasis. The primary gastric cancer specimens showed higher expression of PIK3CA mRNA in comparison with the normal gastric mucosa. The lymph node metastasis tissues displayed the strongest expression of PIK3CA mRNA, which was approximately 5 and 2 folds higher, respectively, than that in normal gastric mucosa and primary gastric cancer tissues ($P < 0.05$), whereas in contrast to distant metastasis, no statistically significant difference was found in PIK3CA mRNA expression (Figure 1A). In addition, the expression and localization of PIK3CA protein were studied immunohistochemically in resected tissues mentioned above. No or weak cytoplasmic staining for PIK3CA appeared in the normal gastric mucosa, while moderate staining was displayed in 7 (35%) of 20 of primary gastric cancer specimens, and moderate or intense staining in lymph node metastasis (10/15 or 67%) and distant metastasis (11/18 or 61%) (Figure 1B, Table 2), which is similar to the corresponding PIK3CA mRNA expression profile. Moreover, statistically significant correlation was discovered between PIK3CA expression and the presence of lymph node metastasis in gastric cancer, while all other clinicopathological factors such as gender, age and differentiation, were statistically irrelevant

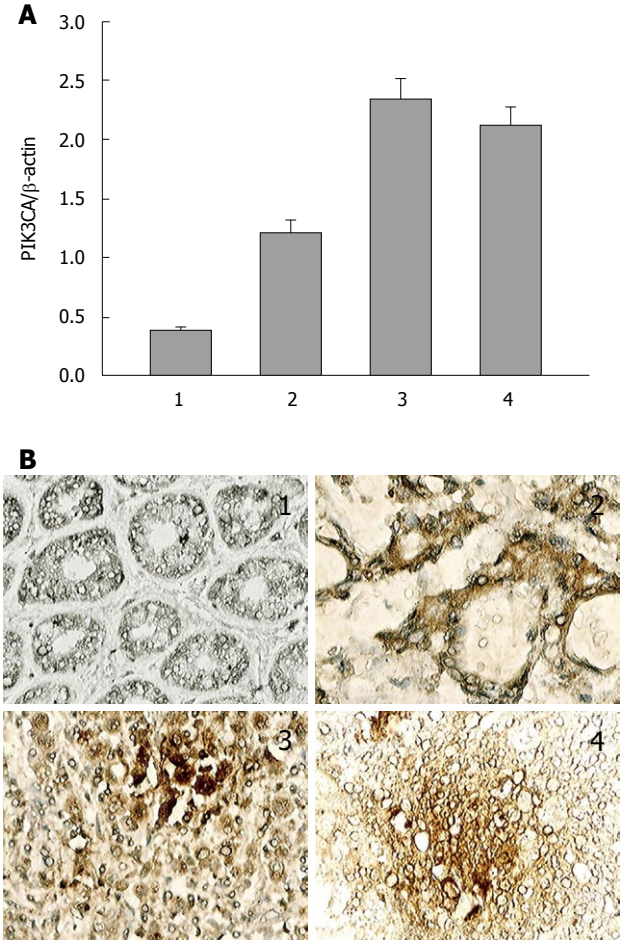

Figure 1 PIK3CA mRNA and protein expression in normal and gastric cancer tissues. A: Expression analyses of PIK3CA mRNA determined by real-time quantitative polymerase chain reaction, values are shown as mean ± SD; B: Immunohistochemical study of PIK3CA (original magnification: × 400). Negative or weak expression of PIK3CA in normal tissues around tumor; Positive expression of PIK3CA in primary gastric cancer tissues. Strong positive expression of PIK3CA was detected in lymph node metastasis and distant metastasis gastric cancer tissues. 1: Normal gastric mucosa; 2: Primary gastric cancer; 3: Lymph node metastasis in gastric cancer; 4: Distant metastasis in gastric cancer.

Table 2 Difference of PIK3CA protein expression among normal gastric mucosa, primary, lymph node metastasis and distant metastasis in gastric cancer

Tissues	PIK3CA staining			Total	*P*
	0-1	2-4	5-6		
Normal	10	0	0	10	0.036[a]
Primary	13	6	1	20	0.015[b]
Lymph node metastasis	5	3	7	15	0.044[c]
Distant metastasis	7	3	6	18	0.537[d]

[a]$P < 0.05$, normal *vs* primary; [b]$P < 0.05$, primary *vs* lymph node metastasis; [c]$P < 0.05$, primary *vs* distant metastasis; [d]$P > 0.05$, lymph node metastasis *vs* distant metastasis, all by Mann-Whitney *U* test.

Table 3 Correlation between PIK3CA expression and clinicopathological characteristics in gastric carcinoma

Variables	PIK3CA expression				
	n	Positive	Negative	χ^2	*P*
Gender					
Male	29	17	12	0.862	0.834[a]
Female	24	11	13		
Age (yr)					
≥ 60	32	16	16	0.256	0.968[a]
< 60	21	12	9		
TNM stage					
Ⅰ, Ⅱ	20	7	13	4.926	0.177[a]
Ⅲ, Ⅳ	33	21	12		
Differentiation					
Well	22	7	15	6.685	0.083[a]
Moderate and poor	31	21	10		
Lymph node metastasis					
Negative	26	8	18	9.982	0.019[a,b]
Positive	27	20	7		
Distant metastasis					
Negative	35	17	18	0.749	0.862[a]
Positive	18	11	7		

[a]χ^2 test; [b]The value is significant.

to the positive staining for PIK3CA (Table 3). These results suggested that up-regulation of PIK3CA expression was likely related to lymph node metastasis in gastric cancer.

Effects of PIK3CA expression on activation of PI3K/Akt signaling pathway

PIK3CA encoding the catalytic subunit p110α of PI3K, is an important signal molecule in PI3K/Akt signaling pathway, which was involved in tumor growth and metastasis[18]. Our studies indicated that normal gastric mucosa had almost no expression of PIK3CA, while lymph node metastasis and distant metastasis had significantly higher PIK3CA expression than the primary gastric cancer tissues, suggesting that PI3K/Akt pathway could be aberrantly activated in gastric cancer metastasis. To test this possibility, we examined the phosphorylation of Akt [p-Akt (Ser473)] and total Akt in normal gastric mucosa, primary foci, lymph node and distant metastasis in gastric cancer. The results revealed that p-Akt (Ser473) was not expressed (or loss) in normal gastric mucosa, and highly expressed in lymph node metastasis and distant metastasis compared

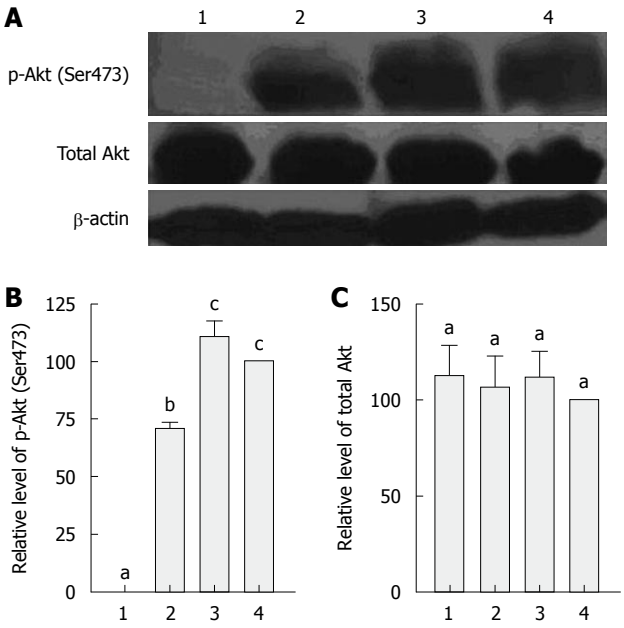

Figure 2 Western blotting analyses of Akt phosphorylation. A: Protein from the indicated tissues was Western blotting with anti-phospho-Akt (Ser473) and anti-Akt to analyze the effect on activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling. β-actin was used as a loading control; B: Relative expression level of p-Akt (Ser473) protein quantified by grey analysis; C: Relative expression level of total Akt protein quantified by grey analysis. p-AKT, phosphorylated Akt; Lane 1: Normal gastric mucosa; Lane 2: Primary gastric cancer; Lane 3: Lymph node metastasis in gastric cancer; Lane 4: Distant metastasis in gastric cancer. [a,b,c]Significant difference was indicated with different lower-case letters ($n = 3$).

with that in primary gastric cancer tissues (Figure 2A and B). However, total Akt expression levels remained nearly unchanged (Figure 2A and C). These findings indicated that up-regulation of PIK3CA expression level contributed to the increased catalytic activity of PI3K p110α, promoting phosphorylation of Akt and overactivation of PI3K/Akt signaling pathway, in which the latter promoted invasion and metastasis in gastric cancer cells.

DISCUSSION

In recent years, although the morbidity and mortality rates of gastric cancer have fallen worldwide, the therapeutic efficacy of advanced gastric cancer is still unsatisfactory. In order to clarify the molecular mechanism of invasion and metastasis in gastric cancer, we investigated the mRNA and protein expression of PIK3CA in different gastric cancer tissues, as well as the effect of PIK3CA expression on activation of PI3K/Akt signaling pathway.

Interestingly, Woenckhaus *et al*[19] pointed out that increased expression of PIK3CA was associated with progression of dysplasia to an invasive squamous cell carcinoma. Akagi *et al*[20] found that PIK3CA mRNA overexpression was highly prevalent in esophageal squamous cell carcinoma samples by quantitative RT-PCR. Additionally, the presence of node metastasis was significantly higher in the group with positive staining for PIK3CA compared with the negative staining group immunohistochemically. Similar to their studies, weak or no expression

of PIK3CA mRNA and protein was discovered in normal gastric mucosa, while strong expression was detected during the progression of primary gastric cancer to lymph node metastasis and distant metastasis in our study. Thus, up-regulation of PIK3CA was most likely linked to tumor invasion and metastasis.

In a previous study, deregulation of PI3K/Akt pathway frequently found in a great number of human malignant tumors was closely related to tumor development[21]. Akt, also known as protein kinase B, the major downstream effector of PI3K, is activated by phosphoinositide-dependent protein kinase 1, which is recruited and phosphorylated by activation of PI3K[22]. Increasing studies have shown that Akt plays an important role in many physiological processes, such as cell growth and proliferation, apoptosis, cell motility and invasion[23,24]. In this study, we found for the first time that p-Akt (Ser473) was lost or not expressed in normal gastric mucosa tissues through Western blotting analysis, while it was highly expressed in metastatic tissues compared with primary gastric cancer tissues. In addition, such observation is in good agreement with the expression profile of PIK3CA. This implied that up-regulation of PIK3CA could increase catalytic activity of PI3K, as suggested by Shayesteh *et al*[25] and Ma *et al*[26], and subsequently overactivated PI3K/Akt pathway to promote metastasis in gastric cancer.

These data, together with our previous findings, demonstrated that up-regulation of PIK3CA and resultant constitutive activation of PI3K/Akt signaling pathway are of primary importance in understanding the process of metastasis in gastric cancer. To date, because of no reliable clinical method available to predict metastasis in gastric cancer, increased expression of PIK3CA is expected to be a potential molecular marker for the early diagnosis of advanced gastric cancer. Meanwhile, PIK3CA, the most proximal pathway component, might be a better target for anticancer drug discovery than distal components such as Akt and mTOR.

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COMMENTS

Background

Invasion and metastasis are the main factors contributing to the death of gastric cancer patients. Currently, no ideal method was available to predict metastasis of gastric cancer in clinic. Many studies have implicated PIK3CA mutations with features of metastasis, but the correlation between PIK3CA expression and metastasis in gastric cancer and its effects on activation of phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (Akt) remains unclear.

Research frontiers

Several molecular pathways are known to play a role in gastric cancer development and progression. Perhaps the most important pathway is the currently discovered PI3K/Akt pathway. Many studies have reported that mutations of the PIK3CA gene encoding the catalytic subunit p110α of PI3K, are contributed to dysregulation of PI3K/Akt pathway.

Innovations and breakthroughs

Previous reports have highlighted the importance of mutations of PIK3CA oncogene in various carcinogenesis. In this study, the authors reported for the first time that up-regulation of PIK3CA and resultant constitutive activation of PI3K/Akt pathway are of primary importance in understanding the process of metastasis in gastric cancer.

Applications

The data presented in this paper, together with the authors' previous findings, suggested that up-regulation of PIK3CA is expected to be a potential molecular marker for the early diagnosis of advanced gastric cancer. Meanwhile, PIK3CA, the most proximal pathway component, might be a better target for anticancer drug discovery than distal components such as Akt and mTOR.

Terminology

PIK3CA gene, encoding the key catalytic subunit p110α of PI3K, is located on chromosome 3q26.3. AKT, a serine/threonine kinase, serving as the major downstream effector of PI3K, regulates many biological processes, such as proliferation, apoptosis and growth.

Peer review

This descriptive study focuses on the important role of PIK3CA in PI3K/Akt pathway in gastric carcinogenesis. The authors demonstrated the data indicating that PIK3CA could be a promising indicator for metastasis from gastric carcinomas. The study is well written.

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BRIEF ARTICLE

Five-year long-term outcomes of laparoscopic surgery for colon cancer

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Abstract

AIM: To perform a meta-analysis to answer whether long-term recurrence rates after laparoscopic-assisted surgery are comparable to those reported after open surgery.

METHODS: A comprehensive literature search of the MEDLINE database, EMBASE database, and the Cochrane Central Register of Controlled Trials for the years 1991-2010 was performed. Prospective randomized clinical trials (RCTs) were eligible if they included patients with colon cancer treated by laparoscopic surgery *vs* open surgery and followed for more than five years.

RESULTS: Three studies involving 2147 patients reported long-term outcomes based on five-year data and were included in the analysis. The overall mortality was similar in the two groups (24.9%, 268/1075 in the laparoscopic group and 26.4%, 283/1072 in open group). No significant differences between laparoscopic and open surgery were found in overall mortality during the follow-up period of these studies [OR (fixed) 0.92, 95% confidence intervals (95% CI): 0.76-1.12, P = 0.41]. No significant difference in the development of overall recurrence was found in colon cancer patients, when comparing laparoscopic and open surgery [2147 pts, 19.3% *vs* 20.0%; OR (fixed) 0.96, 95% CI: 0.78-1.19, P = 0.71].

CONCLUSION: This meta-analysis suggests that laparoscopic surgery was as efficacious and safe as open surgery for colon cancer, based on the five-year data of these included RCTs.

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Key words: Colon cancer; Laparoscopic surgery; Open surgery; Randomized clinical trials; Meta-analysis

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INTRODUCTION

Colon cancer is the third most common malignancy in men and women[1]. Traditionally, cancers of the colon were removed through large abdominal incisions. Since the advent of laparoscopic surgery, it has become clear that patients benefit from a minimally invasive approach in a variety of ways[2]. In 1991, laparoscopic-assisted colectomy (LAC) was first reported[3,4]. Short-term advantages of laparoscopic colorectal surgery compared to conven-

tional surgery are well known, and include less pain, better pulmonary function, shorter duration of postoperative ileus, less fatigue and a better quality of life[5-8].

However, it was uncertain whether there would be a long-term survival difference. Several large trials have been reported which discuss the long-term survival difference. The primary aim of these trials is to test the hypothesis that disease-free survival and overall survival are equivalent, regardless of whether patients receive laparoscopic-assisted or open colectomy. The second aim is to assess the recurrence of cancer. The objective of this systematic review is to assess that in the long-term, laparoscopic-assisted colon resection for cancer is not inferior to open colectomy with respect to cancer survival and recurrence. The main outcome of concern is overall mortality and recurrence.

MATERIALS AND METHODS

Identification and selection of studies

A comprehensive literature search of the MEDLINE database, EMBASE database, and the Cochrane Central Register of Controlled Trials for the years 1991-2010 was performed. Searches were carried out using medical subject headings (MeSH) and free text words in combination with the search strategy for randomised controlled trials (RCT). The following search was adapted for each database: laparoscopy [MeSH], surgery [MeSH], colon [MeSH], colectomy [MeSH], restorative proctocolectomy [MeSH], and colonic neoplasms [MeSH]. Reference lists from the trials were hand-searched to identify further relevant trials. The following selection criteria were applied: (1) study design: RCTs reported with relevant information available; (2) study population: patients with colon cancer; (3) intervention: laparoscopic surgery *vs* open surgery; (4) samples more than 100 patients; and (5) follow-up more than five years.

Quality assessment

Two authors independently evaluated all included trials using a list of selected quality items assessing components of internal validity. Method of randomization, concealment of random allocation, blinding of outcome assessors and reporting of an intention-to-treat analysis were assessed. Trials were considered to be of good quality if they reported on three or four of these quality items, of moderate quality if they reported on one or two items, and of low quality if they reported none of the items. The reporting of this systematic review is in accordance with the QUOROM statement[9].

Statistical analysis

Treatment effects were expressed as risk ratios with corresponding 95% confidence intervals (95% CI). Where possible, outcomes were pooled with a fixed effects model and random effects model. Heterogeneity was assessed using the χ^2 statistic and the proportion of variation due to heterogeneity was expressed as I^2. In the absence of significant heterogeneity ($P > 0.05$ for χ^2), fixed-effects model (inverse variance method) was used, and in the presence of significant heterogeneity ($P < 0.05$ for χ^2) and random effects (DerSimonian-Laird method) models. If substantial heterogeneity was found in the included studies, the result was reported from the random effects model. A P value of 0.05 was used as the cut-off value to determine statistical significance. The meta-analyses were performed by using Review Manager 4.2 provided by The Cochrane Collaboration. If data for meta-analysis was considered inappropriate in the included studies, some outcomes were presented in a descriptive way.

Funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio, however, because there are only three studies included, the funnel plots will not be very helpful and will not be shown in the article.

RESULTS

A total of 66 published articles of RCTs comparing laparoscopically assisted and open surgery for colon cancer were identified. Of these trials, there were 19 articles just with short-term outcomes available and another 17 articles with regard to the colorectal cancer. In addition, 21 articles had not reported relevant information. So there were 9 potential articles left to further review. Of these trials, 4 studies were excluded because there were less than 100 patients included[10-13]. Two studies were reported at a different time. So finally 3 studies involving 2147 patients reported long-term outcome data and were included in the analysis. All of the included studies were published as full articles. Baseline characteristics of included studies are described in Table 1. Quality assessment revealed that all studies were of good or moderate quality (Table 2), indicating that all studies were of reasonable methodological quality; none of the studies had any "fatal" methodological flaws.

In 2009, the COLOR trial[14] published its long-term outcomes after laparoscopic surgery *vs* open surgery. 1076 patients were eligible for analysis (542 assigned open surgery and 534 assigned laparoscopic surgery). Median follow-up was 53 mo (range 0.03-60). The combined 3-year disease-free survival for all stages was 74.2% in the laparoscopic group and 76.2% in the open surgery group ($P = 0.70$). The hazard ratio (HR) for disease-free survival (open *vs* laparoscopic surgery) was 0.92 (95% CI: 0.74-1.15). The combined 3-year overall survival for all stages was 81.8% in the laparoscopic group and 84.2% in the open-surgery group ($P = 0.45$).

In 2008, Lacy *et al*[15] reported the long-term results of a randomized clinical trial of laparoscopy-assisted *vs* open surgery (LAC *vs* OC) for colon cancer. Two hundred and nineteen patients entered the study. The median follow-up was 95 mo. There was a tendency towards higher cancer-related survival ($P = 0.07$, NS) and overall survival ($P = 0.06$, NS) for the LAC group. The regression analysis showed that LAC was independently associated with a reduced risk of tumor relapse (hazard ratio 0.47, 95% CI: 0.23-0.94), death from a cancer-relat-

Table 1 Characteristics of included studies (laparoscopic *vs* open group)

Ref.	Age (yr, median)	Localisation of the tumour	Follow-up	Analyzed (*n*)
COST 2007[16]	70/69	Right/left/sigmoid colon	5 yr	863
Lacy 2008[15]	68/71	Right/left/sigmoid colon	95 mo	219
COLOR 2009[14]	71/71	Right/left/sigmoid colon	53 mo	1076

Table 2 Quality assessment: internal validity of the included randomized trials

Ref.	Randomization allocation	Concealment of allocation	Blinding	Intention-to-treat analysis	Withdrawal and dropouts
COST 2007[16]	Yes	Not clear	Not clear	Yes	Clear report
Lacy 2008[15]	Yes	Adequate	Not clear	Yes	Clear report
COLOR 2009[14]	Yes	Not clear	Not clear	Yes	Clear report

ed cause (0.44, 0.21-0.92) and death from any cause (0.59, 0.35-0.98). So they concluded that LAC is more effective than OC in the treatment of colon cancer. In 2002, Lacy *et al*[5] reported the same study with a median length of follow-up of 43 mo, demonstrating that LAC was more effective for treatment of colon cancer in terms of morbidity, hospital stay, tumor recurrence, and cancer-related survival.

In 2007, Fleshman *et al*[16] published the 5-year data from the COST study group trial. Patients were followed a median of 7 years. Disease-free 5-year survival (OC 68.4%, LAC 69.2%, $P = 0.94$) and overall 5-year survival (OC 74.6%, LAC 76.4%, $P = 0.93$) were similar for the 2 groups. Overall recurrence rates were similar for the 2 groups (OC 21.8%, LAC 19.4%, $P = 0.25$). These recurrences were distributed similarly between the 2 treatment groups. Sites of first recurrence were distributed similarly between the treatment arms (OC: wound 0.5%, liver 5.8%, lung 4.6%, other 8.4%; LAC: wound 0.9%, liver 5.5%, lung 4.6%, other 6.1%). Likewise, in 2004, the three-year outcomes of COST[17] were also reported with the recurrence rate and the overall survival being similar for the two groups. So they concluded that laparoscopic colectomy for curable colon cancer is not inferior to open surgery based on oncologic endpoints.

Overall mortality and cancer-related mortality

All 3 studies reported overall mortality at maximum follow-up. 2147 patients were included in this meta-analysis. The overall mortality was similar in the two groups (24.9%, 268/1075 in the laparoscopic group and 26.4%, 283/1072 in the open group). No significant differences between laparoscopic and open surgery were found in overall mortality during the follow-up period of the study [OR (fixed) 0.92, 95% CI: 0.76-1.12, $P = 0.41$] (Figure 1A). Regarding the cancer-related mortality, only Lacy's study reported this result (16%, 17/106 in laparoscopic group and 27%, 28/102 in open group, $P = 0.07$, NS).

Overall 5-year disease-free survival and overall 5-year survival

Both COLOR and COST trials reported the overall 5-year disease-free survival and overall 5-year survival between laparoscopic and open groups. In the COLOR trial, the overall 5-year disease-free survival and overall 5-year survival were 66.5% *vs* 67.9% and 73.8% *vs* 74.2%, respectively; in the COST trial, the overall 5-year disease-free survival and overall 5-year survival were 69.2% *vs* 68.4% and 76.4% *vs* 74.6%, respectively. As seen in the two large randomized trials, these two outcomes were similar between the two groups.

Overall and local and distant recurrence

All 3 studies reported these outcomes. No significant difference in the development of overall recurrence was found in colon cancer patients, when comparing laparoscopic and open surgery [2147 pts, 19.3% *vs* 20.0%; OR (fixed) 0.96, 95% CI: 0.78-1.19, $P = 0.71$] (Figure 1B). The number of patients that developed a local recurrence at the maximum follow-up of the study was similar after laparoscopic and open surgery, showing that there is no significant difference between laparoscopic and open procedures [2147 pts, 4.0% *vs* 4.4%; OR (fixed) 0.91, 95% CI: 0.59-1.39. $P = 0.66$] (Figure 1C). Similarly, no significant difference in the development of distant metastases was found in colon cancer patients, when comparing laparoscopic and open surgery [2147 pts, 12.8% *vs* 14.0%; OR (fixed) 0.90, 95% CI: 0.70-1.16, $P = 0.41$] (Figure 1D).

DISCUSSION

Colon cancer is one of the most common cancers in both female and male persons. Treatment involves surgical resection of the segment of the bowel containing the tumor and wide tumor-free margins. Lymph nodes in the area are also removed. Conventional surgery is the mainstream treatment of colorectal cancer and has good survival rates for stage-1 tumors. For many people it is now possible to use video-endoscopic surgery (laparoscopy), which may have short term advantages that include less pain, better pulmonary function, shorter time for return of bowel function (duration of postoperative ileus), less fatigue, and improved convalescence, as suggested in a Cochrane systematic analysis on short-term outcomes[18]. This meta-analysis also demonstrated that postoperative duration of hospital stay is less and quality of life may be improved in

A

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 01 overall mortality at maxium follow-up
Outcome: 01 overall mortality

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	102/435	108/428		39.62	0.91 (0.66-1.24)
Lacy 2008[15]	38/106	50/102		15.54	0.58 (0.33-1.01)
COLOR 2009[14]	128/534	125/542		44.84	1.05 (0.79-1.39)
Total (95% CI)	1075	1072		100.00	0.92 (0.76-1.12)
Total events	268	283			

Test for heterogeneity: $\chi^2 = 3.50$, $df = 2$ ($P = 0.17$); $I^2 = 42.9\%$
Test for overall effect: $Z = 0.82$ ($P = 0.41$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

B

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 02 recurrence at maxium follow-up
Outcome: 01 overall recurrence

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	84/435	93/428		43.66	0.86 (0.62-1.20)
Lacy 2008[15]	19/106	29/102		14.00	0.55 (0.29-1.06)
COLOR 2009[14]	105/534	92/542		42.34	1.20 (0.88-1.63)
Total (95% CI)	1075	1072		100.00	0.96 (0.78-1.19)
Total events	208	214			

Test for heterogeneity: $\chi^2 = 5.13$, $df = 2$ ($P = 0.08$); $I^2 = 61.0\%$
Test for overall effect: $Z = 0.37$ ($P = 0.71$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

C

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 02 recurrence at maxium follow-up
Outcome: 02 local recurrence

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	10/435	11/428		24.13	0.89 (0.37-2.12)
Lacy 2008[15]	7/106	10/102		21.20	0.65 (0.24-1.78)
COLOR 2009[14]	26/534	26/542		54.67	1.02 (0.58-1.77)
Total (95% CI)	1075	1072		100.00	0.91 (0.59-1.39)
Total events	43	47			

Test for heterogeneity: $\chi^2 = 0.58$, $df = 2$ ($P = 0.75$); $I^2 = 0\%$
Test for overall effect: $Z = 0.44$ ($P = 0.66$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

D

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 02 recurrence at maxium follow-up
Outcome: 03 distant recurrence

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	74/435	82/428		52.86	0.86 (0.61-1.22)
Lacy 2008[15]	8/106	14/102		10.17	0.51 (0.21-1.28)
COLOR 2009[14]	56/534	54/542		36.97	1.06 (0.71-1.57)
Total (95% CI)	1075	1072		100.00	0.90 (0.70-1.16)
Total events	138	150			

Test for heterogeneity: $\chi^2 = 2.15$, $df = 2$ ($P = 0.34$); $I^2 = 7.0\%$
Test for overall effect: $Z = 0.82$ ($P = 0.41$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

Figure 1 Meta-analysis on overall mortality (A), overall recurrence (B), local recurrence (C) and distant recurrence (D) at maximum follow-up.

the early postoperative course. Furthermore, the risk of postoperative morbidity is decreased by the laparoscopic approach, namely because of a reduced surgical morbidity.

However, the procedure is complex and for colon cancer the long-term results on survival are not known. There are several large RCTs published and several systematic reviews performed to assess the difference between the laparoscopic and open approach. In 2007, Bonjer and his colleague[19] performed a meta-analysis of trials randomizing patients with colon cancer to laparoscopically assisted or open colectomy to determine whether laparoscopic colectomy for cancer is oncologically safe. Patients included in this analysis had at least 3 years of complete follow-up data. Of 1765 patients, 229 were excluded, leaving 796 patients in the laparoscopically assisted arm and 740 patients in the open arm for analysis. Three-year disease-free survival rates in the laparoscopically assisted and open arms were 75.8% and 75.3%, respectively. The 3-year overall survival rate after laparoscopic surgery was 82.2% and after open surgery was 83.5%. Disease-free and overall survival rates for stages Ⅰ, Ⅱ, and Ⅲ evaluated separately did not differ between the 2 treatments. So they concluded that laparoscopically assisted colectomy for cancer is oncologically safe. Again in 2007, Kahnamoui and his colleagues[20] published a systematic review on laparoscopic surgery for colon cancer. The results of this review suggest that, although there is no definitive answer, overwhelming evidence presently indicates that laparoscopic colon cancer resection is as safe and efficacious as the conventional open technique. In 2008, Kuhry and his colleagues[21] published a Cochrane systematic review of randomised controlled trials on the long-term outcomes of laparoscopic surgery for colorectal cancer with the median follow-up from 19-59 mo. No significant difference in tumour recurrence after laparoscopic and open surgery for colon cancer was observed (3 RCTs, hazard ratio for tumour recurrence in the laparoscopic group 0.86; 95% CI: 0.70-1.08). Similarly, in colon cancer patients, no significant differences in overall mortality were found (2 RCTs, hazard ratio for overall mortality after laparoscopic surgery 0.86; 95% CI: 0.86-1.07). So they also concluded that laparoscopic resection of carcinoma of the colon is associated with a long-term outcome that is similar to that after open colectomy.

All 3 systematic reviews demonstrated that laparoscopic colon cancer resection is as safe and efficacious as the conventional open technique in terms of 3-year survival and 3-year disease-free survival and recurrence.

The main objective of this present meta-analysis is to demonstrate that in the long run, laparoscopic colon cancer resection is also as safe and efficacious as open surgery, especially for overall 5-year survival and mortality and recurrence. So the duration of follow-up of all included studies was more than 60 mo, as shown in the table of patient characteristics. There are several characteristics of the three included trials. First, all 3 included trials had a large sample size, especially COST trial (863) and COLOR (1076) trial. Second, the quality of the 3 trials is very high. Third, all of the patients only had colon adenocarcinoma. Additionally, the follow-up was very long. So the combined results from the 3 studies should be convincing. 2147 patients were included in this meta-analysis. The overall mortality was similar in the two groups (24.9%, 268/1075 in the laparoscopic group and 26.4%, 283/1072 in the open group) with no significance detected, as was the overall recurrence regardless of local or distant recurrence. Both the overall 5-year survival (about 74%) and 5-year disease-free survival (about 68%) were similar between the two groups, and no significant difference was demonstrated. From these results, laparoscopic colon cancer resection was demonstrated to be as safe and efficacious as the open surgery, as we expected.

In conclusion, compared to open surgery, laparoscopically assisted colectomy has been demonstrated to have short term advantages that include less pain, better pulmonary function, shorter time for return of bowel function (duration of postoperative ileus), less fatigue, improved convalescence, and more importantly reduced surgical morbidity and shorter duration of hospital stay. From the 3-year long-term data, laparoscopic surgery was also demonstrated to be as safe and efficacious as the conventional open approach. Our present work again demonstrated that, in terms of long-term outcomes of trials a the median follow-up more than 5 years, laparoscopic surgery is also as safe and efficacious as the conventional open approach. In addition, laparoscopic resection is associated with a modest additional cost, compared with open surgery. So regardless of the short-term outcomes or the long-term outcomes or even the cost-effectiveness, we could conclude that laparoscopic surgery is not inferior to the conventional open approach. From these analyses above, we think that for colon adenocarcinoma, laparoscopic assisted colectomy should be the preferred choice as appropriate.

COMMENTS

Background

Short-term advantages of laparoscopic colorectal surgery compared to conventional surgery are well known, however, it was uncertain whether there would be a long-term survival difference. Several large trials has been reported which report on long-term survival differences.

Research frontiers

The objective of this systematic review is to assess that in the long term, laparoscopic-assisted colon resection for cancer is not inferior to open colectomy with respect to cancer survival and recurrence.

Innovations and breakthroughs

This meta-analysis suggests that laparoscopic surgery was as efficacious and safe as open surgery for colon cancer, based on the five-year data of the included randomized clinical trials.

Applications

For colon adenocarcinoma, laparoscopic assisted colectomy should be the preferred choice as appropriate.

Peer review

The study was well structured and gives interesting information about the present status of laparoscopy and colon cancer. The main strength is the longer follow up of patients and the criteria to be included. I think this manuscript should be interesting for surgeons dealing with this disease.

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BRIEF ARTICLE

Diagnosis of bile duct hepatocellular carcinoma thrombus without obvious intrahepatic mass

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Abstract

AIM: To study the diagnosis of hepatocellular carcinoma (HCC) presenting as bile duct tumor thrombus with no detectable intrahepatic mass.

METHODS: Six patients with pathologically proven bile duct HCC thrombi but no intrahepatic mass demonstrated on the preoperative imaging or palpated intrahepatic mass during operative exploration, were collected. Their clinical and imaging data were retrospectively analyzed. The major findings or signs on comprehensive imaging were correlated with the surgical and pathologic findings.

RESULTS: Jaundice was the major clinical symptom of the patients. The elevated serum total bilirubin, direct bilirubin and alanine aminotransferase levels were in concordance with obstructive jaundice and the underlying liver disease. Of the 6 patients showing evidence of viral hepatitis, 5 were positive for serum alpha fetoprotein and carbohydrate antigen 19-9, and 1 was positive for serum carcinoembryonic antigen. No patient was correctly diagnosed by ultrasound. The main features of patients on comprehensive imaging were filling defects with cup-shaped ends of the bile duct, with large filling defects presenting as casting moulds in the expanded bile duct, hypervascular intraluminal nodules, debris or blood clots in the bile duct. No obvious circular thickening of the bile duct walls was observed.

CONCLUSION: Even with no detectable intrahepatic tumor, bile duct HCC thrombus should be considered in patients predisposed to HCC, and some imaging signs are indicative of its diagnosis.

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Key words: Hepatocellular carcinoma; Obstructive jaundice; Bile duct tumor thrombus; Diagnosis; Diagnostic imaging

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INTRODUCTION

Obstructive jaundice associated with hepatocellular carcinoma (HCC) is not common, with an incidence of 0.5%-13% in patients with HCC[1-11]. This type of HCC is also known as icteric type of HCC (IHCC)[5-7]. Bile duct tumor thrombus (BDTT) is the leading cause of obstruc-

tion in IHCC. It has been shown that HCC is smaller in patients with biliary tumor thrombi than in those without biliary tumor thrombi, with a mean tumor size of 3.8 ± 2.1 cm *vs* 6.7 ± 4.6 cm[8]. Several studies[9-11] concerning IHCC reported that primary hepatic parenchymal tumor is detectable in most patients with IHCC while no obvious intrahepatic tumor is detectable in only 2.9%-25.0% of patients with biliary HCC thrombi. We encountered 6 patients with this special type of IHCC. Postoperative pathologic examinations "surprisingly" proved that the bile duct nodules leading to obstructive jaundice were HCCs. However, neither intrahepatic mass nor portal vein thrombus was identified on the preoperative imaging or even during explorative surgery. This type of IHCC is very rare and difficult to diagnose, and only few cases have been occasionally reported[12-17], without their features summarized. We retrospectively analyzed the clinical and imaging data about 6 patients with emphasis laid on the diagnostic imaging correlated with pathologic and surgical data, and clinical features and imaging signs that might lead to the diagnosis.

MATERIALS AND METHODS

Ethics

This study was approved by our institutional review board. Informed consent was not obtained from the patients as this was a limited, anonymous retrospective review of patient data.

Patients

Six patients including 5 men and 1 woman at the age of 47-64 years were confirmed with bile duct HCC by surgery and histology between January 2000 and November 2008 at our hospital. Their medical records were thoroughly reviewed and cross-checked.

Jaundice was the predominant symptom of the patients, and presented as an initial symptom of 4 patients. The time from the onset of jaundice to admission was 7 d-3 mo (median 1 mo). The other main clinical symptoms were fatigue, right upper quadrant abdominal pain or upper abdominal pain, abdominal distension, loss of appetite and loss of weight. Liver function reserve was Child grade A and B in 4 and 2 patients, respectively.

Methods

Laboratory data about the patients before surgery were recorded and analyzed.

Each patient underwent two or more preoperative diagnostic imaging procedures, including transabdominal ultrasonography (US), computed tomography (CT) with plain scan and arterial and portal phase contrast enhanced scans, magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC). All available imaging data including diagnosis reports and images were retrospectively reviewed by two radiologists with more than 5 years of experience in abdominal imaging. A consensus was reached with the main findings or signs recorded.

Surgical records and pathologic reports were also reviewed and correlated with the major findings or signs on comprehensive imaging.

RESULTS

Blood test

The levels of total serum bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), hepatitis B surface antigen (HBsAg), α-fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA) of each patient are listed in Table 1.

US

Transabdominal US was performed, showing dilatated intrahepatic ducts with nodules in hilar bile ducts but no intrahepatic mass. The intraluminal nodules were hypoechoic, slightly hyperechoic, and mixed echoic in 4, 1, and 1 patients, respectively. Three and 1 patients were diagnosed as hilar cholangiocarcinoma and choledocholithiasis, respectively. Further evaluation was needed in 2 patients.

CT

CT with pre-contrast scan and dual-phase contrast-enhanced scan was performed for 5 patients, showing dilated bilateral intrahepatic ducts with an intraductal nodule obstructing the hilar bile duct and/or common bile duct, but no tumor thrombus in the portal vein or systemic vein and no obvious mass in the hepatic parenchyma. These intraductal nodules were relatively mild hypodense to the hepatic parenchyma on pre-contrast images. During the arterial phase, they showed different degrees of enhancement and were relatively isodense or mildly hyperdense to the hepatic parenchyma. The enhancement of intraductal nodules was relatively lower in portal phase than that of hepatic parenchyma. In three lesions with the longest diameter greater than 3.0 cm, the intraluminal nodules appeared as cast moulds in the dilated ducts without obvious thickening of the walls. Non-enhanced sludge, which was mildly hyperdense in the bile, was observed in the common bile duct of 2 patients (Figure 1). The sludge was found to be tumor debris or hemorrhage of tumor at surgery. CT showed signs of liver cirrhosis in 4 patients, such as splenomegaly, varices, heterogeneously attenuated liver with lacelike fibrosis and regenerative nodules, and irregular or nodular liver surface. A small amount of ascites was present in 1 patient.

MRI combined with MRCP

Conventional non-enhanced MRI combined with MRCP was performed for 3 patients, showing no mass in the hepatic parenchyma or portal vein. MRCP images showed moderate-severe dilatation of bilateral hepatic ducts with columnar or plugged filling defect of bile ducts in the hilar area. The filling defects were hypointense on T_1-weighted

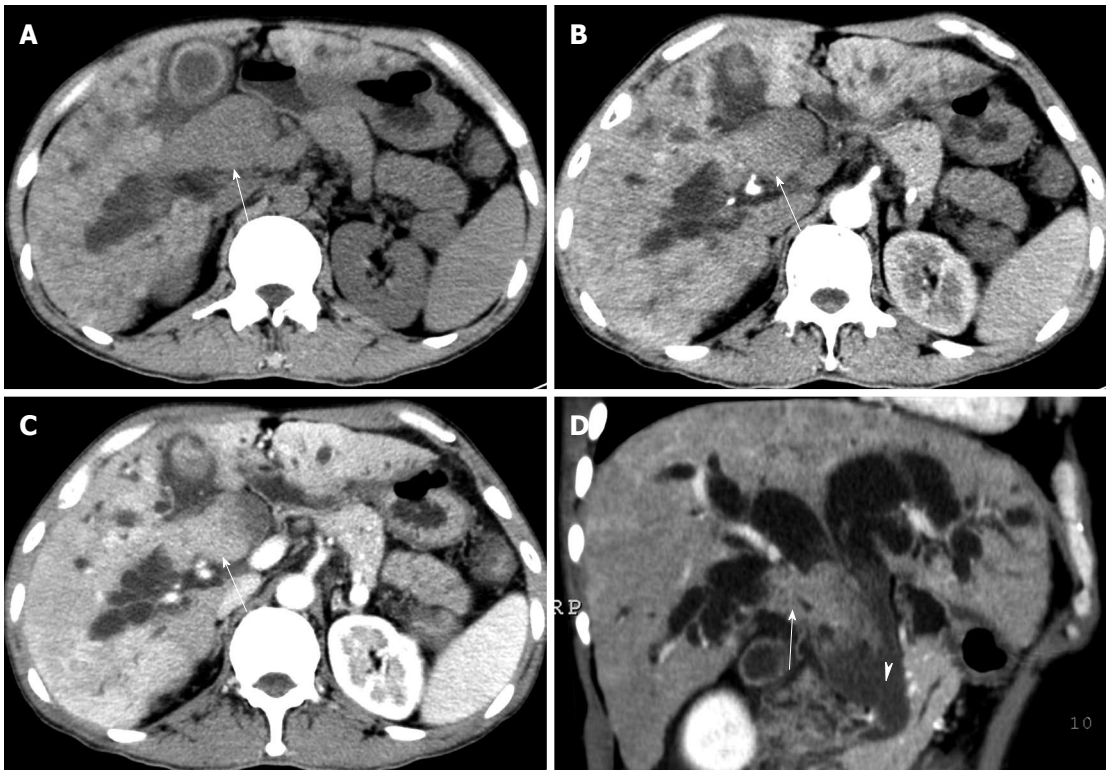

Figure 1 Axial computed tomography images of case 1 at plain scan (A), early arterial phase (B), portal phase (C), and coronal oblique plane reformation image at portal phase (D) showing irregular tumor thrombi in right hepatic, common hepatic and common bile ducts (arrows). The tumor thrombus was mild hypointense on plain scan with enhancement in early arterial phase. Neither portal vein thrombus nor hepatic parenchymal mass was identified. Heterogeneously attenuated liver with lacelike fibrosis and regenerative nodules due to cirrhosis could be observed. The common bile duct was filled with hemorrhage and debris (arrowhead), which was not enhanced and mild hyper-dense in the bile.

Table 1 Blood test

Patient No./sex/age (yr)	TBIL (μmol/L)	DBIL (μmol/L)	ALT (U/L)	HBsAg	AFP (ng/mL)	CA19-9 (U/mL)	CEA (μg/L)
1/M/48	364.4	145.7	99.4	Positive	159	123.1	2.82
2/F/64	265.1	110.2	190.9	Positive	12.4	> 575	3.24
3/M/47	268.6	146.4	127.4	Positive	263	465.7	7.10
4/M/51	277.3	156.1	137.2	Positive[1]	> 1210	Unknown	Unknown
5/M/53	373.9	153.6	133.8	Positive	Negative	183.1	1.52
6/M/55	344.2	199.3	204.0	Positive	10.7	164.9	3.90

Normal value is less than 8.1 ng/mL for α-fetoprotein (AFP), less than 37 U/mL for carbohydrate antigen 19-9 (CA19-9), and less than 5 μg/L for carcinoembryonic antigen (CEA), respectively. [1]Also positive for hepatitis E virus-IgG. HBsAg: Hepatitis B surface antigen; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; TBIL: Total serum bilirubin.

images and iso or mild hyperintense on T_2-weighted images. The intraluminal nodule was originated from the left hepatic duct and extended downward into the common bile duct of 1 patient accompanying a short T_1 signal in surrounding bile duct and gallbladder due to intraluminal hemorrhage of tumor confirmed at surgery (Figure 2). Debris as a sludge-like filling defect was observed in the common bile duct of another patient.

ERCP

ERCP was performed for 1 patient, showing a smooth oval filling defect in the upper common bile, common and right hepatic ducts with dilated intrahepatic ducts (Figure 3A). The cup-shaped filling defect caused dilatation of the bile duct. Gallbladder was not visualized because of obstruction by the tumor.

PTC

PTC was performed for 1 patient, showing an oval smooth intraluminal filling defect in common and right hepatic ducts with dilated intrahepatic ducts (Figure 3B). Both ends of the filling defect were cup-shaped.

Findings during surgery

Tumor thrombi in bile ducts and evident hepatic cholestasis were found in 6 patients during surgery. Typical liver cirrhosis was found in 4 patients. Diffuse HCC was not considered because none of them had evidence of

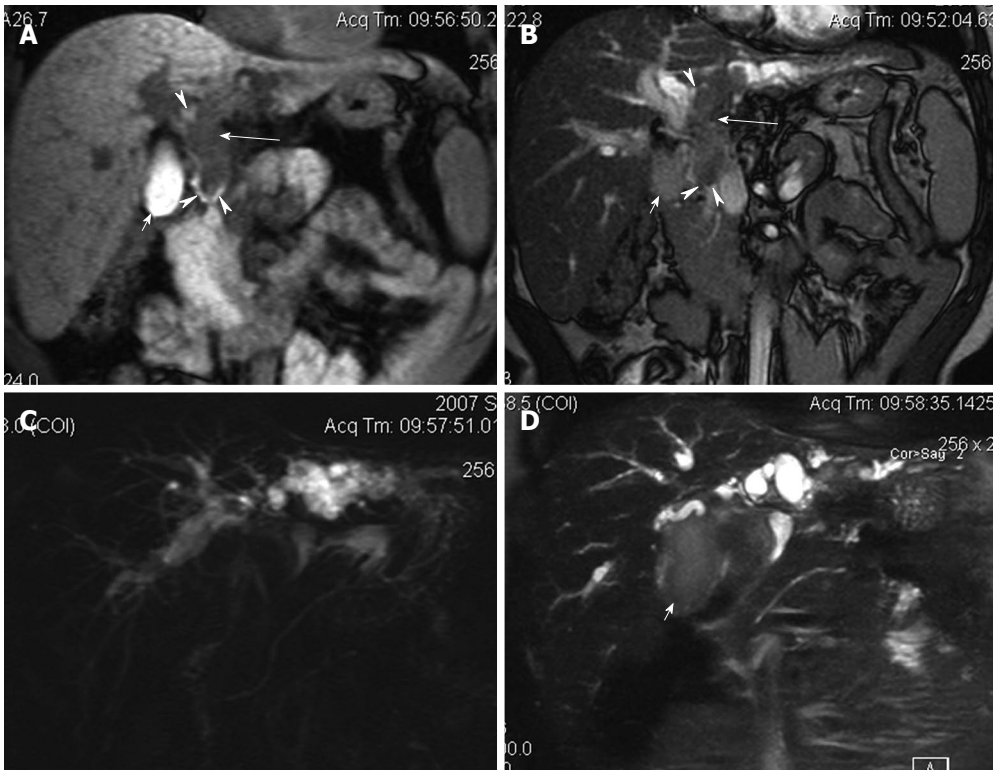

Figure 2 Magnetic resonance and magnetic resonance cholangiopancreatography images of Case 2. A: Coronal T1 weighted image showing hypo-intense tumor thrombus (large arrows) in left hepatic, common hepatic and common bile ducts; B: Coronal T2 weighted image showing the tumor thrombus as iso- to slight hyper-intense; C: Thick slice magnetic resonance cholangiopancreatography image showing bilateral dilated intrahepatic ducts with "vanished" common hepatic and common bile ducts; D: Thin slice magnetic resonance cholangiopancreatography image showing filling defect in the hilar. Neither portal vein thrombus nor hepatic parenchymal mass is identified. The signal intensity of liver is heterogeneous due to cirrhosis. Blood clots or hemorrhage can be observed in gallbladder (small arrows), common hepatic and common bile ducts (arrowheads), which are hyper-dense on T1 weighted image, and slight hyper-intense on T2 weighted image.

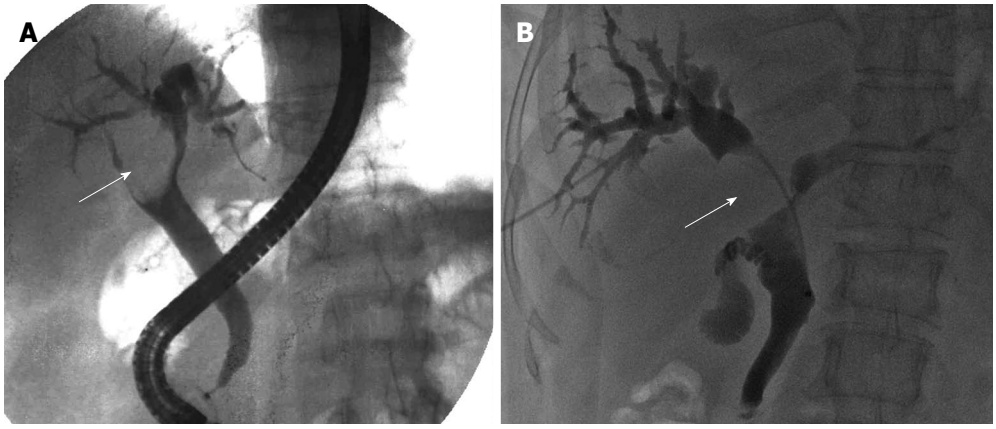

Figure 3 Endoscopic retrograde cholangiopancreatography image of case 3 (A) and percutaneous transhepatic cholangiography image of case 4 (B) showing tumor thrombus (arrows) in right hepatic duct and common hepatic duct as a smooth oval filling defect on endoscopic retrograde cholangiopancreatography image and a smooth oval filling defect on percutaneous transhepatic cholangiography image, respectively.

tumor invasion of the portal vein or system vein. No obvious intrahepatic mass was palpated in all patients. Diffuse miliary peritoneum metastasis was observed in 1 patient and thought to be infiltration of the tumor in hilar duct. The tumor thrombi were dark brown, dark red or yellowish brown in color, and soft or slightly elastic, relatively friable, and extremely vascular tending to bleed even on light touch. Most of them were easily separated from the bile duct walls. Sludge-like debris or small blood clots were found in the common bile ducts of 4 patients and blood-stained bile was found in the intrahepatic ducts of 2 patients. Removal of tumor thrombi was attempted in 6 patients and was successful without active hemorrhage in 4 patients. Partial hepatectomy was performed for 2 patients, and aborted in 4 patients due to the poor liver function reserve or peritoneal metastasis. Further exploration after clearance of thrombi revealed relatively smooth internal walls of common bile duct (CBD) and

common hepatic duct (CHD). The resected liver tissue revealed a small hepatic parenchymal tumor in each, 0.5 cm × 1.0 cm and 0.8 cm × 1.5 cm in size.

Pathology

The pathologic reports of intrabiliary thrombi revealed HCC but no variants of cholangiocarcinoma, mixed type of HCC or cholangiocarcinoma in 6 patients. Of the 6 patients, 2 had poorly-differentiated HCC, 3 had poorly-moderately differentiated HCC, and 1 had moderately-differentiated HCC, which accompanied hemorrhage or necrotic tissue in most of the 6 patients.

DISCUSSION

Portal vein tumor thrombus (PVTT) is frequently seen in HCC patients. However, HCC presented as biliary duct tumor thrombus (BDTT) is a relatively rare entity. Intrahepatic tumor or PVTT is evident in most of patients with HCC presented as BDTT, yet few patients with HCC thrombi in the bile duct but without any detectable intrahepatic mass or PVTT have been reported[9-17]. In this circumstance, it is difficult but still important to establish the correct diagnosis, especially to differentiate it from cholangiocarcinoma or diffuse-type HCC. Because the therapeutic plan for HCC presented as BDTT may be substantially different from that for cholangiocarcinoma and diffuse-type HCC, surgery can often be offered when the disease is still localized[1,2,10-20], while percutaneous transhepatic cholangial drainage (PTCD) combined with transcatheter arterial chemoembolization (TACE) serves as an effective alternative therapy especially when the tumor is unresectable[1,6,7]. We retrospectively analyzed the clinical and imaging data about these patients, and found that some features might be helpful for the diagnosis.

Jaundice is the predominant clinical presentation of this disease[1-21]. Causes of obstructive jaundice in this type of HCC include intraluminal growth of tumor leading to obstruction of intra or extrahepatic ducts, tumor tissue fragments and/or hemorrhages or blood clots due to necrosis, bleeding, and detachment of intraductal tumors, giving rise to the obstruction, which are similar to the reported findings in IHCC[3].

Apart from jaundice, there are also other non-specific symptoms such as fatigue, abdominal pain, abdominal distension and loss of appetite. A differential diagnosis between this and other common diseases causing obstructive jaundice such as cholangiocarcinoma and choledocholithiasis is essential.

In this study, all the 6 patients were positive for the markers of chronic viral hepatitis. The proportion of liver cirrhosis was relatively high with typical liver cirrhosis found in 4 patients. The elevated serum ALT level in our patients might be associated with the underlying disease. The majority of patients were middle-aged or old males. These features were also found in common types of HCC.

Serum tumor markers may be helpful in the diagnosis of this disease[22]. Positive serum AFP supports the diagnosis of HCC, while CEA level is frequently elevated in patients with cholangiocarcinoma. In this study, the positive ratios for AFP and CA19-9 were high, suggesting that positive AFP and CA19-9 support the diagnosis. However, positive CA19-9 may also frequently be seen in cholangitis, bile duct stones and biliary or pancreatic tumors leading to obstructive jaundice[23]. Thus, its value for differential diagnosis has to be further evaluated in studies with more cases.

Several diagnostic imaging methods play an important role in the diagnosis of obstructive jaundice[24-31]. The location and extent of obstruction can be reliably demonstrated using proper techniques, and the cause of obstruction can often be inferred by analyzing the morphology of obstruction site and other relevant signs. Transabdominal US is the most widely used and usually the initial tool to evaluate obstructive jaundice. However, none of our patients was correctly diagnosed by transabdominal US, and most of them were misdiagnosed as cholangiocarcinoma. Tamada *et al*[29] reported that intraductal US (IDUS) can distinguish between tumor thrombi caused by HCC and polypoid type cholangiocarcinoma. However, IDUS has not been widely accepted because it is invasive and requires special equipments and specific expertise. CT, MRI combined with MRCP, ERCP and PTC are also widely used to evaluate obstructive jaundice. Jung *et al*[30] compared the CT features of HCC invading bile ducts with those of intraductal papillary cholangiocarcinoma, and found that the presence of parenchymal mass is the distinct difference between them. Since no parenchymal mass was detectable on cross-sectional images in our patients, we searched for other signs leading to the diagnosis. After analyzing the intrabiliary lesions on comprehensive images with surgical correlation, we found that the following features may be helpful in the diagnosis of this special type of IHCC.

First, the tumor thrombi are typically intraluminal polypoid lesions with irregular or smooth surface depending on the presence of necrosis. Lesions can be either small or larger. Small lesions are localized in 1 or 2 ducts while large lesions can form "biliary duct casts" and extend inferiorly. Because of rapid growth of the tumor with no apparent fibrosis of the duct wall, the ducts can often be expanded into a fusiform shape at the site of lesion. Cup-shaped filling defects can also be observed in dilated ducts around the lesion on cholangiographic images due to the expanded growth pattern of the lesion.

Second, the tumor thrombi are hypervascular. It is well known that HCC is a hypervascular tumor. Detection of vascularity in bile duct thrombi can reliably rule out the diagnosis of bile duct stones, and differentially diagnose between HCC and cholangiocarcinoma. The bile duct thrombi with the characteristic early enhancement pattern on dual-phase contrast-enhanced CT[30] or dynamic contrast-enhanced MRI[28] are important to differentiate HCC from cholangiocarcinoma. The results of this study support this finding. The hypervascularity of bile duct tumor thrombi was confirmed during surgery in this study. It was reported that color Doppler sonography can also effectively detect tumor vascularity of bile duct HCC thrombi[31].

Third, intraluminal fragments or blood clots may

present. Because the tumor thrombi are loose, fragile and prone to necrosis, detachment of parts of the lesions and hemorrhage may frequently occur, and those that are relatively large can lead to free thrombi in the ducts. CT can demonstrate irregular or sludge-like lesions in the ducts with no enhancement. MRI and MRCP may be even superior over CT in demonstrating such lesions[28]. Hemorrhagic lesions or blood clots have a high signal on T_1WI, and a low signal on T_2WI. Debris in bile appears as sludge in bile ducts and gallbladder.

Fourth, no apparent circular thickening of the duct walls or constriction of the ducts is present. Cholangiocarcinoma is often associated with the thickening of bile duct walls, often leading to constriction of nearby ducts. No apparent thickening of the duct walls, especially no circular thickening of the walls, was observed in our patients, and ducts were compacted rather than constricted or narrowed due to the tumor.

Fifth, no portal vein thrombus is present. It might be due to the relatively early stage of the disease in our patients, and it is critical for differentiating it from diffuse-type HCC.

Sixth, cirrhosis of the background liver may support the diagnosis. A relatively high percentage of cirrhosis was observed on preoperative images and during surgery in our patients. "Downstream duct dilatation", a sign standing for the dilatated bile duct below the level of intraluminal nodule, has been described by Jung *et al*[30] in patients with intraductal cholangiocarcinoma, which is thought to be related to mucin produced by the tumor. However, "downstream duct dilatation" was also present in 2 out of 6 patients in our case study, which was contributed to the obstruction by blood clots or fragments in the common bile ducts.

The reasons why HCC is present as intrabile duct tumor thrombi without detectable primary hepatic tumor are as follows. The tumor may originate from cancerization of ectopic hepatocytes in the bile duct wall[17], or the primary tumor is just too small to be identified, or the tumor located at the origin of or close to the intrahepatic duct grows intraluminally and stretchs inferiorly. Although no primary hepatic tumor was demonstrated on preoperative imaging or palpated during operation in our patients, the resected tissues revealed small hepatic tumors in 2 patients. Moreover, since deeply seated small hepatic tumor is hard to palpate during intraoperative exploration, especially in patients with marked cirrhosis, it is hard to rule out the potentiality of small primary intrahepatic HCC in the other 4 patients who did not undergo partial hepatic resection. So, it is recommended that intraoperative ultrasonography (IOUS) should be performed to find the potential intrahepatic tumor or to determine the resection level before the operator decides to perform the resection.

If this disease is suspected, it is still important to look for more sensitive techniques such as CT during arterial portography (CTAP) and superparamagnetic iron oxide (SPIO)-enhanced MRI to find possible primary tumors. CTAP is generally accepted as the most sensitive technique to detect small HCC, but it is only performed for selected patients due to its invasiveness. SPIO-enhanced MRI has emerged as another effective technique to detect small HCC, but its value in evaluating bile duct tumor has not yet fully investigated. Unless the clinicians or the radiologists take HCC into consideration, these techniques can be first adopted in the diagnosis of obstructive jaundice. Thus, our study may help the clinicians and radiologists to consider this disease before such techniques are applied.

There are some limitations in our study. First, it is a retrospective analysis of a limited number of cases. Second, although dynamic contrast-enhanced MRI is used as a conventional technique for the diagnosis of HCC in our hospital, it has not been routinely performed for the evaluation of obstructive jaundice. Third, contrast studies with other types of HCC or other tumors with intraluminal growth are not available due to the limited number of cases. Further study is needed to verify the diagnostic value of the features listed.

COMMENTS

Background

Hepatocellular carcinoma (HCC) thrombus in the bile duct is a rare cause of obstructive jaundice. Although it is rarely encountered, its correct diagnosis, especially differentiating it from other causes of biliary obstruction such as cholangiocarcinoma, is very important. Usually, the presence of primary intrahepatic tumors is the key to its diagnosis. However, since few cases of HCC thrombi in the bile duct with no detectable intrahepatic mass have been reported, its diagnosis is even difficult.

Research frontiers

Six patients with this rare disease were reported. Their clinical and imaging data were retrospectively analyzed with a review of the literature. Some clinical features and imaging signs that may favor the diagnosis were summarized. The study may be helpful for a better understanding of the disease, especially for its diagnosis.

Innovations and breakthroughs

Little is known about the diagnosis of this rare disease. More accurate diagnosis were introduced in this study by describing their clinical features and imaging signs.

Applications

This research may evoke the attention of clinicians to the diagnosis of bile duct hepatocellular carcinoma thrombi without an intrahepatic tumor demonstrated on the diagnostic imaging. If certain clinical features and imaging signs are presented, the diagnosis of the disease can be considered.

Peer review

The manuscript presents an interesting series of patients with HCC presented as biliary tract obstruction leading to jaundice. The clinical and imaging data help find the features that differentiate this tumor from cholangiocarcinoma.

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CASE REPORT

Laparoscopic wedge resection of synchronous gastric intraepithelial neoplasia and stromal tumor: A case report

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Abstract

Synchronous occurrence of epithelial neoplasia and gastrointestinal stromal tumor (GIST) in the stomach is uncommon. Only rare cases have been reported in the literature. We present here a 60-year-old female case of synchronous occurrence of gastric high-level intraepithelial neoplasia and GIST with the features of 22 similar cases and detailed information reported in the English-language literature summarized. In the present patient, epithelial neoplasia and GIST were removed *en bloc* by laparoscopic wedge resection. To the best of our knowledge, this is the first reported case treated by laparoscopic wedge resection.

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Key words: Laparoscopy; Stomach neoplasm; Gastrointestinal stromal tumor; Gastrectomy; Synchronous neoplasm

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INTRODUCTION

Synchronous occurrence of epithelial neoplasia and gastrointestinal stromal tumor (GIST) in the stomach is uncommon. Only few case reports can be found in the literature[1-16]. We present here a case of synchronous occurrence of gastric high-level intraepithelial neoplasia and GIST in the body of stomach, close to the cardia. Epithelial neoplasia and GIST were removed *en bloc* by laparoscopic wedge resection. To the best of our knowledge, this is the first reported case treated by laparoscopic wedge resection. In addition, we also summarized the features of 22 similar cases with detailed information reported in the English-language literature.

CASE REPORT

A 60-year-old woman was admitted to our department in June 2009 because of epigastric pain for three months. She had no fever, nausea or vomiting, hematemesis or melena, and weight loss. Physical examination showed no abnormalities. Blood biochemistry was within the normal range. Computed tomography (CT) of the abdomen with intravenous contrast demonstrated a soft tissue mass measuring 5 cm × 5 cm in size with a clear borderline near the lesser curvature of the gastric body, which was consistent with a GIST (Figure 1). Gastroscopy revealed a mucosal ulcer about 1 cm in diameter located in the lesser curvature of the stomach, 3 cm away from the cardia (Figure 2). Histological examination of the specimen from the ulcer showed high-level intraepithelial neoplasia with positive *Helicobacter pylori*.

Figure 1 Computed tomography scan demonstrating a soft tissue mass (arrow) near the lesser curvature of the gastric body.

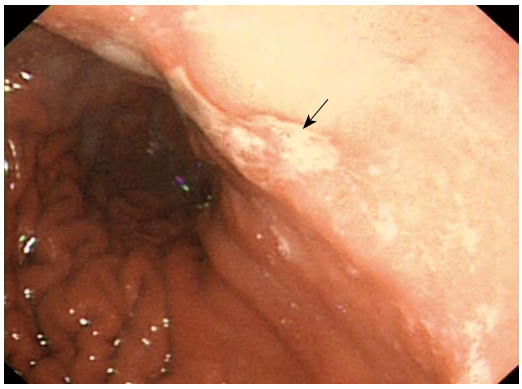

Figure 2 Gastroscopy revealing a mucosal ulcer (arrow) located in the lesser curvature.

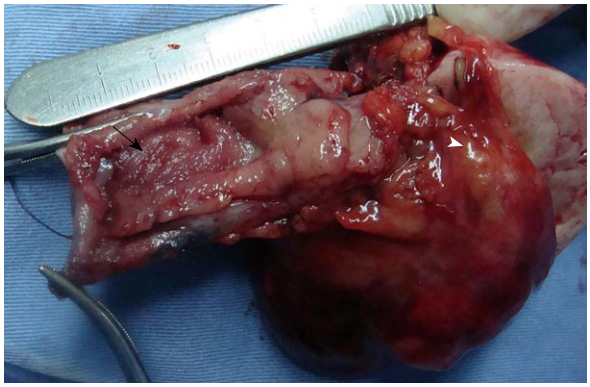

Figure 3 Resected specimens of mucosal ulcer (arrow) and GIST (arrowhead).

During laparoscopic exploration, an extramural pedunculated mass, approximately 5 cm in diameter, was located in the lesser curvature of the gastric body. By intraoperative gastroscopic injection of methylene blue, the mucosal ulcer was localized proximate to the extramural tumor, with 2 cm in between. Laparoscopic wedge resection of the two lesions was performed with triple endoscopic linear staplers (Endocutter 60 staple, green cartridge; Ethicon, Endo-Surgery, Cincinnati, OH, USA) (Figure 3). Intraoperative frozen section of the resected margins was

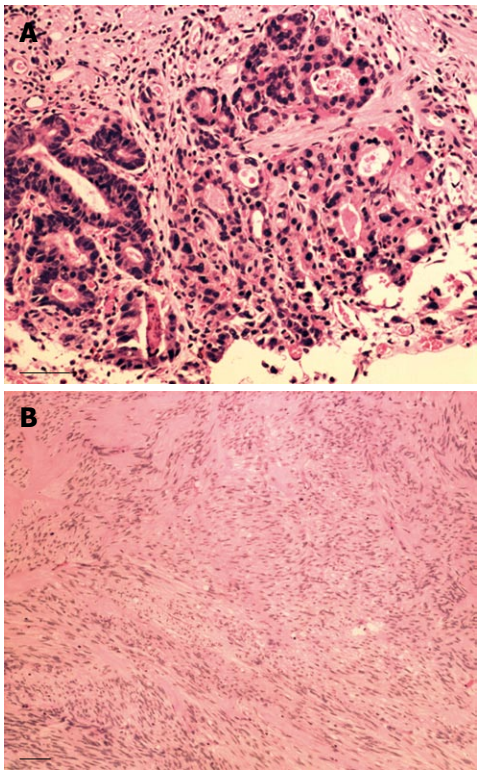

Figure 4 Histological features of high-level intraepithelial neoplasia (A) (HE stain, 200 ×) and gastrointestinal stromal tumor (B) (HE stain, 100 ×). Scale bar = 100 μm.

free of tumor. The operation time was 150 min and intraoperative bleeding was 50 mL. The postoperative course was uneventful, and the patient was discharged 4 days later. She was followed up and abdominal CT and upper gastrointestinal imaging 6 mo after operation showed no signs of recurrence.

Histopathological examination of the mucosal ulcer revealed high-grade intraepithelial neoplasia (Figure 4A) without lymph node metastasis (0/8), while the extramural mass was verified as a stromal tumor consisted of spindle to ovoid-shaped mesenchymal cells arranged in interlacing bundles or sheets (Figure 4B). The cells demonstrated eosinophilic cytoplasm and single elongated nuclei with a moderate level of mitotic activity (3 mitoses per 50 HPF, H&E stain). Immunohistochemical staining was positive for CD117 (Figure 5A) and CD34 (Figure 5B) but negative for SMA, S-100 and Desmin.

DISCUSSION

The term of GIST was introduced by Mazur *et al*[17] in 1983 in order to indicate a distinct heterogeneous group of mesenchymal neoplasms of spindle or epithelioid cells with varying differentiation. GIST occurs from the lower esophagus to the anus, with its most common site in the stomach. However, simultaneous occurrence of GIST and epithelial tumor in the stomach is uncommon. To the best of our knowledge, 44 cases have been reported in the English-language literature[1-16]. The largest published study consisted of 22 cases[16], but without detail information.

Table 1 Summary of previous synchronous gastric epithelial tumors and gastrointestinal stromal tumors in the stomach

No.	Source	Sex/ age (yr)	Epithelial tumor				GIST			Surgical procedure
			Location	Size (cm)	Appearance	Histology	Location	Size (cm)	Appearance	
1	Maiorana *et al*[1]	F/81	Cardia	4	Exophytic	AC	Fundus	5	Intramural mass	Partial gastrectomy
2	Maiorana *et al*[1]	F/79	Antrum	2	Erosion	AC	Pylorus	6	Submucosal mass	Partial gastrectomy
3	Maiorana *et al*[1]	M/75	Antrum	4	Ulcer	AC	Antrum	5	Submucosal mass	Total gastrectomy
4	Maiorana *et al*[1]	F/79	Pylorus	1.2	Ulcer	AC	Corpus	5	Subserosal nodule	Total gastrectomy
5	Maiorana *et al*[1]	M/79	Antrum	2	Ulcer	AC	Corpus	0.6	Subserosal nodule	Total gastrectomy
6	Maiorana *et al*[1]	M/69	Corpus	0.6	Sessile polyp	Carcinoid	Corpus	5	Submucosal nodule	Resection of submucosal nodule
7	Andea *et al*[2]	F/73	Antrum	0.6	Nodule	Carcinoid	Fundus	1.2	Intramural nodule	Antrectomy + wedge resection
8	Kaffes *et al*[3]	M/78	Antrum	Unknown	Slightly raised	AC	Corpus	1.5	Serosal nodule	Total gastrectomy
9	Liu *et al*[4]	M/70	Cardia + corpus	8	Ulcerative	AC (collision)	Cardia + corpus	8	Ulcerative tumor	Total gastrectomy
10	Bircan *et al*[5]	M/71	Antrum	5.7	Ulcerovegetative	AC	Corpus	0.5	Subserosal nodule	Total gastrectomy
11	Bircan *et al*[5]	M/77	Corpus	7.5	Exophytic	AC	Cardia	0.6	Submucosal nodule	Total gastrectomy
12	Wronski *et al*[6]	F/64	Antrum	5	Unknown	AC	Corpus	2	Unknown	Unknown
13	Wronski *et al*[6]	M/66	Antrum	1	Unknown	AC	Corpus	1	Unknown	Unknown
14	Lin *et al*[7]	F/70	Antrum	1.7	Depressed	AC	Fundus	1.1	Sessile polyp	Subtotal gastrectomy
15	Uchiyama *et al*[8]	M/74	Antrum	1.5	Elevated	AC	Corpus	0.8	Extramural nodule	LADG + wedge resection
16	Lee *et al*[9]	M/82	Corpus	1.5	Ulcer	AC	Corpus	9.5	Transmural tumor	Palliative wedge resection
17	Salemis *et al*[10]	F/78	Antrum	6.5	Ulcerative	AC	3 cm to AC	1	Nodular lesion	Total gastrectomy
18	Villias *et al*[11]	M/78	Antrum	Unknown	Ulcer	AC	3.5 cm to AC	0.9	Subserosal nodule	Subtotal gastrectomy
19	Kountourakis *et al*[12]	F/72	Unknown	Unknown	Unknown	AC	Unknown	1.8	Unknown	Subtotal gastrectomy
20	Hsiao *et al*[13]	M/75	GEJ	0.8	Polyp-like	AC	Near AC	3.3	Serosal nodule	Proximal gastrectomy + distal esophagectomy
21	Bi *et al*[14]	F/73	Fundus	4	Ulcerovegetative	AC (collision)	Fundus	4	Ulcerovegetative	Proximal subtotal gastrectomy
22	Ozgun *et al*[15]	M/78	Antrum	Unknown	Ulcer	AC	Opposite to AC	10	Extramural mass	Total gastrectomy

AC: Adenocarcinoma; LADG: Laparoscopic assisted distal gastrectomy; GEJ: Gastroesophageal junction; GIST: Gastrointestinal stromal tumor.

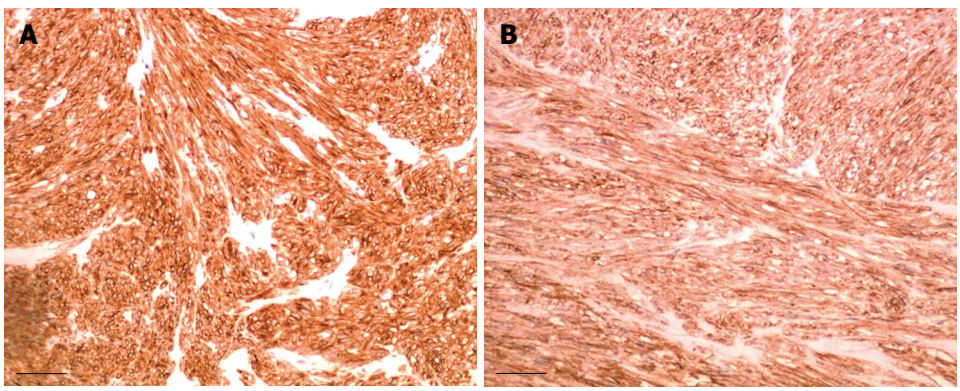

Figure 5 Over-expression of CD117 (A) and CD34 (B) (200 ×). Scale bar = 100 μm.

The remaining 22 cases (12 males and 10 females) at the age of 64-82 years (mean 74.6 years) are listed in Table 1. Of the 22 cases, 20 had adenocarcinoma and 2 had carcinoid.

The simultaneous development of gastric epithelial and stromal tumors, especially two cases of collision tumor composed of gastric adenocarcinoma intermingled with primary GIST[4,14], indicating that such an occurrence is intrinsically connected. An interesting hypothesis is that a single carcinogenic agent can interact with 2 neighboring tissues, inducing the development of tumors of different histotypes in the same organ, and experimental evidence for this possibility has been provided[18,19]. Oral administration of N-methyl-N9-nitro-N-nitrosoguanidine induces the development of gastric adenocarcinomas in rats[18]. When it is used in combination with other agents that alter the gastric mucosal barrier, such as aspirin or stress, leiomyosarcoma develops in conjunction with epithelial tumor[19].

Although many surgeons have realized the possibility of simultaneous development of gastric epithelial and stromal tumors, it is still difficult to diagnose it before operation. In our reviewed cases, simultaneous gastric adenocarcinoma and GIST were confirmed only in 1 case by histological examination before operation[7]. To increase the preoperative diagnostic rate of synchronous tumors, enhanced abdominal CT scan, gastroscopy and endoscopic ultrasonography have been recommended. Careful exploration of residual stomach intraoperatively is also important to avoid missing GIST when it is too small to be found by image examination.

It has been reported that laparoscopic surgery for early gastric cancer and GIST is safe, valid, and minimally invasive[20,21]. However, rare reports are available on laparoscopic resection of synchronous gastric epithelial tumor and GIST. In our reviewed cases, only 1 case was treated by laparoscopic procedure (laparoscopy-assisted distal gastrectomy + laparoscopic wedge resection)[8]. In our case, complicated lymphadenectomy was not needed for either gastric high-level intraepithelial neoplasia or GIST located in the same region with only 2 cm in distance, that makes laparoscopic wedge resection a optimal choice for the patient. Because of the close location of the lesions to the cardia, care should be taken not to injure the esophagocardial junction while firing the stapler. Intraoperative gastroscopy is a simple and effective procedure for the complete excision of tumors and intactness of esophagocardial junction.

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Haemodynamic and renal effects of tadalafil in patients with cirrhosis

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Abstract

A recent report introduced the phosphodiesterase-5 inhibition by vardenafil as a novel treatment of portal hypertension in patients with cirrhosis. In the herein presented "letter to the editor", the administration of tadalafil did not influence portal haemodynamics but impaired systemic haemodynamics in patients with cirrhosis. Our observations concur with the results of a report in a previous issue of *World Journal of Gastroenterology* (October 2008). Moreover, tadalafil adversely affected renal function in patients with decompensated liver disease.

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Key words: Tadalafil; Portal hypertension; Cirrhosis; Ascites; Phosphodiesterase-5 inhibition

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TO THE EDITOR

We read with interest the article by Clemmesen *et al*[1] in a previous issue of *World Journal of Gastroenterology* (October 2008) regarding the effects of sildenafil in patients with cirrhosis and hepatic venous pressure gradient (HVPG) above 12 mmHg. Sildenafil had no effects on HVPG but significantly reduced the mean arterial pressure. We would like to add our experience with the use of tadalafil, a long-acting phosphodiesterase-5 (PDE-5) inhibitor[2], in treatment of patients with cirrhosis.

Six patients with and 6 patients without ascites and oesophageal varices (Child-Pugh class A/B/C: 6/0/0 and 0/2/4, respectively) were studied at baseline and 2 h after oral administration of 10 mg of tadalafil. All patients were included after written informed consent was obtained from them and after the local scientific-ethical committee approved the study. The inclusion criteria were the same as in the study of Clemmesen *et al*[1]. Portal vein velocity (PVV) and portal flow volume (PFV) were evaluated as described by Deibert *et al*[3]. Cardiac output (CO) detected by Doppler ultrasound, mean arterial pressure (MAP) measured with an automatic sphygmomanometer, and systemic vascular resistance (SVR) expressed as the ratio MAP/CO were also evaluated. All patients received a continuous infusion of dextrose water at a rate of 2 mL/min for 4 h before and after the administration of tadalafil to

Table 1 Effects of tadalafil on portal and systemic haemodynamics and renal function in patients with cirrhosis (mean ± SE)

	Compensated (n = 6)		P^1	Decompensated (n = 6)		P^1	P^2
	Baseline	2 h		Baseline	2 h		
PVV (m/s)	0.103 ± 0.016	0.102 ± 0.019	0.9	0.186 ± 0.011	0.18 ± 0.015	0.8	0.6
PFV (L/min)	0.611 ± 0.116	0.543 ± 0.125	0.2	1.194 ± 0.169	1.175 ± 0.198	0.7	0.5
MAP (mmHg)	93.9 ± 3	87.5 ± 2.9	0.02	84.8 ± 2.4	76.9 ± 2	0.001	0.02
CO (L/min)	5.56 ± 0.23	5.7 ± 0.24	0.04	6.91 ± 0.3	7.35 ± 0.25	0.002	0.03
SVR (dynes.sec.cm^{-5})	1708 ± 116	1555 ± 101	0.02	1243 ± 84	1056 ± 59	0.001	0.03
ClCre (mL/min)	98.6 ± 8.1	95.6 ± 7.4	0.09	71.6 ± 2.4	64 ± 2.8	0.001	0.01
UNaV (μmol/min)	102 ± 15.9	97 ± 13.7	0.09	29.6 ± 7.3	20.6 ± 4.6	0.02	0.01

[1]*vs* baseline values; [2]*vs* basal and final results in two groups of patients. PVV: Portal vein velocity; PFV: Portal flow volume; MAP: Mean arterial pressure; CO: Cardiac output; SVR: Systemic vascular resistance; ClCre: Creatinine clearance; UNaV: Urinary sodium.

sustain diuresis, and urine was collected over the two periods of time for estimation of creatinine clearance (ClCre) and sodium excretion.

Tadalafil did not significantly change the PVV and PFV but significantly reduced the MAP and SVR and significantly increased the CO in both study groups (Table 1). More significant systemic haemodynamic changes together with a significant decrease in ClCre and natriuresis were noted in the patients with decompensated cirrhosis.

Our observations concur with the results of Clemmesen *et al*[1] and previous series of compensated[4] or mixed compensated and decompensated patients with cirrhosis[5], showing that PDE-5 inhibition by sildenafil has no effect on portal pressure and impairs systemic haemodynamics. Furthermore, the present results confirm those of Thiesson *et al*[6] in that PDE-5 inhibition may adversely affect renal function and natriuresis in patients with cirrhosis and ascites, possibly due to deterioration of the hyperdynamic state. Although a recent report introduced PDE-5 inhibition by vardenafil as a novel treatment of portal hypertension, the present and previous data[1,4,5] strongly question the portal hypotensive efficacy and safety of PDE-5 inhibitors in patients with cirrhosis.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 40 October 28, 2010

EDITORIAL

5011 Small-for-size syndrome in adult-to-adult living-related liver transplantation
Gruttadauria S, Pagano D, Luca A, Gridelli B

5016 Real-time histology with the endocytoscope
Singh R, Chen Yi Mei SL, Tam W, Raju D, Ruszkiewicz A

TOPIC HIGHLIGHT

5020 Quality of life after surgery of the alimentary tract
Scarpa M

5024 Health related quality of life in inflammatory bowel disease: The impact of surgical therapy
Umanskiy K, Fichera A

5035 Quality of life after laparoscopic and open colorectal surgery: A systematic review
Bartels SAL, Vlug MS, Ubbink DT, Bemelman WA

REVIEW

5042 Breastfeeding and chronic HBV infection: Clinical and social implications
Petrova M, Kamburov V

ORIGINAL ARTICLE

5047 Daily genetic profiling indicates JAK/STAT signaling promotes early hepatic stellate cell transdifferentiation
Lakner AM, Moore CC, Gulledge AA, Schrum LW

5057 Increased TGF-β3 in primary biliary cirrhosis: An abnormality related to pathogenesis?
Voumvouraki A, Koulentaki M, Tzardi M, Sfakianaki O, Manousou P, Notas G, Kouroumalis E

BRIEF ARTICLE

5065 Hepatic lipogranulomas in patients with chronic liver disease: Association with hepatitis C and fatty liver disease
Zhu H, Bodenheimer HC Jr, Clain DJ, Min AD, Theise ND

5070 Retransplantation for graft failure in chronic hepatitis C infection: A good use of a scarce resource?
Rowe IA, Barber KM, Birch R, Curnow E, Neuberger JM

5077 Partially covered *vs* uncovered sphincterotome and post-endoscopic sphincterotomy bleeding
Katsinelos P, Paroutoglou G, Kountouras J, Chatzimavroudis G, Zavos C, Terzoudis S, Katsinelos T, Fasoulas K, Gelas G, Tzovaras G, Pilpilidis I

5084 Survival of geriatric patients after percutaneous endoscopic gastrostomy in Japan
Suzuki Y, Tamez S, Murakami A, Taira A, Mizuhara A, Horiuchi A, Mihara C, Ako E, Muramatsu H, Okano H, Suenaga H, Jomoto K, Kobayashi J, Takifuji K, Akiyama K, Tahara K, Onishi K, Shimazaki M, Matsumoto M, Ijima M, Murakami M, Nakahori M, Kudo M, Maruyama M, Takahashi M, Washizawa N, Onozawa S, Goshi S, Yamashita S, Ono S, Imazato S, Nishiwaki S, Kitahara S, Endo T, Iiri T, Nagahama T, Hikichi T, Mikami T, Yamamoto T, Ogawa T, Ogawa T, Ohta T, Matsumoto T, Kura T, Kikuchi T, Iwase T, Tsuji T, Nishiguchi Y, Urashima M

5092 HNF-4α determines hepatic differentiation of human mesenchymal stem cells from bone marrow
Chen ML, Lee KD, Huang HC, Tsai YL, Wu YC, Kuo TM, Hu CP, Chang C

5104 Intraoperative radiofrequency ablation combined with ^{125}iodine seed implantation for unresectable pancreatic cancer
Zou YP, Li WM, Zheng F, Li FC, Huang H, Du JD, Liu HR

5111 Temporary self-expanding metallic stents for achalasia: A prospective study with a long-term follow-up
Cheng YS, Ma F, Li YD, Chen NW, Chen WX, Zhao JG, Wu CG

5118 Resistance of *Helicobacter pylori* to antibiotics from 2000 to 2009 in Shanghai
Sun QJ, Liang X, Zheng Q, Gu WQ, Liu WZ, Xiao SD, Lu H

5122 Inhibition of KIT RNAi mediated with adenovirus in gastrointestinal stromal tumor xenograft
Wang TB, Huang WS, Lin WH, Shi HP, Dong WG

CASE REPORT

5130 Soft tissue sarcoma with metastasis to the stomach: A case report
Dent LL, Cardona CY, Buchholz MC, Peebles R, Scott JD, Beech DJ, Ballard BR

5135 Local recurrence of hepatocellular carcinoma after radiofrequency ablation
Liao WJ, Shi M, Chen JZ, Li AM

World Journal of Gastroenterology
Volume 16 Number 40 October 28, 2010

Contents

ACKNOWLEDGMENTS	I	Acknowledgments to reviewers of *World Journal of Gastroenterology*
APPENDIX	I	Meetings
	I-IV	Instructions to authors
AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
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EDITORIAL

Small-for-size syndrome in adult-to-adult living-related liver transplantation

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Abstract

Small-for-size syndrome (SFSS) in adult-to-adult living-related donor liver transplantation (LRLT) remains the greatest limiting factor for the expansion of segmental liver transplantation from either cadaveric or living donors. Portal hyperperfusion, venous pathology, and the arterial buffer response significantly contribute to clinical and histopathological manifestations of SFSS. Here, we review the technical aspects of surgical and radiological procedures developed to treat SFSS in LRLT, along with the pathophysiology of this condition.

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Key words: Adult-to-adult living-related liver transplantation; Small-for-size syndrome; Liver resection; Liver transplantation

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INTRODUCTION

Segmental liver transplantation based on cadaveric splitting or living-related donation has been developed as a valuable treatment for patients with end-stage liver disease. It is also a means of overcoming the shortage of organs, and mortality on the waiting list. However, small-for-size syndrome (SFSS) remains the greatest limiting factor for the expansion of segmental liver transplantation from either cadaveric or living donors[1]. If the volume of the engrafted liver is significantly less than standard liver weight in patients with end-stage liver disease who are undergoing partial liver transplantation, the excessive portal venous inflow might cause early portal hypertension[2,3] and increased morbidity and mortality due to SFSS[4]. Previous data have suggested that, in recipients of adult-to-adult living-related liver transplantation (LRLT), one of the most challenging tasks is to match a good size graft, balancing the clinical condition of the sick recipient and the safety of the healthy donor. More recently, emphasis has been placed not only on the evaluation of the ratio between donor and recipient liver volume, but also on the degree of portal hypertension and the stage of the liver disease in the recipient.

PATHOPHYSIOLOGY

In the initial experience with the use of left graft for adult liver transplantation, recipients of small-for-size grafts developed SFSS, characterized by prolonged cholestasis with elevated serum bilirubin, coagulopathy, rises in cytolytic

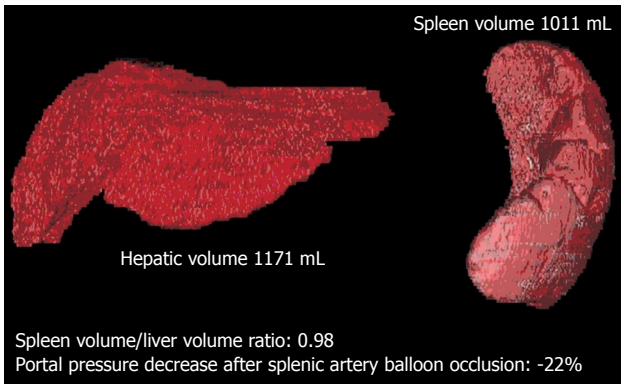

Figure 1 Effects of splenic artery occlusion on portal pressure.

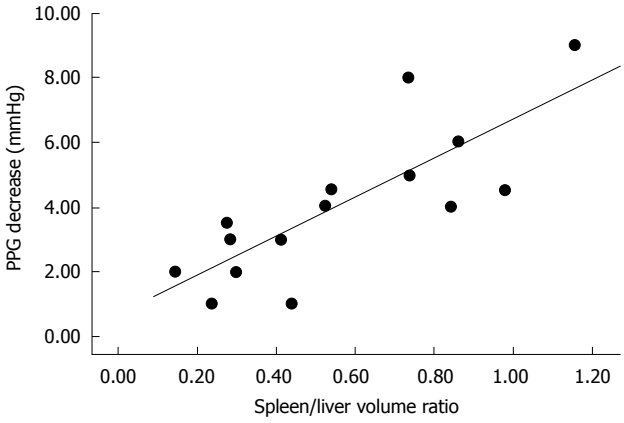

Figure 2 Correlation between spleen liver volume ratio and decrease in portal pressure after splenic artery occlusion. $P < 0.001$, $R = 0.82$. PPG: Portal pressure gradient.

enzymes, ascites, and in severe cases, gastrointestinal bleeding, which occurred within the first week after transplantation[5-7]. In the most severe cases, this syndrome can progress to acidosis, hypoglycemia, encephalopathy, renal failure and septic shock unless retransplantation is promptly considered. Although recipients with low, or absent, portal hypertension could require less hepatic mass from the living donor, underestimation of the useful size-match between recipient and living donor can generate poor results. The pathogenesis of SFSS has yet to be fully clarified. Earlier data have suggested that a graft-to-recipient weight ratio (GRWR) of < 0.8% or a liver volume < 30% of standard estimated volume is a risk factor for the development of SFSS. Recent data, however, have suggested that the exposure of a small graft to persisting hyperdynamic circulation and high portal blood inflow induces impairment of liver regeneration, and hepatic dysfunction[2-4,8-10]. Furthermore, the high portal blood inflow causes a compensatory decrease in arterial blood flow. This phenomenon, known as the "buffering response", is due to a reciprocal compensatory regulation between portal vein and hepatic artery inflow, and might contribute to a worsening of the graft injury. Marcos *et al*[11] has reported that balanced portal venous and hepatic arterial inflow, together with adequate venous blood drainage through hepatic veins, are crucial factors in a successful adult-to-adult LRLT. In 2006, for the first time, our group demonstrated that, in patients with cirrhosis and severe portal hypertension, occlusion of the splenic artery causes a significant reduction in portal pressure, which is directly related to spleen volume and indirectly to liver volume[10]. We have found that reduction of portal pressure after splenic artery occlusion is strictly related to the spleen liver volume ratio (SLVR) that is calculated by multidetector computed tomography (MDCT), which suggests that splenic artery embolization (SAE) can be proposed as a mini-invasive technique to treat or prevent SFSS, by decreasing portal graft perfusion in patients with an SLVR > 0.5 (Figures 1 and 2). This concept is at the center of our strategy for performing early SAE for the treatment of SFSS after LRLT. In light of our data, we believe that regardless of the presence of GRWR > 0.8, the presence of an elevated SLVR could be a risk factor for the development of SFSS, and that early treatment is crucial to preventing a cascade of events that might lead to graft failure or need for retransplantation. A liver graft-to-recipient spleen size ratio has been confirmed as a novel predictor of portal hyperperfusion syndrome in living donor liver transplantation[12,13]. A previous study has suggested that abnormalities in the intragraft gene expression, including endothelin-1 overexpression and plasma nitric oxide level reduction, could negatively affect portal hemodynamics after reperfusion and promote SFSS.

In summary, SFSS has a multifactorial genesis, in which the combination of donor factors (graft volume and quality of the graft, presence of hepatic artery steal syndrome) and recipient factors (portal hyperperfusion, SLVR and stage of liver disease) lead to allograft dysfunction after partial liver transplantation. Recently, Demetris *et al*[14] have shown that SFSS is characterized by early findings, including portal vein sinusoidal endothelial denudation and focal hemorrhage in the portal tract connective tissue, and poor hepatic arterial flow and vasospasm, which in severe cases, leads to functional de-arterialization, ischemic cholangitis, and parenchymal infarction. Late sequelae in grafts that survive the initial events include small portal vein branch thrombosis with occasional luminal obliteration or recanalization, nodular regenerative hyperplasia, and biliary strictures. In light of these findings, it has been suggested that portal hyperperfusion, venous pathology, and the arterial buffer response significantly contribute to clinical and histopathological manifestations of SFSS (Figure 3)[14].

THERAPY

Surgery

Several surgical strategies for reducing portal blood inflow and portal pressure after partial liver transplantation or extended liver resection have been proposed. Historically, a decrease in portal hypertension has been obtained by mesocaval or portocaval shunts[15], portal vein banding, splenic artery ligation[3,4,8,9] and splenectomy[8,9]. All these procedures, however, have been used without a firm hemodynamic basis and without a clear documentation of efficacy.

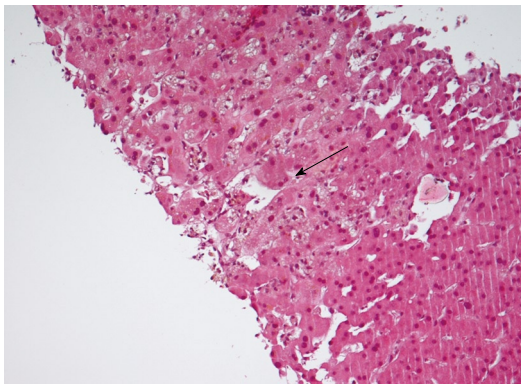

Figure 3 Clinical and histopathological manifestations of small-for-size syndrome. The image shows centrilobular sinusoidal dilatation (arrow) and marked lobular hepatocanalicular cholestasis. Centrilobular atrophic hepatocytes can also be seen. There was moderate ductular proliferation within the portal tracts (not seen). These findings, in absence of bile duct mechanical problems, were mostly consistent with small-for-size syndrome.

SAE

Early diagnosis of SFSS by recognition in the immediate post-transplant period, and the beneficial effects of decreasing splenic venous inflow on portal hypertension have prompted our group to investigate the efficacy of performing early SAE in patients with SFSS. While other groups have routinely performed late SAE[16], we have found the early approach safer and more satisfactory.

In our experience, as previously published[17], Doppler ultrasound and 64-slice MDCT (VCT; GE Medical Systems) have been performed before the procedure, to confirm the absence of vascular complications and to measure liver and spleen volumes. Antibiotic prophylaxis was administered to every patient. The procedure was performed under conscious sedation and local anesthesia with a right femoral artery approach (Figure 4). No major complications occurred, except in one patient who developed a massive splenic colliquation (which was twice drained percutaneously because of persistent fever and abdominal pain), and who then underwent abdominal wash out. In this case, distal embolization was performed using Gelfoam (Pfizer, Belgium) sponge and particles of Contour (Boston Scientific, Cork, Ireland) (Figure 5). In all the other cases, embolization was performed at a proximal level using metallic coils and/or an Amplatzer Vascular Plug (Figure 6).

In this series, we confirmed what was evident in our previous study on cirrhotic patients undergoing occlusion of the splenic artery. In fact, there is clearly a contribution of splenic flow to portal hyperperfusion, therefore, early SAE seems to relieve the partial graft from the deleterious effect of this portal overflow. These data, however, need to be confirmed in controlled studies. SAE also has beneficial effects on post-transplant pancytopenia (Figure 7). It has been recently suggested that, in high-risk patients, preoperative SAE could potentially contribute to improved outcome.

Pharmacological treatment

Various attempts have been made with drugs to control the excessive portal blood inflow to a small graft. This earlier stage of impairment is treated with the use of continuous

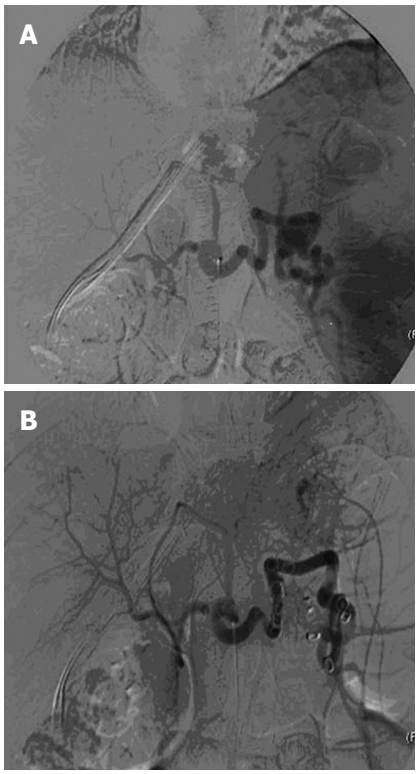

Figure 4 Right lobe living-related donor liver transplantation in a 50-year-old woman with clinical diagnosis of small-for-size syndrome. A: Celiac arteriogram shows poor filling of hepatic artery, enlarged splenic artery and splenomegaly, which suggested associated splenic artery steal syndrome; B: Celiac arteriogram after splenic artery embolization with coils showed increased hepatic artery flow.

intravenous infusion of octeotride or somatostatin and oral consumption of selective β-blockers. The efficacy of these treatments needs to be confirmed in randomized studies.

Splenic artery steal syndrome

Splenic artery steal syndrome (SASS) is a relatively uncommon cause of graft arterial hypoperfusion; a condition that results from a diversion of celiac blood flow into the splenic artery. SASS usually occurs in patients who, before liver transplantation, have severe portal hypertension, hyperdynamic circulation, splenomegaly and enlarged splenic artery. An increase in intrahepatic resistance, due to rejection or hepatitis recurrence, can worsen SASS, which favors the diversion of blood flow away from the hepatic artery. The left gastric artery or gastroduodenal artery might also contribute to the steal phenomenon. SASS either occurs early or as late as several weeks after transplantation. Patients might have impaired liver function tests, cholestasis, ischemic damage of bile ducts or acute graft failure. The diagnosis of SASS is confirmed by selective angiography of the celiac trunk. Angiographic findings include enlarged splenic artery in comparison to the diameter of the hepatic artery, and early filling of splenic artery associated with delayed and dim filling of the hepatic artery (Figure 8). A variety of surgical procedures are performed to increase hepatic artery perfusion, including ligation or banding of the splenic artery, splenectomy, and insertion of a vascular graft from the abdominal aorta

Figure 5 Spleen necrosis treated with conservative therapy. Post-living-related donor liver transplantation status in an 18-year-old man. Splenic artery embolization (SAE) was performed with coils and Amplatzer Vascular Plug to increase the hepatic artery blood flow after recanalization with administration of intravenous recombinant tissue plasminogen activator (rt-PA- alteplase) (case slide 23). A: Two days after SAE, multidetector computed tomography (MDCT) showed patchy areas of severe hypodensity in the spleen; B: One month after SAE, MDCT showed swelling of the spleen, with homogeneous hypodensity in almost all the parenchyma, which reflected severe necrosis; C: One year later, MDCT showed significant spontaneous reduction in spleen volume.

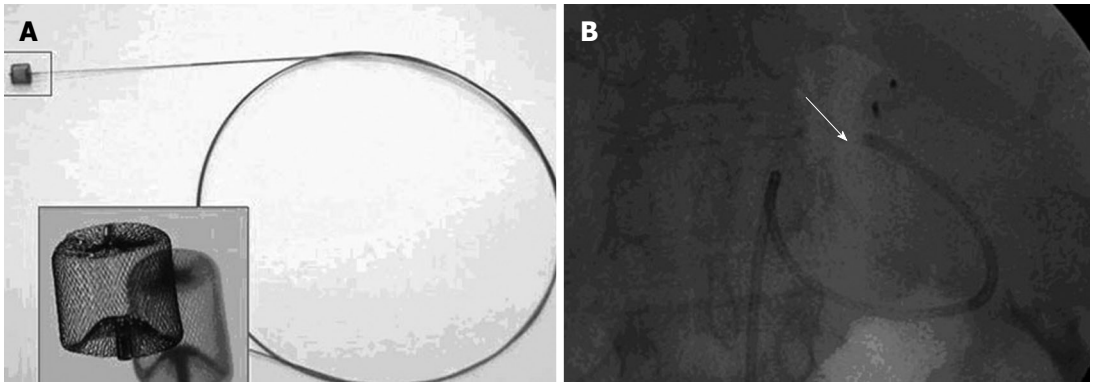

Figure 6 Splenic artery embolization using Amplatzer Vascular Plug. The Amplatzer Vascular Plug (AVP) is a self-expandable occlusion device made from nitinol mesh wires. The AVP comes preloaded and attached to a stainless steel delivery cable. The AVP ranges from 4 to 16 mm in diameter, and 7-8 mm in length. The AVP is FDA-approved and is currently the embolic device that we prefer for splenic artery embolization.

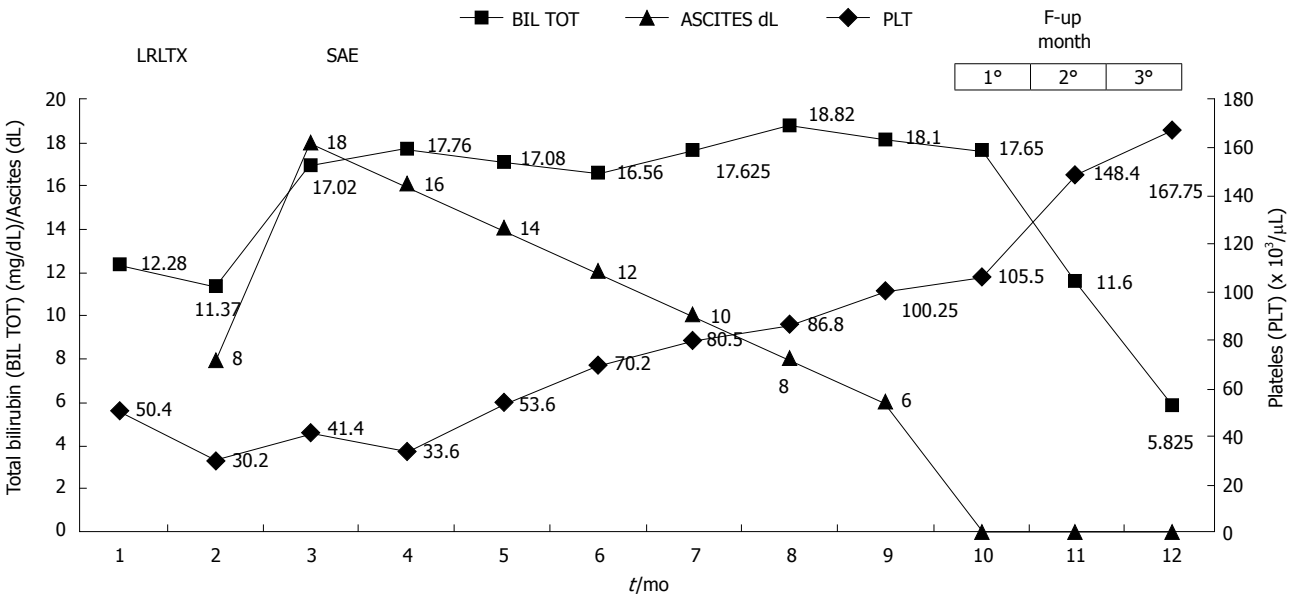

Figure 7 Trend of total bilirubin, ascites, and platelet count after splenic artery embolization. SAE: Splenic artery embolization; LRLT: Living-related donor liver transplantation.

into the hepatic artery. SAE is a non-surgical therapeutic option for SASS.

Figure 8 Splenic artery steal syndrome. Status after right lobe living-related donor liver transplantation for hepatitis-C- and hepatitis-B-virus-related cirrhosis in a 62-year-old woman. A: Celiac arteriogram showed enlarged splenic artery and splenomegaly with no visualization of the hepatic artery; B: Celiac arteriogram after splenic artery embolization with metallic coils showed increased hepatic arterial flow, with opacification of the intrahepatic branches.

CONCLUSION

The transplant community is actually focused on the possibility of detecting predictive factors based on simple biochemical and imaging assessments that could allow physicians to treat those patients at risk of early graft dysfunction immediately after surgery. Although prevention of SFSS should be the goal, how to best manage this once it is established is unclear. In the meantime, as illustrated in Figure 7, we suggest that SAE is a safe and prompt treatment option for patients in whom SFSS is recognized after adult-to-adult LRLT.

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EDITORIAL

Real-time histology with the endocytoscope

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Abstract

Endoscopic Imaging has progressed tremendously over the last few decades. Novel imaging technologies such as high-resolution and high-magnification white light endoscopy, narrow band imaging, optimal band imaging, autoflourescence imaging and optical coherence tomography not only aid the endoscopist in detecting malignant or pre-malignant lesions but also assist in predicting histology. Recently, the introduction of Endocytoscopy (EC) and Confocal Endomicroscopy has taken us into a new realm of diagnostic endoscopy. With the ability to magnify up to 1000 ×, cellular structures can be visualized in real-time. This advance in technology could potentially lead to a paradigm shift negating the need to obtain biopsies. EC is, however, still in the early stages of development and further research needs to be carried out before it can be accepted as standard practice. This review will focus on the diagnostic utility of the Endocytoscope.

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Key words: Endocytoscopy; Advanced endoscopy imaging; Magnification endoscopy; Real-time histology

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INTRODUCTION

Recent advances in endoscopic technology have pushed the boundaries of diagnostic endoscopy further, enabling more accurate and better lesion recognition and characterization. Endocytoscopy (EC) is the latest innovation in the ever-expanding armamentarium of devices available to the endoscopist. This technology uses the principles of light microscopy, producing a clear and in-focus image of a thin layer of tissue located within a biologically thick sample. This is possible using a high-power fixed focus objective lens that provides ultra-high magnification images of surface morphology at cellular resolution.

EQUIPMENT AND TECHNIQUE

The EC device manufactured by Olympus Medical Systems Co., Tokyo, Japan either comes as a probe-based system or one that can be incorporated into the endoscope. Both devices are prototypes and are currently not available commercially. The probe-based Endocytoscope consists of a catheter-type device 380 cm in length, measuring 3.2 mm in diameter and is available at 2 levels of magnification. The lower magnification, the XEC 300 Endocytoscope,

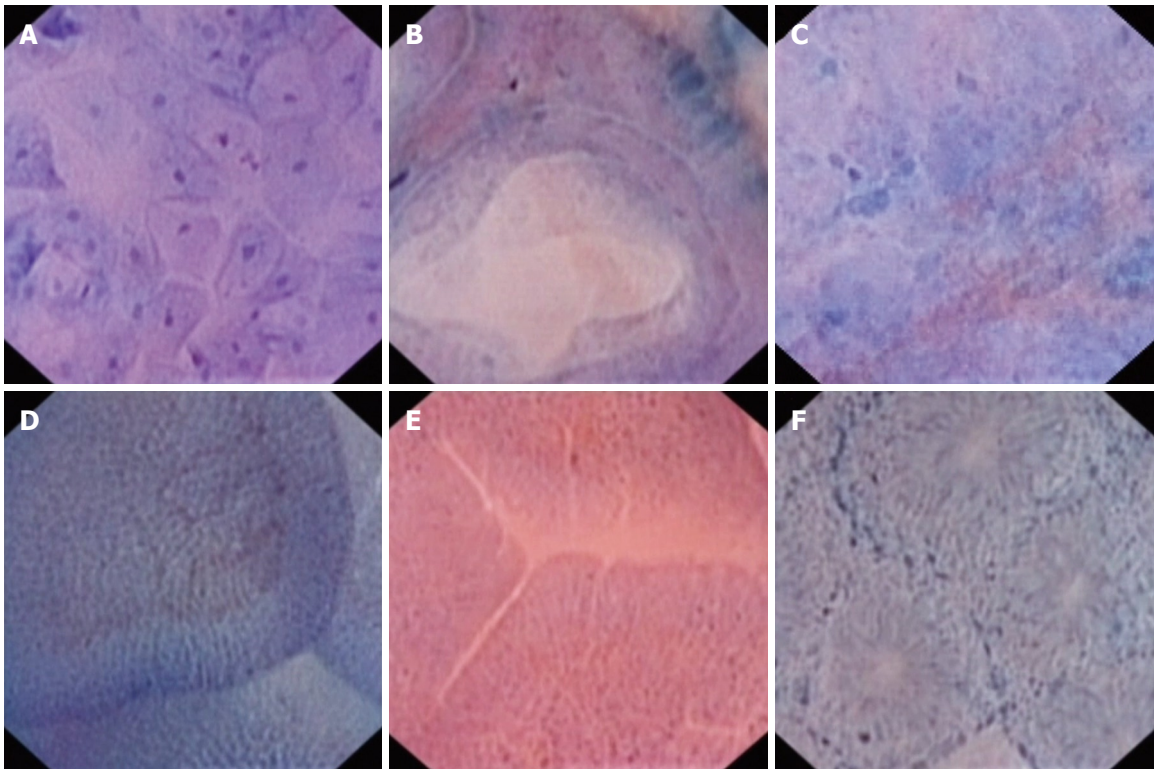

Figure 1 The various endocytoscopic images seen in the gastrointestinal tract. A: Normal esophageal mucosa on endocytoscopy (EC): Squamous cells can be seen with nuclei that have a regular shape and size. There is good nuclei:cytoplasm ratio; B: Normal Barrett's mucosa on EC: Regular glandular structure can be observed with homogenous intestinal metaplasia cells containing uniform appearing nucleus bordering the glands; C: EC in Barrett's esophagus with intramucosal cancer: There is total loss of the glandular pattern and markedly increased cellular density. The nuclei appear pleomorphic and enlarged; D: EC in normal mucosa in the duodenum: The villi can be clearly visualized with presence of regular appearing villous capillaries. Numerous nuclei can also be seen within each villi; E: In a patient with Marsh 3 biopsy-proven celiac disease, the mucosa appears atropic with complete absence of any villous structure. There is a "cracked mud" appearance and capillaries are distinctly absent in contrast to the normal pattern; F: EC performed on normal colonic mucosa reveals uniform appearing glands which are regularly arranged. Each gland is lined by epithelium and they are arranged in a radial fashion with the colonic crypt seen centrally.

has a magnification capability of 450 × and a field of view of 300 μm × 300 μm whilst the higher magnification, the XEC120 Endocytoscope, enables magnification up to 1125 × with a field of view of 120 μm × 120 μm; both based on a 19-inch-high resolution monitor. These instruments should be passed into the working channel of a therapeutic endoscope which has a channel diameter of 3.7 mm (Olympus GIF-IT240). Alternatively, the integrated-type device incorporates the Endocytoscope into the endoscope itself. This system also comes in 2 different configurations, the upper (103 cm), (XGIF-Q260EC1) endoscope and the lower (133 cm) (XCF-Q260EC1) endoscope. Both of these devices have a magnification capability of 580 × and a field of view of 400 μm × 400 μm. The depth of penetration of both the probe-based and integrated device is limited to 30 μm.

Similar to the endoscope, the Endocytoscope is connected to a light source and a video processor. Thus, for visualization of images in real-time, it is necessary to utilise 2 processors simultaneously. As in conventional histopathology, vital staining is used to further elucidate cellular detail. Prior to staining, the mucosal surface should be vigorously flushed with water and a mucolytic agent (such as 10% N-acetyl cysteine). To maintain stability of the Endocytoscope when it approximates an area of interest, a plastic cap is attached to the tip of the endoscope prior to commencement of the procedure. Various dyes have been used to stain the mucosa. In a recent study regarding the optimal dye and concentration needed, 60 s of exposure of the mucosa to 1% methylene blue for the esophagus and 0.25% toluidine blue for the stomach and the colon were found to be the best staining techniques[1]. Recently, another technique aptly called the double staining technique which utilizes 1% methylene blue (to stain the nucleus) and 0.1% crystal violet (to stain both the nucleus and the cytoplasm) has been used to approximate hematoxylin and eosin staining seen in conventional histology.

OBSERVATIONS

Esophagus

In the esophagus, staining is optimal with methylene blue. With the low power Endocytoscope (XEC 300), two to three layers of cells can be observed. The nuclei are regular in size and shape and exhibit a low nuclear to cytoplasmic ratio (Figure 1A). With the high power Endocytoscope (XEC 120), nucleoli can also be observed. In an *ex-vivo* pilot study of esophageal squamous cell carcinomas (SCC), EC images closely correlated with histologic results for both cancerous and normal esophageal squamous cells although the quality of some images was deemed

inferior[2]. In another study on *in-vivo* diagnosis, EC demonstrated increased density of the cells with loss of cellular uniformity in esophageal SCC[3]. The nuclei in SCC were also of different shapes and sizes. Prominent nucleoli were noted on the higher magnification Endocytoscope and the nucleus to cytoplasm ratio appeared altered. In Barrett's esophagus, the nuclei are uniform and the cell density is low. These cells are arranged radially forming a regular glandular and crypt structure (Figure 1B). In cancer, the cell density is high with loss of the glandular pattern whilst the crypts are destroyed and the nuclei may appear pleomorphic[4] (Figure 1C).

Stomach

The gastric mucosa with its multiple folds is generally more difficult to observe with EC. Benign gastric mucosa exhibits regularly arranged tubules and nuclei. In gastric cancer, irregular branched and destroyed tubules are noted and cells show nuclear pleomorphism. EC has also been successful in *ex-vivo* demonstration of *Helicobacter pylori* (*H. pylori*)[5]. This study was performed after preparing cultures from gastric mucus obtained from patients with gastric ulcers. Live *H. pylori* could be observed directly using the Endocytoscope.

Duodenum

EC can clearly visualize the villi in the duodenum with the presence of regular capillaries and regularly placed nuclei (Figure 1D). In Celiac disease, the mucosa appears atrophic with complete absence of the villi. There is a "cracked mud"-like appearance and capillaries are distinctly absent (Figure 1E). EC at 450 × magnification has been shown to accurately identify mucosal histopathology in advanced Celiac disease[6]. The investigators identified four criteria which were significant predictors of Marsh Ⅲ pathology: low number of villi per visual field (< 3), confluence of villi, irregular epithelial lining and inability to delineate loop capillaries. They were, however, unable to identify any salient features which were good predictors of early morphological changes in Celiac disease.

Colon

EC allows clear visualization of the mucosa and microvasculature of the colon[7]. The EC images of normal mucosa reveal uniform glands arranged neatly in a radial or hexagonal fashion around crypts with borders which are demarcated by microvasculature observed as red blood cells circulating through the arterioles (Figure 1F). In hyperplastic polyps, the glands are serrated with foamy changes in the cytoplasm and star-like pits. In low grade adenomatous polyps, homogenous tubular glands with regular fusiform nuclei which have uniform polarity can be visualized. High grade adenomas have nuclei on the luminal side of the gland with disordered polarity. The glands also tend to be irregularly branched. Sasajima *et al*[8] reported that it was possible to distinguish neoplastic from non-neoplastic lesions with EC. Conventional histology and EC had high concordance values in the diagnosis of colorectal neoplasia with accuracies of 93.3% and a κ score of 0.91, suggesting substantial agreement. EC also allows *in-vivo* real-time visualization of blood flow in rectal microvasculature[7] and clear visualization of the dysplasia in aberrant crypt foci (ACF) in the colon and rectum[9]. In dysplastic ACF, crypt contours are polygonal, the lumen is linear and the nuclei are elongated with pseudostratification toward the luminal half of the crypt. In this study, histology confirmed low grade dysplasia with a sensitivity of 91.4% and specificity of 100%.

DISCUSSION

Recent promising developments in wide field technologies such as Narrow Band Imaging, Optimal Band Imaging and High-Resolution High-Definition White Light Endoscopy have increased the capability of the endoscopist for visualizing and detecting pathology. EC and Confocal Endomicroscopy (CLE) are novel techniques which enable lesions which are already detected to be investigated further, thus allowing real-time visualization of cellular detail. Both of these are in essence 'point biopsy' techniques. Apart from using a totally different technology, CLE differs from EC in that it requires different staining agents (intravenous fluorescein and occasionally topical acriflavin). The resolution of the images is, however, superior although they do appear in black and white unlike those of the EC. CLE also enables the mucosa to be assessed up to a depth of 250 μm. Similar to the EC, it is available either as a probe-based system or integrated into the endoscope.

There are some key challenges which need to be addressed before EC can be accepted into routine practice. Extensive preparation is required prior to Endocytoscope assessment such as intensive washing of the mucosa, as well as staining which can be very labour intensive. The area to be assessed by the Endocytoscope is very small and the time spent studying it has not yet been looked into. Peristalsis, respiratory and cardiac movement artefacts can also make the assessment of lesions technically challenging. Being a new technique, standardized criteria describing the various morphological appearances of the different lesions encountered should be set in place. Systematic training for the novice endoscopist in recognition of these new patterns should then follow.

The future, though, does appear to be bright for the Endocytoscope. Whilst histopathology remains the gold standard for diagnosis of lesions, it involves a multistep process which may take a few days before the final conclusion(s) can be reached. EC simulates histology in real-time and has potential to ultimately replace conventional histopathology. In the shorter term however, this technology may assist the endoscopist in targeting biopsies based on some of the characteristic features described above. It may have the capability of having a very high negative predictive value which could be clinically relevant, especially in patients undergoing surveillance for Barrett's esophagus or ulcerative colitis where random biopsies normally have a very poor yield. Real-time histologic diagnosis may ultimately allow endoscopists to make

decisions in a one stop approach, where lesions can be assessed and management decisions made depending on findings all in a single session. This may ultimately lead to an overall reduction in cost.

In conclusion, EC is a promising novel technology which enables *in vivo* assessment of cellular detail. Further large scale multicentre randomized controlled trials are needed to confirm the true utility of this technology. Issues such as training, reproducibility as well as cost-effectiveness need to be addressed before EC can be accepted into routine clinical practice. Numerous studies are presently underway assessing this exciting new technology and results with regard to its true clinical benefit are eagerly awaited.

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TOPIC HIGHLIGHT

Dr. Marco Scarpa, PhD, *Series Editor*

Quality of life after surgery of the alimentary tract

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Abstract

In recent decades, patient-reported outcomes have become important in clinical medicine. Nowadays, health-related quality of life (HRQOL) is considered a primary outcome in many clinical trials, and it is often the major criterion for judging treatment success. At the beginning of the 21st century, morbidity and mortality rates after surgery of the alimentary tract have dropped dramatically and they can no longer be considered the only outcome measures to determine the success of a surgical procedure. QOL can yield a definitely more patient-orientated measure of outcome that provides us with a more formal measure of the patient's judgment and desires, which can influence treatment decisions. Nevertheless, despite a very large number of published papers on HRQOL, there is some skepticism on the value of HRQOL and other patient-related outcomes. Therefore, this topic highlight aims to assess how QOL after surgery of the alimentary tract is covered in the medical literature. Different reviews have analyzed the topic according to different points of view: benign and malignant disease; curative and palliative treatment; open and minimally invasive surgical approach; traditional and newly introduced surgical procedures. This topic highlight does not aim to cover all the possible diseases or different surgical procedures, but it does describe the different approaches in order to give the reader a broad spectrum of analysis of QOL after surgery. This quick overview could stimulate the reader to form his/her own opinion about how to use this primary outcome measure.

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Key words: Patient-reported outcomes; Health-related quality of life; Esophageal cancer; Gallbladder stones; Ulcerative colitis; Crohn's disease; Colonic diverticular disease; Colorectal cancer; Rectal prolapse

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INTRODUCTION

In recent decades, patient-reported outcomes have become important in clinical medicine. Nowadays, health-related quality of life (HRQOL) is considered a primary outcome in many clinical trials, and it is often the major criterion for judging treatment success[1]. Evidence of improved QOL life before approving therapeutic interventions is often demanded by regulatory agencies, insurance companies and third-party payers, because many authorities now regard it as a key measurement in clinical trials[2,3]. However, most of the QOL measures and studies that measure QOL lack a proper definition or conceptualization of what QOL really is[4]. Despite the explosion of interest in QOL, the consensus regarding its limits or the optimal method for measuring it is somehow still debated[5].

QOL IN CLINICAL SETTINGS

In 1947, the World Health Organization (WHO) defined health as "a state of complete physical, mental and social

well-being and not merely the absence of disease"[6]. These domains of physical, emotional and social well-being are incorporated by this definition into the concept of QOL[7]. Although some authors have rejected the romantic/holistic WHO definition[8], many have used it as the basis for conceptualizing QOL. The notions that QOL should be assessed by or considered from the patient's point of view, that it can fluctuate over time, and that there are cross-cultural differences in how it is defined, have also been accepted. In the literature, health status and QOL are often confused or poorly distinguished, and both terms are used for the instruments that are used to measure them. Defining QOL is difficult because it is an abstract, complex and often highly individualized concept. For these reasons, it is agreed that, in a clinical setting, QOL analysis should be limited to HRQOL. In fact, HRQOL reflects an attempt to restrict the complex concept of QOL to those aspects of life specifically related to individual health, and potentially modified by healthcare[9]. The paradox is that HRQOL, when assessed in patients, is focused on health (as it is commonly argued in textbooks and journals), but it is rather focused on disease[5]. Current QOL questionnaires have been developed for and validated with ill people. The psychological predicament and consequently the concept of QOL of ill people might differ considerably from that of healthy or normal people. Specifically, patients have something socially and personally undesirable, namely an illness and the psychological state, thus, questionnaire responses can be affected by the patients' abnormal physiological state[7].

QOL AFTER SURGERY OF THE ALIMENTARY TRACT

Why could be it useful to measure QOL after surgery of the alimentary tract? As clearly and exhaustively explained by McLeod in 1999, the traditional outcome measures for assessing the outcome of a surgical procedure have been morbidity and mortality[5]. During the 20th century these measures were appropriate because most gastrointestinal surgical procedures were associated with high complication and operative mortality rates[5]. Thus, survival was of utmost importance in assessing the success of a surgical procedure, and consequently, the decision to adopt an operative technique was largely based on operative mortality and long-term survival[5]. At the beginning of the 21st century, operative mortality rates have dropped dramatically, and fortunately, this parameter has become of limited use as an outcome measure to discriminate between two surgical techniques, or determine the value of a surgical technique as compared with medical therapy[5]. Therefore, morbidity and mortality can no longer be considered the only outcome measure and alternative ones should be adopted.

PHYSIOLOGICAL PARAMETERS AND QOL

Physiological parameters can be adopted and compared to discriminate between two surgical techniques. However, measuring QOL can yield a definitely more patient-orientated measure of outcome that provides us with a more formal measure of the patient's judgment and desires, which can influence treatment decisions. In fact, although physiological outcomes are easier to measure, they might not necessarily correlate with patients' perception of their status[5]. Clinical experience suggests that some patients rate their overall QOL as quite good when they are clearly very ill. In aggregate patient samples, regression analyses have shown that objective health variables and self-reported health variables do not fully explain the variance of global QOL[10,11]. In one of our studies that has focused on QOL in patients with Crohn's disease, who underwent ileo-colonic resection, the main predictor of HRQOL after surgery was clinical and surgical recurrence, but the variability of the HRQOL score explained by these two clinical parameter was relatively low[12]. In fact, physiological outcomes provide information to the clinicians but are of limited interest to patients, and often correlate poorly with well-being. Furthermore, even though two patients might be in the same state of health, their perceptions of their QOL can be different. After reconstructive surgery for ulcerative colitis, for example, surgeons usually assess outcome in terms of stool frequency[13]. However, evidence suggests that these physiological parameters do not always correlate with patients' perceived QOL and satisfaction with the outcome of their surgery[5]. In fact, several complex indexes have been developed to assess function or disease activity but measurement of functional status alone is of limited value. In fact, not only the physical domain is involved in the patient's well-being, but also the psychosocial domain might have an impact on QOL. A famous example is the work of Brickman *et al*[14], who have shown that, after an adaptation period of 1 year, paraplegic accident victims and lottery winners reported practically the same level of overall well-being; a phenomenon known as the well-being paradox. Apparently, an overall judgment of QOL includes not only physical and psychosocial components, but also a component of coping[15].

LIMITS OF QOL MEASUREMENT

QOL analysis can have its limits. Despite a very large number of published papers on HRQOL, there is some skepticism on the value of HRQOL and other patient-related outcomes[16]. In fact, very often, the intrinsic characteristics of the questionnaires (reliability, validity *etc.*) are well defined criteria, and no recommendations are made about interpretation of HRQOL results; especially regarding the clinical significance of a change in HRQOL that leads to a therapeutic approach. Furthermore, although some scientific societies have created working groups to debate the role of HRQOL in clinical research, the true value of HRQOL evaluations in clinical trials has not yet been completely defined[17]. Moreover, different questionnaire can lead to different results with the same subset of patients[18]. In the clinical setting, this intrinsic subjectivity has been always regarded with suspicion and skepticism that is exemplified by a comment made by Wulff: "Scientists

may use rating scales and visual analogue scales to measure pain, and they may even invent scoring systems quantifying types of handicaps; but when they talk about measuring quality of life they have gone too far"[19]. This statement reflects a model of medicine and human experience in which objective facts are clearly distinguished from subjective values[19]. Nevertheless, in our opinion, QOL analysis is an extremely powerful instrument to evaluate the outcome of patients that can give unexpected and important insight about how patients cope with surgery. At the same time, it is also a delicate tool that should be handled with care and it should not be abused. Conclusions should be drawn only after a careful choice of questionnaires and after adequate analysis of the results and the literature.

AIM OF THE TOPIC HIGHLIGHT

By taking these precautions into account, analysis of QOL after surgery of the alimentary tract is mandatory if we want to answer correctly the main questions that patients ask when they are about to undergo surgery: "How will I feel after the operation; what will my life be like?" These sorts of questions are even more important if surgery is proposed for patients who have experienced failure of medical therapy. Therefore, this topic highlight aims to analyze patient-related outcomes of surgery of the alimentary tract with a specific focus on postoperative QOL.

OUTLINE OF TOPIC HIGHLIGHT

The first review from the University of Padova, Italy, has analyzed the impact of surgery for diverticular disease on HRQOL[20]. Various studies have suggested that diverticular disease has a negative impact on HRQOL, which affects bowel function and general health. Nevertheless, several studies have observed a significant improvement in QOL and, in particular, of the social function domain following elective sigmoid resection in the majority of patients[20]. However, both surgery-related complications and disease activity have a significant impact on patients' HRQOL[20]. Finally, no significant differences in HRQOL between different laparoscopic and open colectomy procedures for diverticular disease were revealed in the non-randomized population. On the contrary, the only prospective, double-blind randomized study that has compared laparoscopic and open colectomy has found that laparoscopic colectomy seemed to reduce major postoperative complication rates and achieved better HRQOL scores.

The second review was a systematic review from the University of Amsterdam, The Netherlands, which aimed to examine the latest evidence of QOL in patients after laparoscopic or open colorectal surgery. The clinical heterogeneity among the included studies limited the possibility of performing a proper meta-analysis of the data. In fact, virtually every study used different QOL instruments and did not present exact data. Furthermore, the recruited patients were treated for a range of different disorders. Therefore, it was impossible to perform the statistical analyses or a meaningful meta-analysis. Future randomized trials that compare open and laparoscopic surgery are needed, and should be well-designed, sufficiently powered, and focus on QOL, particularly shortly after the operation, i.e. within 1 wk, in which time, most of the differences are likely to occur. This systematic review suggests that, when introducing and comparing new surgical procedures, the standardization of questionnaires and the timing should be strongly encouraged[16].

The third review was from the Venetian Oncology Institute of Padova, Italy, and aimed to assess the long-term HRQOL of patients with esophageal cancer who underwent curative surgery. The HRQOL of these patients was compared to the established norms, and the evolution of HRQOL during follow-up after esophageal resection was described. Even in this case, the clinical heterogeneity limited the possibility of performing a complete meta-analysis of the data. However, the standardization performed by the European Organization for Research and Treatment of Cancer (EORTC) allowed the investigators to overcome this problem, at least partially. In fact, EORTC QLQ30 and optical emission spectrometry 18 were the most commonly used questionnaires. Therefore, although based on low-level evidence from uncontrolled studies, this systematic review showed a trend for improvement of the generic and disease-specific HRQOL in the first 12 mo follow-up after esophageal resection. Nevertheless, in long-term survivors, the pooled physical function, role physical, social function, vitality, and general health perception scores were lower than the general population norms.

The fourth review was from the University of Chicago, United States, and it analyzed the available data in the literature regarding HRQOL in patients with ulcerative colitis or Crohn's disease after surgery. Although these two diseases might have some similarities in their management, clearly their impact on QOL is different. The authors concluded that not a single HRQOL instrument, general or inflammatory bowel disease (IBD)-specific, satisfactorily covers all of the critical criteria of reproducibility, reliability, validity, ease of use, responsiveness to change, and meaningfulness of results. Only by using a combination of general and IBD-specific instruments is it possible to capture and properly evaluate HRQOL prospectively in interventional studies of IBD patients. Only by utilizing the appropriate instruments and by integrating and thoroughly analyzing the results is it possible to capture accurately the complexity of HRQOL in IBD.

The fifth review was from the University of Leuven, Belgium, aimed to evaluate QOL in patients affected by obstructed defecation syndrome (ODS), which is one of the most complex clinical problems in colorectal surgery, who underwent stapled transanal rectal resection. Limited data exist on QOL following this surgical procedure. Therefore, other patient-reported outcomes played a major role: Cleveland Continence Scale obstructed defecation syndrome-score (ODS-score), or a modified ODS score were used, as well as patient satisfaction after the procedure. However, patient satisfaction is a patient-related outcome measure, but it could be greatly influenced by factors such as, the personal relationship between the patient and the

nurse/doctor. For this reason, we agree with Arpinelli and Bamfi[16], and we believe that patient satisfaction should be considered as a less important indicator than HRQOL.

The sixth review was from the Venetian Oncology Institute of Padova, Italy, and aimed to assess the long-term HRQOL in patients with esophageal cancer who underwent palliative endoscopic surgery. In such a situation, where the indication for treatment is not to improve the survival but to ameliorate poor QOL, it is essential that QOL should be measured to determine whether the therapeutic intervention has been worthwhile[5].

The seventh review was from the Venetian Oncology Institute of Padova, Italy and the San Giovanni Hospital of Bellinzona, Switzerland, and aimed to investigate the QOL in adults after cholecystectomy. This study revealed that there has been only a limited number of studies that have reported on HRQOL following cholecystectomy, and these studies usually have used generic instruments (i.e. SF-36 and Gastrointestinal Quality of Life). Patients with symptomatic cholelithiasis and low surgical risk achieve the best HRQOL results after laparoscopic cholecystectomy, whereas patients with asymptomatic cholelithiasis or high surgical risk usually do not seem to have the same improvement. HRQOL perception is usually better for those patients who have undergone minimally invasive surgery compared to open surgery. However, there is no validated disease-specific HRQOL questionnaire for use in the context of hepatobiliary or pancreatic disease, and further studies are warranted.

CONLUSION

In conclusion, this topic highlight aimed to assess how QOL after surgery of the alimentary tract has been covered in the medical literature. Different reviews have analyzed the topic from different points of view: benign and malignant diseases; curative and palliative treatment; open and minimally invasive surgical approaches; and traditional and newly introduced surgical procedures. This topic highlight did not aim to cover all the possible diseases or surgical procedures, but to show different approaches in order to give the reader a broad spectrum of QOL analysis after surgery. This brief overview could stimulate the reader to form his/her own opinion about how to use this primary outcome measure.

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TOPIC HIGHLIGHT

Dr. Marco Scarpa, PhD, *Series Editor*

Health related quality of life in inflammatory bowel disease: The impact of surgical therapy

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Abstract

Over the past 30 years, health related quality of life (HRQOL) has developed into a scientific index of subjective health status. Measurement of HRQOL is now clearly a mandatory component in evaluating interventions and management of medical and surgical diseases. In designing comprehensive and meaningful clinical studies particular attention ought to be made of measures of HRQOL. This is clearly very important in inflammatory bowel disease. Both ulcerative colitis (UC) and Crohn's disease (CD) have a major impact on HRQOL. The chronic and unrelenting nature of these diseases, the often early age of onset, and the impact on social and sexual aspects of life significantly change patient's perception, body image and quality of life. This manuscript is an overview of the available published data on HRQOL in UC and CD patients focusing on the impact of surgical therapy. While these two diseases may have some similarities in their management, clearly their impact on quality of life and the effects of are significantly different. Hence we are presenting the data separately.

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Key words: Inflammatory bowel disease; Quality of life; Gastrointestinal surgery; Surgical outcome

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INTRODUCTION

Over the past 30 years, health related quality of life (HRQOL) has developed into a scientific index of subjective health status. Measurement of HRQOL is now a mandatory component in evaluating interventions and management of medical and surgical diseases. In designing comprehensive and meaningful clinical studies particular attention ought to be made of measures of HRQOL. This is clearly very important in inflammatory bowel disease (IBD). Both ulcerative colitis (UC) and Crohn's disease (CD) have a major impact on HRQOL. The chronic and unrelenting nature of these diseases, the often early age of onset, and the impact on social and sexual aspects of life significantly change patient's perception, body image and quality of life. Many IBD patients will require surgery at some point in their disease course. With the evolution of newer surgical treatment modalities, it is paramount to establish the effect of surgical interventions on HRQOL. This in turn can allow both the patients and the practitioners to have an

understanding of the anticipated changes in HRQOL when selecting a treatment plan.

Even though CD and UC share several clinical and therapeutical similarities, their impact on HRQOL is significantly and vastly different. This is clearly evident from the available data in the literature[1]. For CD, fluctuations in HRQOL are common and strongly correlate with the activity of the disease. Surgery, often only temporarily, may improve it. While surgery is curative for UC, medical and surgical interventions in CD are palliative at best. It is, therefore, expected to see an improvement in HRQOL with definitive surgical treatment in UC as compared to patients with active UC[2].

The currently available HRQOL instruments are a global measure of the patient's perceptions, illness experience, and functional status that incorporates social, cultural, psychological, and disease related factors[3]. Measurements of HRQOL are a quantification of the patient's subjective perception of physical, emotional, and social function. Typically these measurements include social and sexual activity, ability to work, attend school, sports and recreation and finally body image. Multiple HRQOL instruments, both general and IBD specific instruments, have been utilized in literature[3-12].

It is obvious, by reviewing the literature, that there is no single general or IBD specific HRQOL instrument that satisfactorily covers all of the critical aspects of a comprehensive assessment of quality of life in IBD. Only by using a combination of general and IBD specific instruments is it possible to capture and properly evaluate HRQOL prospectively in interventional studies of IBD patients.

This is a comprehensive overview of the available literature on HRQOL in patients with UC and CD with an emphasis on the effect of surgical therapy on HRQOL. We took into account the effects of various surgical therapies on HRQOL in patients with IBD. While the instruments used in various studies might be individually inadequate to capture the complexity of the impact of these therapies on patients with IBD, we sought to integrate the available data to provide a comprehensive and accurate assessment. For the many reasons mentioned above, we present the data separately. While these two diseases may have some similarities in their management, clearly their impact on quality of life is significantly different.

SURGICAL THERAPY AND HEALTH RELATED QUALITY OF LIFE IN ULCERATIVE COLITIS

Physicians treating UC patients currently have at their disposal an array of medical and surgical options. The patients with UC who become refractory to medical management ultimately are referred for surgical evaluation. It is at the time of surgical consultation that an extensive discussion of the available surgical options takes place. The decision to choose one operation *vs* another is highly individualized and rests on the discussion between the patient and the treating physician. This decision is largely based on the extensive evaluation of the risks and benefits of various approaches as well as expected changes in quality of life, body image, sexual and reproductive function. Consideration is also given to the age of the patient. While younger patients are more concerned with body image, long term pouch function, sexual and reproductive health, older patients often fear the possibility of becoming incontinent, and dealing with potential complications.

HRQOL in UC patients: surgical vs medical treatment

There is a general consensus among physicians that UC refractory to medical management requires surgical intervention with colectomy. The issue becomes more complex in patients who are currently in remission, but are troubled by flares with the need for frequent hospital admissions. There is a paucity of studies addressing HRQOL in this situation. Cohen *et al*[2] evaluated intravenous cyclosporine (CSA) as a medical alternative to colectomy in severe, steroid-refractory UC patients. Quality-of-life analyses were conducted using the IBDQ, a visual-analog scale (VAS), and the Oresland scale. The patients treated with CSA reported a better ability to sleep, better stool consistency, less abdominal or rectal pain (VAS), and fewer daytime, nighttime (Oresland), and daily trips to the toilet (VAS) than the surgical patients. The mean number and rate of hospitalizations within the first year was also lower in the CSA patients. Surgical patients, however, reported fewer initial visits to their treating physician and less medication use (Oresland). Sagar *et al*[13] compared HRQOL in UC patients after a restorative proctocolectomy with ileal pouch anal anastomosis (IPAA) with UC patients on long-term medical treatment. The authors developed a scoring system based on a questionnaire described by Oresland *et al*[14]. It consisted of questions that related to bowel function, work, social life, and sexual activity. The questionnaire also aimed to identify restrictions the condition had imposed on diet, leisure, and social pursuits. The technique of restorative proctocolectomy with IPAA included both mucosal proctectomy and pull through IPAA in the earlier part of the series and a stapled anastomosis constructed 1.5 to 2.0 cm above the dentate line in the later phase. Each patient received a temporary ileostomy that was closed 8 to 12 wk later. The authors found that HRQOL after IPAA was good and appeared to be no worse than that of patients with long-standing UC on medical treatment in remission. Although the frequency of bowel movements was greater in patients with IPAA than in patients with medically treated colitis, patients with pouches experienced significantly less urgency on defecation, fewer symptoms of anxiety or depression, and were less restricted socially than their medically treated counterparts. Patients with medically treated colitis required less antidiarrheal medication but many continued to require steroids. This study suffers from obvious limitations of

comparing patients who chose to continue with medical management versus those who elected to proceed with IPAA.

HRQOL after total proctocolectomy with end ileostomy

Despite the wide acceptance of IPAA as the definitive surgical treatment of UC, a total proctocolectomy (TPC) and conventional end ileostomy (EI) is still considered a viable alternative when an IPAA is contraindicated. Although the patient is left with a permanent, incontinent stoma, it is a safe operation with a low complication rate. To evaluate HRQOL of patients who had this operation compared with that of the general population Camilleri-Brennan and Steele[15] conducted a mail survey using quality of life questionnaire SF-36 version 2 (SF-36 Ⅱ)[16,17]. Despite this being a generic questionnaire designed for comparing quality of life across patient populations and disease groups it allowed the authors to compare the TPC and EI patient scores to those obtained from the general population in United Kingdom of similar age and gender. The authors evaluated the difference between patients and the general population for all dimensions and summary scores including physical function, role limitations due to physical problems, energy and vitality, body pain, role limitations due to emotional problems, social function, mental health, general health perception, physical component summary and mental component summary. The scores directly relating to physical well-being as well as the Physical Component Summary were similar to the general population. Similar results were also achieved for the energy and vitality dimension and the pain scores. The scores in the mental health and role-emotional dimensions, as well as in the social functioning dimensions, and general health perceptions were similar to that of the general population. This study suggested that despite the presence of a permanent ileostomy HRQOL was very similar to that of the general population. The results clearly underscore the notion supported by other authors that perceived negative impact of the ileostomy does not appear to affect HRQOL. Therefore, TPC and EI remains a viable option for the patients requiring surgery for UC and should always be brought to discussion when counseling the patient regarding surgery.

Patients with fulminant UC or those refractory to medical management frequently require urgent or emergency colectomy. While colectomy clearly improves the quality of life there are no studies to address the changes in HRQOL following emergency colectomy. This is likely due to the fact that patients who are failing medical management or becoming progressively ill cannot be adequately captured by surveys. This in turn results in HRQOL evaluations being based predominantly on the patient populations undergoing elective surgery for UC.

HRQOL after ileorectal anastomosis

Total colectomy with ileorectal anastomosis (IRA) was a rather common operation for UC before the development of IPAA. It was primarily offered to patients who were not willing to have a permanent ileostomy. Because IRA is a less complex operation with comparatively low morbidity it is still performed in selected UC patients. da Luz Moreira *et al*[18] reviewed their experience with IRA to determine the fate of the rectum, functional results and quality of life after ileorectal anastomosis in UC. Seventy four UC patients after an IRA were matched by age, sex and follow up duration with 66 patients who underwent IPAA. The patients were contacted by telephone to evaluate functional outcomes and HRQOL. Functional outcomes were assessed by determining the number of bowel movements per day, daytime and nighttime seepage, incontinence, urgency and use of protective pads. Quality of life was determined by Cleveland Clinic Global Quality of Life (CGQOL) score. Thirty patients (44%) continued to have a functioning IRA after a median follow-up of 11 years. The rectum was removed in 46 patients. The indication for completion proctectomy included refractory proctitis, rectal dysplasia, and cancer. The patients with IRA had significantly fewer bowel movements per day and less nighttime seepage but had greater urgency than those with IPAA. Even though HRQOL was similar between groups, patients with IRA had significantly more dietary and work restrictions. The authors concluded that while IRA is not the definitive operation for patients with ulcerative colitis, a significant number of patients were able to keep their rectum after 10 years within acceptable functional outcome and the quality-of-life.

HRQOL after continent ileostomy

Continent ileostomy (CI) was initially described by Nils Kock in 1969[19]. Since then, the Kock pouch became an option for patients for whom the only alternative was an end ileostomy. It was developed and modified to provide physical and psychosocial benefits over the end ileostomy but had many complications, mainly attributed to the valve mechanism, which maintains continence. Nessar *et al*[20] reviewed the Cleveland Clinic continent ileostomy experience comparing HRQOL in CI patients and those whose Kock reservoir failed, was removed and converted to an EI. Results were evaluated using the continent ileostomy surgery follow-up questionnaire and the Cleveland Global Quality of Life (CGQL) scale. Patients with an EI were more than twice as likely to report social, work, and sexual restrictions and to require a higher antidiarrheal medication and fiber intake compared with patients with CI. A higher percentage of patients with CI reported having a better appetite. Patients with CI reported less abdominal pain than the EI group, and rated a higher score for overall happiness. CGQL measurements were better on all scales as well as the summary scale in the CI group. With experience, early and late complications of the technique have declined, however the procedure did not gain a wide acceptance by the surgical community. At present, continent ileostomy is performed only by dedicated surgeons in a few specialized centers.

HRQOL after IPAA

Early in the evolution of the IPAA there was some skepticism whether IPAA would offer advantages over medical management alone. In an elegant prospective study Berndtsson and Oresland[21] evaluated HRQOL before and after restorative proctectomy with IPAA. The authors used the General Quality-of-Life according to Kajang (GQL) instrument, as well as the Visual Analogue Scale (VAS), and a modified disease specific Olbrisch adjustment scale (OAS). These instruments were supplemented by a set of open ended questions. The authors subdivided the patients based upon the presence or absence of ileostomy prior to IPAA. The results indicated that the operation did not influence the general quality-of-life as measured by specific quality-of-life instruments or a visual analogue scale. In the open ended questions, however, the patients reported improved relations with their friends. Even though there was no clear improvement in HRQOL IPAA gave patients greater freedom in role function, improved body image, and reduced the negative effects caused by colitis or life with ileostomy.

Our group evaluated the functional outcomes and HRQOL in a large prospective series of patients who underwent IPAA[22]. A total of 391 consecutive patients were included in the study. The ileoanal anastomosis was constructed either by hand sewn or stapled technique. When a hand sewn anastomosis was selected a complete mucosectomy was performed. A protecting ileostomy was fashioned in the majority of patients. HRQOL was assessed by a validated questionnaire that consisted of two parts. The first part consisted of a list of questions designed to assess the use of pharmacological aids and diet restrictions to reduce the number of bowel movements and improve the quality of continence. The second part included questions related to oral intake, sleep pattern, bowel activity, and daily continence over one week. The patients were asked to complete the questionnaire at 3, 6, 9, 12, 18, and 24 mo after the surgical procedure and yearly thereafter. Over a 10 year follow up the patients who underwent IPAA had on average six bowel movements in a 24-h period. The majority of the patients were able to postpone a bowel movement until a convenient time. Only 18% of patients were able to consistently distinguish flatus from stool and this finding was similar between handsewn and stapled IPAA. The percentage of fully continent patients was higher following stapled anastomosis. Complete daytime and nighttime continence was achieved by 53% to 76% of patients and improved over time. Five years following IPAA 81.4% of patients judged their quality-of-life was much better or better. An impressive 97% of patients reported overall satisfaction and overall adjustment following IPAA as being excellent or good. Our results were similar to those reported by the Cleveland Clinic[23]. In their study, the authors used a validated instrument short form 36 (SF36) as well as their own scoring system Cleveland Global Quality-of-Life (CGQL). The quality-of-life was shown to increase two years following IPAA and there was no deterioration thereafter. The fecal continence improved from 75.5% before surgery to 82.4% following surgery. Even though there was late deterioration of continence, it never became worse than the preoperative function.

Based on these results and the results of other groups, the double stapled IPAA became the preferred anastomotic technique except in situations where mucosectomy is required because of dysplasia or malignancy[24]. In these situations a hand sewn anastomosis is constructed. Anastomotic leak, a dreaded complication of IPAA, has been evaluated by Lian *et al*[25] comparing hand-sewn and stapled anastomosis. The primary endpoint of the study was pouch failure. Functional outcomes were determined by the number of daytime and nighttime bowel movements, incontinence, urgency, and daytime and nighttime seepage. HRQOL was assessed with the CGQL. The authors concluded that the functional outcomes following stapled anastomotic technique were significantly better compared to those occurring after handsewn anastomotic leak.

Despite various instruments used to evaluate the HRQOL, there appears to be significant disparity in results based on the instrument used. In an interesting study by Scarpa *et al*[26] the authors addressed the fact that there appears to be distinct types of HRQOL outcomes depending on the type of the instrument used. The analysis based on CGQL reports excellent long-term functional results of IPAA suggesting that an IPAA offers patients long-term HRQL comparable to that of healthy controls. Conversely, other researchers report long-term HRQOL similar only to that of patients with mild UC or remission disease activity. The authors used a cohort of patients consisting of patients with UC, healthy controls, and those who underwent IPAA. A combination of Italian version of the CGQL and the Padova Inflammatory Bowel Disease Quality of Life (PIBDQL) were administered to all patients. According to the Italian CGQL the patient with IPAA had scores similar to healthy controls and patients with mild UC or UC in remission, while the PIBDQL scores of patients who underwent IPAA were significantly worse than those of healthy controls and similar to those of patients with UC in remission or mild UC. The authors concluded that PIBDQL had significantly better discriminative ability compared to the Italian CGQL and, therefore, allowed to more accurately evaluate HRLQOL.

HRQOL in IPAA in patients with compromised anal sphincter

Poor anal sphincter function has always been considered a contraindication to IPAA. However, in selected patients with sphincter defects that are continent to liquid stools an IPAA should still be considered. Gearhart and colleagues[27] prospectively evaluated 42 women with anorectal manometry and endo-anal ultrasound. Even though all patients were continent at the time of evaluation, endo-anal ultrasound revealed significant sphincter defects in 19 of them. Of these, 4 individuals had significant sphincter defects that involved both internal and

external anal sphincter, a complication of an obstetric trauma. The findings of the endo-anal ultrasound correlated with anal physiology studies that revealed significantly decreased resting pressures, squeeze pressures, and shorter anal canal length. All patients underwent an IPAA (40 stapled, 2 handsewn). The participants were surveyed postoperatively with Cleveland Clinic Florida scale (Wexner score), Fecal Incontinence Severity Index (FISI), or Fecal Incontinence Quality of Life (FIQL) scale comparing those with or without sphincter defects. The authors did not find a correlation between the size of the sphincter defects and incontinence. Despite the fact that almost all responders reported episodes of seepage, there was no significant difference found in Wexner score, FISI, or FIQL between the patients with and without sphincter defects. Interestingly, out of 4 patients who reported perfect continence, 2 had significant preoperative sphincter defects. Six patients were dissatisfied with the functional outcome of the IPAA and said they would not undergo this procedure again. Of these patients three had significant sphincter defects. This study demonstrates that, despite careful selection of the candidates for an IPAA, approximately 1/4 of the individuals will suffer from various degrees of stool leakage and up to 5% of them will undergo pouch excision. The authors believe, however, that women with known sphincter defects can obtain a satisfactory continence following an IPAA. Although this study suffers from small size and has limited statistical power, it suggests that when used selectively in highly motivated patients restorative proctocolectomy with an IPAA can provide a satisfactory HRQOL even in those patients who have a sphincter defect but are fully continent preoperatively.

HRQOL following IPAA failure

Lepistö *et al*[28] evaluated HRQOL after pouch failure. From a cohort of 486 patients the authors identified 26 patients. The overall pouch failure in the study was 5.3%. The patients who suffered from pouch failure, controls with functioning pouches as well as normal controls were sent an SF-36 quality-of-life questionnaire. The pouch was excised in 24 patients, 2 patients died from IPAA related complications. Reasons for pouch failure were fistulae, pouchitis, fecal incontinence, CD, perianal pain, and major anastomotic disruption. Compared with a healthy population, patients in the failure group reported significantly worse physical function, social functioning, energy level and the physical role function. All of these findings were statistically significant. The scores for general health, emotional well-being, bodily pain, emotional role function did not differ significantly between the two groups. Similarly, when compared to a control group with well functioning IPAA, the pouch failure group had significantly lower scores for physical function, energy level, and physical role function. Pouch excision following restorative proctocolectomy and ileal pouch anal anastomosis is a rather distressing experience for the patients. Tan and colleagues[29] compared HRQOL in IPAA patients and required pouch excision with patients with an initial TPC and EI. A questionnaire addressing bowel symptoms, systemic symptoms, functional impairment, social empowerment, and emotional impairment was used. Mean scores for bowel and systemic symptoms, as well as for functional, social and emotional impairment, were lower following pouch excision. The difference, however, was only statistically significant for bowel symptoms. This particular difference was due to frequency of emptying of the ileostomy bag and perception of this being disruptive of family life. The higher ileostomy output was a result or shorter length of small bowel due to a pouch loss.

HRQOL for salvage surgery in failed IPAA

In selected patients with pouch complications such as recurrent strictures, pouch emptying disorders, long efferent limb of an S-pouch and disabling pouchitis salvage pouch surgery can be performed. Baixauli *et al*[30] assessed functional outcomes, HRQOL and pouch salvage rates from a prospectively maintained database. In addition the patients were asked to complete a self administered structured questionnaire during their follow-up visits. Assessment of HRQOL was performed using the CGQL score. A total of 40% of patients with pouch failure were eventually diagnosed with CD. Those patients with CD who retained their pouches had similar HRQOL and functional scores to those with UC. Of 101 patients with an attempted pouch salvage procedure, 13 had to have the pouch excised. The overall 5-year pouch salvage rate was 74% (79% in UC and 53% in CD). No differences were seen between those having repeat ileal pouch-anal anastomosis procedure for septic or non-septic indications, or whether using a new or repaired pouch. In those patients in whom new pouch had to be constructed high-frequency of defecation was noted, in addition to increased number of bowel movements at night. However this was similar to those patients who had their pouches repaired and reanastomosed. Despite the complications associated with initial IPAA, 97% of patients said they would undergo repeat ileal pouch-anal anastomosis again, and 99% would recommend it to others. The authors concluded that salvage procedure for failed IPAA is a valid alternative to pouch excision in cases where pelvic sepsis is controlled.

HRQOL in IPAA at end of the age spectrum

Older patients have not been traditionally considered good candidates for IPAA. However, in selected septuagenarians IPAA can be a valid option. Delaney *et al*[31] evaluated functional outcomes and HRQOL in 17 patients older than 70 at the time of surgery using CGQL scoring system. The patients' responses were also collected using SF-36 analysis comparing it with United States population 65 years of age and older. The authors found no significant difference between patients with IPAA older than 70 years and healthy individuals older than 65 years. In particular there was no difference in mental and physical components. Unfortunately, in their

series 4 patients (23.5%) had major morbidity and 1 patient died of septic complications of an intra-abdominal abscess. The overall quality-of-life, health and levels of energy and happiness were perceived as good by the patients. Most patients would recommend IPAA to others and 82% said that they would undergo pouch surgery again. While the results of the HRQOL and functional outcomes were encouraging, the authors underscored the importance of patient selection.

At the other end of the spectrum is IPAA performed in children younger than 18 years of age. In the study by Lillehei *et al*[32] the authors included 75 children with UC who underwent IPAA. The results compare favorably to those reported in literature in regards to stool frequency and fecal continence. The average number of daily bowel movements was 5. The rates of daytime and nighttime fecal continence were found to be excellent. Ninety percent of the patients were able to distinguish flatus from stool. The rate of pouchitis in this young patient population was, however, quite high. Of 75 children with UC, 35 (47%) had at least one episode of pouchitis and 10 patients required prolonged treatment. Pouchitis had a significant impact on HRQOL in this group, but the reasons for such a high incidence in pediatric population remain unknown.

HRQOL after laparoscopic IPAA

With the advent of laparoscopic surgery, there was a gradual shift to performing IPAA with laparoscopic assistance. Our group reported on a long-term functional results in a prospective observational study on laparoscopic assisted IPAA[33]. The patients were asked to complete a previously validated two-part questionnaire used by our group. The overall average number of daily bowel movements was similar between laparoscopic and open groups. There was a trend in the laparoscopic group to report improved stool consistency with mostly pasty bowel movements. There were no significant differences in fecal continence between the groups. In the laparoscopic group only 20.8% compared to 21.9% of patients in the open group reported some degree of incontinence. Significantly fewer patients in the laparoscopic group used pads during the daytime and nighttime. The quality-of-life was similar between the groups. The majority of the patients rated their quality of life as better or much better compared to before their IPAA or before ileostomy closure. Similar findings were reported by other groups. Larson *et al*[34] reported the Mayo Clinic experience comparing laparoscopic and open IPAA. Three separate surveys were mailed to the patients: the validated SF-8, female sexual function index (FSFI), and international index of erectile function (IIEF). In addition the authors used non-validated body and cosmetic evaluation instrument. Similar to our study the authors found the quality-of-life to be comparable between open and laparoscopic patients. Short-term benefits were noted in the laparoscopic group; however, long-term outcomes likely reflect the function of the pouch and not the surgical technique used. Alterations in female sexual function were noted to be similar whether laparoscopic or open IPAA was performed. The male sexual function was less affected.

Because laparoscopic surgery has advantages over open surgery by primarily improving appearance and body image, but not included in the quality-of-life questionnaire, Dunker *et al*[35] developed a body image questionnaire consisting of a body image scale and cosmetic scale. Similar to the previous studies, no significant differences were found in functional outcomes and in HRQOL. Satisfaction with cosmetic result, however, was significantly higher in laparoscopic assisted group as compared to the conventional group. Accordingly, body image scores were higher in the laparoscopic assisted group when compared with open group, although this difference was not significant. Even though the functional impact on HRQOL in IPAA has not been shown to be significantly different between laparoscopic and open group, better patient acceptance and improved cosmesis combined with the decreased risk of hernia formation makes laparoscopic IPAA the preferred surgical approach to UC at the present time.

Sexual function and reproductive health after an IPAA

Proctocolectomy with IPAA is frequently performed in young and active men and women. Sexual dysfunction is a potential risk for both men and women who undergo pelvic surgery. Damage to the autonomic nerves can occur resulting in impotence, retrograde ejaculation in men or vaginal dryness and dyspareunia in women. Davies and colleagues[36] prospectively evaluated male and female sexual function before and after IPAA. The authors used the IIEDF, a validated instrument that evaluates erectile function, orgasmic function, sexual desire, intercourse satisfaction, and overall satisfaction. The female sexual function was evaluated with the FSFI which addresses six domains of female sexual function (desire, arousal, lubrication, orgasm, satisfaction, and pain). The authors asked 59 patients to complete a preoperative questionnaire. These patients were then mailed questionnaires at 6 and 12 mo after surgery. There were 33 men and 26 women who underwent IPAA. The IIEDF scores in men did not change following IPAA while in women the FSFI scores significantly improved following surgery. The authors noted a high proportion of women with abnormal sexual function preoperatively (73.1%). This proportion decreased significantly to 25% at 12 mo after surgery. Improved overall physical well-being after surgery has been suggested as the reason for such improvement. On the other hand, dyspareunia, vaginal dryness, and incontinence have been reported in a small portion of women. In contrast to previous studies that used retrospective surveys relying on the patients' recall of their preoperative sexual function, Davies and colleagues used a validated tool and eliminated the recall bias by administering a prospective survey. This study, however, didn't address the effect of complications such as anastomotic leaks on sexual function.

Despite advances in operative technique, proctocolectomy with IPAA is still associated with a significant

decrease in female fertility[37]. Because many women with UC are diagnosed at a young age, it has been reported that 45% of them will attempt to become pregnant following surgery. In the study by Olson and colleagues[38], the cumulative incidence of pregnancy in women following conventional IPAA was only 36% whereas 80% of women in the general population and 90% of women with UC who had not had surgery were successful in becoming pregnant. It is thought that the infertility problems are likely due to adhesion formation following rectal dissection. This notion is supported by the fact that patients who undergo total abdominal colectomy with either an IRA or an EI have essentially unchanged fertility compared to patients with UC who did not undergo surgery. While many strategies to preserve fertility have been proposed, such as oophoropexy and use of anti-adhesion substances in the pelvis, currently there is no evidence that they have a proven benefit. There is still insufficient data on the impact of the laparoscopic approach on fertility and fecundity. For women with active disease in need of a colectomy the recommendation is to perform a colectomy with an end ileostomy and defer an IPAA until a woman completes her family.

Pregnancy following IPAA has been shown to be safe and not associated with increased maternal or fetal complications. Additionally, pregnancy does not result in pouch related complications. Most authors advocate a caesarian section and discourage vaginal delivery. This is based on the fact that sphincter disruption that might occur during vaginal delivery can result in significant deterioration of the functional results of IPAA particularly in patients who already have liquid stools. This recommendation is reflected by 38% to 78% rate of caesarian sections following an IPAA compared to 22% average in North America. In a study by Hahnloser and colleagues[39] a total 135 female patients were prospectively followed. Seventy two percent of them had uncomplicated pregnancies and were able to carry to term. The pregnancy complications in IPAA patients did not occur more frequently than in the general population. In this study 56% of women gave birth vaginally after IPAA. The authors noted that vaginal delivery did not have significant impact on long-term pouch function compared to women who had a cesarean section. After delivery pouch related complications in patients with IPAA increased. It is unclear whether the increased pouch related complication rate was due to the delivery or to the natural history of the pouch. Despite this finding the authors recommended vaginal delivery unless there are obstetrical concerns that mandate a cesarean section. The summary of included articles for HRQOL in UC was shown in Table 1.

SURGICAL THERAPY AND HEALTH RELATED QUALITY OF LIFE IN CROHN'S DISEASE

There are many characteristics of CD that make HRQOL measurements of particular importance in this entity, such as the recurrent and panintestinal nature of the disease, the young age of onset, impact on productivity, body image and sexual life[40]. While it is important to correlate HRQOL with disease activity, it is also important to recognize that traditional measurements of disease activity correlate poorly with patient assessment in CD[41]. HRQOL has been shown to correlate with disease activity in CD in a variety of studies. Standard disease activity indices, such as the Crohn's Disease Activity Index (CDAI) and the Harvey Bradshaw index[42], have been shown to correlate with IBDQ[43] the short IBDQ[44], the Korean-translated IBDQ[45], the TTOT, the Spanish IBDQ[46] and the Dutch-translated IBDQ[47].

Furthermore, when evaluating HRQOL in CD patients after surgery it is important to consider the type of surgery performed. A difference in HRQOL is expected between CD patients undergoing a simple ileocolic resection versus having a permanent end ileostomy after a total proctocolectomy. Due to the importance of HRQOL in CD, formal HRQOL questionnaires are now routinely included as secondary outcomes in clinical trials. This review will focus on HRQOL in surgically treated CD patients, comparing it with medically treated CD patients, and correlating it with CD activity[42].

It is important to keep in mind that CD patients have been shown to have significantly impaired HRQOL compared to healthy controls[48]. Even patients in the least severe health state, who are in remission and receiving minimal medical therapy, have a measurable decrease in HRQOL relative to healthy individuals[49,50]. If HRQOL scores in CD patients are compared with those of patients with other chronic diseases, they are worse than that of individuals with severe angina, but better than those patients with chronic renal failure undergoing haemodialysis, or those with ulcerative colitis before a colectomy[51].

Surprisingly there are very few studies that compare HRQOL in medically and surgically treated CD patients. We speculate that it is due to the heterogeneity of these patient populations that it is very difficult to compare taking into account the multiple sites of disease, the extreme variability of severity and type of associated complications and the multiple surgical options available. Thirlby *et al*[52] in a series of 36 primary CD patients showed postoperative improvement at various time points using the Health Status Questionnaire (HSQ)[53]. Preoperative measures of HRQOL of the patients were low, with values well below the general population in all 8 scales of the HSQ. Postoperatively, HRQOL measures improved significantly to reach scores equal to the general population in most parameters, for example the average raw scores for general health (74 in the general population *vs* 54 and 73 preoperatively and postoperatively, respectively, in the study group). Tillinger *et al*[54] (which included utility studies) showed an advantage at 3 and 6 mo, but not at 24 mo postoperatively in the presence of chronic disease. In this prospective study 16 CD patients (mostly with terminal ileal diseases) were investigated within 1 wk before surgery and 3, 6 and 24 mo postoperatively. CDAI decreased significantly after surgery and 10

Table 1 Summary of included articles for health related quality of life in ulcerative colitis

Study	Patient population	HRQOL instruments	Study conclusions
Cohen *et al*[2]	Steroid-refractory UC patients treated with CSA *vs* colectomy	IBDQ visual-analog scale, Oresland	CSA patients had similar HRQOL compared to colectomy group. CSA can be alternative to surgery in selected patients
Sagar *et al*[13]	UC patients after IPAA *vs* UC patients on long-term medical treatment	based on a questionnaire by Oresland *et al*	HRQOL after IPAA is no worse than that of patients with long-standing UC on medical treatment in remission
Camilleri-Brennan *et al*[15]	UC patients with TPC and EI *vs* general population	SF-36 version 2 (SF-36II)	HRQOL of TPS and EI patients very similar to that of the general population. TPC and EI remains a viable option for patients with UC
da Luz Moreira *et al*[18]	UC patients with IRA *vs* IPAA	CGQL	HRQOL similar between groups, but IRA is inferior to IPAA because of dietary and work restrictions
Nessar *et al*[20]	CI (Kock) *vs* EI	CGQL	HRQOL significantly better with CI, but complications are common in CI group
Berndtsson *et al*[21]	UC patients before and after IPAA	GQL, VAS, OAS open ended questions	IPAA had no impact on HRQOL, but improved relations with friends, freedom in role function, and body image reduced the negative effects caused by colitis or life with ileostomy.
Michelassi *et al*[22]	10 yr prospective study of UC patients with IPAA	Two part questionnaire	Excellent long term functional outcomes after double stapled IPAA
Fazio *et al*[23]	UC patients with IPAA	SF 36 CGQL	HRQOL increase following IPAA; no deterioration with time.
Lian *et al*[25]	UC patients with hand sewn *vs* stapled IPAA complicated by anastomotic leak	CGQL	Functional outcomes following anastomotic leak better in stapled IPAA compared to handsewn IPAA
Scarpa *et al*[26]	UC patients, UC patients after IPAA, and normal controls	Italian CGQL *vs* PIBDQL	PIBDQL has significantly better discriminative ability compared to the Italian CGQL
Gearhart *et al*[27]	UC female patients with sphincter defects	FISI, FIQL Wexner score	IPAA can provide a satisfactory HRQOL in patients with sphincter defect who are fully continent preoperatively
Lepistö *et al*[28]	HRQOL after pouch failure *vs* well functioning IPAA	SF-36	IPAA failure group with significantly lower scores for physical function, energy level, and physical role function
Tan *et al*[29]	UC patients following pouch excision *vs* initial TPC and EI	SF-36	HRQOL similar between the pouch excision and initial IPAA groups
Baixauli *et al*[30]	IPAA patients with pouch failure	CGQL	CD is common cause of pouch failure. HRQOL similar in UC or CD pouch failure patients
Delaney *et al*[31]	IPAA patients 70 yr and older	CGQL	Good HRQOL, health, levels of energy and happiness. However, IPAA associated with high rate of morbidity and mortality
Lillehei *et al*[32]	Pediatric UC patients with IPAA	Standardized questionnaire	Excellent functional outcomes. High rate of pouchitis
Fichera *et al*[33]	Laparoscopic *vs* open IPAA	Two part questionnaire	HRQOL was similar between the groups. Better patient acceptance and improved cosmesis in laparoscopic IPAA
Larson *et al*[34]	Laparoscopic *vs* open IPAA	FSFI, IIEF body and cosmetic evaluation	HRQOL comparable between open and laparoscopic patients
Dunker *et al*[35]	Laparoscopic *vs* open IPAA	Body image questionnaire	HRQOL similar between groups
Davies *et al*[36]	Prospective evaluation of sexual function before and after IPAA	FSFI, IIEDF	Improved sexual function in women. Unchanged sexual function in men
Ording Olsen *et al*[38]	Reproductive health of women with UC undergoing IPAA *vs* UC *vs* general population		Significant decrease in fecundity (36% *vs* 80%-90%) following IPAA compared to UC and general population
Hahnloser *et al*[39]	Pregnancy following IPAA *vs* general population		No increase in pregnancy complications following IPAA. Vaginal delivery not contraindicated

HRQOL: Health related quality of life; UC: Ulcerative colitis; CSA: Cyclosporine A; VAS: Visual-analog scale; IPAA: Ileal pouch anal anastomosis; EI: End ileostomy; TPC: Total proctocolectomy; CGQOL: Cleveland Clinic Global Quality of Life; CI: Continent ileostomy; IRA: Ileorectal anastomosis; CGQL: Cleveland Global Quality of Life; PIBDQL: Padova Inflammatory Bowel Disease Quality of Life; GQL: General Quality-of-Life according to Kajang; FISI: Fecal Incontinence Severity Index; FIQL: Fecal Incontinence Quality of Life; IIEF: International index of erectile function.

patients remained in remission for 24 mo. Two patients had postoperative relapses and went into remission after prednisolone treatment. Four patients developed chronic active disease. HRQOL was significantly improved in all patients 3 and 6 mo postoperatively. Except for the 4 patients with chronic active disease, all other 12 patients also had significantly improved HRQOL after 24 mo. Thus, in patients with active CD, HRQOL appears to improve in the immediate post-operative period, but not in the long term. This is consistent with the natural history of CD, which gradually recurs postoperatively in most patients[55,56].

Meyers at Mount Sinai interviewed 51 patients with CD 5-10 years after elective surgery[57]. Patients were asked to retrospectively assess 5 areas of psychosocial functioning (personal relations, school and job performance, recreation, sexual function, and body image) 6 mo preoperatively, 1 year postoperatively, and at the time of interview. As expected, patients reported significantly less severe symptoms at interview than preoperatively with large differences given the fact that this was a surgical series; 100% of patients were symptomatic at surgery and only approx-

imately half at follow-up, which may support an effect of the intervention itself. Exceptions were patients with an ileostomy and patients with recurrent illness. Overall 92% felt the surgery had been helpful[57].

The study by Casellas *et al*[49] looked at the impact of disease activity on HRQOL in the surgical CD patient. In this study the determining factor was whether the patient had active disease and not whether he or she had undergone previous CD, in part confirming Tillinger's findings[54]. They looked at the outcomes of 29 CD patients in remission with a previous bowel resection compared with 42 clinically active CD patients and 48 patients with medically induced remission. The control group was composed of 63 healthy individuals. HRQOL was measured by IBDQ, the Psychological General Well Being Index[58], and the EuroQol[59]. Not surprisingly they showed that active CD patients scored the lowest on the IBDQ. Both operated and non operated inactive CD patients had lower HRQOL scores than controls in overall IBDQ and in all 5 domains. They concluded that HRQOL is impaired in active CD and improves during remission irrespective of whether it had been achieved medically or surgically.

Cooper and colleagues reported 42 patients with CD sparing the rectum undergoing colectomy and ileorectal anastomosis[60]. Problems with this study were the high mortality rate (7%) due to anastomotic breakdown and the need for reoperation (48%). Unrestricted social activity and regular employment was reported by 85% of the 14 patients questioned who continued to have a functioning anastomosis 1 to 14 years postoperatively.

In a provocative study by Halme, 98 patients treated for CD for a mean period of ten years showed a substantial difference in HRQOL in favor of patients with large bowel involvement treated with proctocolectomy and ileostomy compared to all other treatment groups (ileocecal resection, colectomy with ileorectostomy, and colostomy with proctectomy). Furthermore the ability to work was greatest in the nonsurgical group and the ileostomy group[61].

Restorative proctocolectomy with IPAA is generally contraindicated in patients with preoperative diagnosis of CD. On the other hand, in approximately 10% of patients with a diagnosis of UC, signs and symptoms of CD may develop in the pouch. CD following IPAA can manifest itself as inflammation of the pouch with fibrostenotic or fistulizing characteristics. Shen and colleagues[25] evaluated 73 patients with CD of the pouch. Twenty five of them were found to have inflammatory CD, 17 fibrostenotic, and 31 fistulizing disease. HRQOL was assessed using three instruments: Cleveland Clinic global quality-of-life, the irritable bowel syndrome quality-of-life, and the short inflammatory bowel disease questionnaire. The irritable bowel syndrome quality-of-life tool was used to address the functional aspects of symptoms in patients with IPAA because the patients with IBD frequently have IBS-like symptoms. The symptomatology correlated closely with the disease phenotype. Patients who developed inflammatory CD in their pouch predominantly suffered from diarrhea and/or pain. Those with fibrostenotic disease had predominantly obstructive symptoms. Patients with fistulizing CD of their pouches had significant fistular drainage symptoms. There was no statistically significant difference in HRQOL scores between the three phenotypes of CD when adjusted for disease activity. Fistulizing CD appeared to be associated with a higher risk of pouch failure. The risk factors for development of CD in the pouch include intentional construction of IPAA in patients with known CD, patients with indeterminate colitis at the time of IPAA, or patients with long-standing pouchitis. Patients who were diagnosed with CD of the pouch often required long-term maintenance therapy. The majority of the patients responded to infliximab infusion. A total 33% of patients with CD of the pouch required pouch excision. The remaining 67% had an adequate pouch function. Overall the patients who developed CD following an IPAA have a perception of the poor quality of life since it requires sustained pharmacologic therapy, sometimes surgery, and is complicated by frequent exacerbations and gastrointestinal and extra intestinal complications.

While in UC the use of validated HRQOL instruments in surgical studies is routine, the vast majority of studies addressing surgical treatment of CD are uncontrolled, and very few used a validated HRQOL instrument. Assessment of the surgical management of CD is greatly limited by the use of physician rated scales that arbitrarily assign patients to levels of HRQOL. Physician ratings cannot truly measure HRQOL, which is by definition the patient's subjective opinion. The insensitivity of these scales provides quite limited support for the value of surgery and no convincing argument for the superiority of any particular surgical approach.

Thus in IBD it is misleading to place too much confidence in uncontrolled studies. Any chronic illness that waxes and wanes and often recurs will yield large discrepancies in the assessment of severity, which depend largely on the time of sampling. A comparison of current function to preoperative function will predictably show better function currently because the time when a person chooses palliative surgery presumably marks a nadir in satisfaction and function, as opposed to the more arbitrarily determined date of follow-up when some proportion of patients will be in remission by chance. Further controlled studies are needed to better understand HRQOL in IBD.

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TOPIC HIGHLIGHT

Dr. Marco Scarpa, PhD, *Series Editor*

Quality of life after laparoscopic and open colorectal surgery: A systematic review

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Abstract

This study was a systematic review of the available evidence on quality of life in patients after laparoscopic or open colorectal surgery. A systematic review was performed of all randomized clinical trials (RCTs) that compared laparoscopic with open colorectal surgery. Study selection, quality assessment and data extraction were carried out independently by two reviewers. Primary endpoint was quality of life after laparoscopic and open colorectal surgery, as assessed by validated questionnaires. The search resulted in nine RCTs that included 2263 patients. Short- and long-term results of these RCTs were described in 13 articles. Postoperative follow-up ranged from 2 d to 6.7 years. Due to clinical heterogeneity, no meta-analysis could be conducted. Four RCTs did not show any difference in quality of life between laparoscopic or open colorectal surgery. The remaining five studies reported a better quality of life in favor of the laparoscopic group on a few quality of life scales at time points ranging from 1 wk to 2 years after surgery. In conclusion, based on presently available high-level evidence, this systematic review showed no clinically relevant differences in postoperative quality of life between laparoscopic and open colorectal surgery.

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Key words: Quality of life; Colorectal surgery; Laparoscopy; Colonic neoplasms; Colonic diseases; Inflammatory bowel diseases

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INTRODUCTION

Since the introduction of laparoscopic surgery in the early 1990s, several multicenter randomized clinical trials (RCTs) have established that laparoscopy is a safe and feasible approach in colorectal surgery. These studies have focused on benign diseases such as diverticulitis and ulcerative colitis (UC), pre-malignant diseases like familial adenomatous polyposis (FAP)[1,2], and malignant diseases, mostly colorectal carcinoma[3-5]. Advantages of laparoscopic surgery include shorter postoperative hospital stay, less peri-

operative blood loss, less postoperative pain and cosmetic advantages. Long-term follow-up will most probably show less incisional hernias and adhesions. However, no sufficient data are available yet. Morbidity and oncologic follow-up have been reported to be similar for open and laparoscopic colorectal surgery[4-6]. Disadvantages are the prolonged operating time, the higher costs and the need for an experienced surgeon, because it takes at least 20 procedures to come through the learning curve[7,8].

After colorectal surgery for malignancy, many patients experience a combination of physical and emotional problems for a long period of time. Symptoms such as fatigue, pain and disturbed bowel function, as well as problems in social and role functioning, inevitably affect the patients' wellbeing. Assessment of self-reported quality of life is therefore increasingly important in clinical trials, and also when considering the higher costs for laparoscopy and its cost-effectiveness. In addition, in cancer trials, it has been shown that assessing quality of life could contribute to improved treatment[9]. In 2008, Dowson *et al*[10] performed a systematic review that included studies published up to March 2007 on quality of life following laparoscopic and open surgery. The authors however concluded that there was a lack of data and a need for further research[10]. Over the past 3 years, more trials on quality of life after open or laparoscopic colorectal surgery have been published, therefore, an update of this systematic review was required.

The aim of this systematic review was to examine the latest evidence of quality of life in patients after laparoscopic or open colorectal surgery.

SEARCH STRATEGY

A literature search of the following electronic databases was conducted: PubMed, Cochrane Central Register of Controlled Trials, and EMBASE (all from January 1980 to April 2010). The key words used were: [colon (MeSH) OR colon OR colonic OR colorectal OR rectal OR mesorectal OR rectoanal OR anorectal OR rectum (MeSH) OR rectum OR colectomy (MeSH) OR colectomy] AND [minimal* AND invasive OR laparoscopy (MeSH) OR laparoscop* OR laparotomy (MeSH) OR laparotom*] AND [quality of life (MeSH) OR quality of life].

No limits as to language were applied. Additionally, a hand search was performed of the references of relevant studies. Two reviewers (SB and MV) independently selected studies on the basis of their titles and abstracts. Studies were included if they were an RCT that compared laparoscopic and open colorectal surgery for malignant or benign disease, and contained comparative data on quality of life, either as primary or secondary endpoints. If studies reported on similar patient data, the study with the largest sample size was included. Exclusion criteria were: clinical comparative studies, case series, case reports, reviews, letters, or abstracts. In case of disagreement between the two reviewers, a third reviewer (WB) was involved.

DATA EXTRACTION

The results of each included trial were extracted onto a form that contained the following items: methodological aspects of the trial (i.e. randomization, concealment of allocation, blinding, follow-up, intention to treat, possible selective reporting, other possible bias), inclusion and exclusion criteria, patient characteristics, details on the surgical procedures, primary and secondary endpoints, instruments, timing, and results of the quality of life measurements. All quality of life results were extracted at any time interval, as well as preoperative baseline characteristics and short- and long-term postoperative follow-up data.

ASSESSMENT OF METHODOLOGICAL QUALITY

The methodological quality of the RCTs was assessed using "The Cochrane Collaboration's Tool for Assessing Risk of Bias"[11]. This tool assesses the quality of RCTs by addressing items such as: the methods of randomization, concealment of allocation, blinding, drop-out rate, intention to treat, and other forms of potential bias. Again, this assessment was made by two reviewers independently (SB and MV).

OUTCOME MEASURE: QUALITY OF LIFE INSTRUMENTS

Studies were included if at least one of the following validated quality of life instruments was used: European Organization for Research and Treatment of Cancer (EORTC)-QLQ-C30; EORTC-QLQ-C38; Short Form-36 (SF-36); Gastro Intestinal Quality of Life Index (GIQLI); Quality of Life Index (QLI); EuroQoL-5D (EQ-5D); Symptom Distress Scale (SDS) and Global QoL. A summary of the four most commonly used questionnaires is given below.

The EORTC-QLQ-C30 questionnaire has been developed by the Quality of Life Department of the EORTC. This is a self-reported patient questionnaire that included: five functional scales (physical, role, emotional, social, and cognitive); three symptom scales (fatigue, nausea and vomiting and pain); a global health status/QoL scale; and six single items (dyspnea, insomnia, appetite loss, constipation, diarrhea and financial difficulties)[12]. The EORTC-QLQ-C38 is an extra module used specifically for colorectal cancer. This questionnaire consists of 38 items that cover symptoms and side effects related to different treatment modalities, body image, sexuality and future perspective[13]. The SF-36 consists of 36 items within eight dimensions: psychological functioning; role limitations due to physical problems; pain; general health perceptions; energy/vitality; social functioning; role limitations due to emotional problems and mental health[14]. Lastly, the GIQLI assesses bowel-

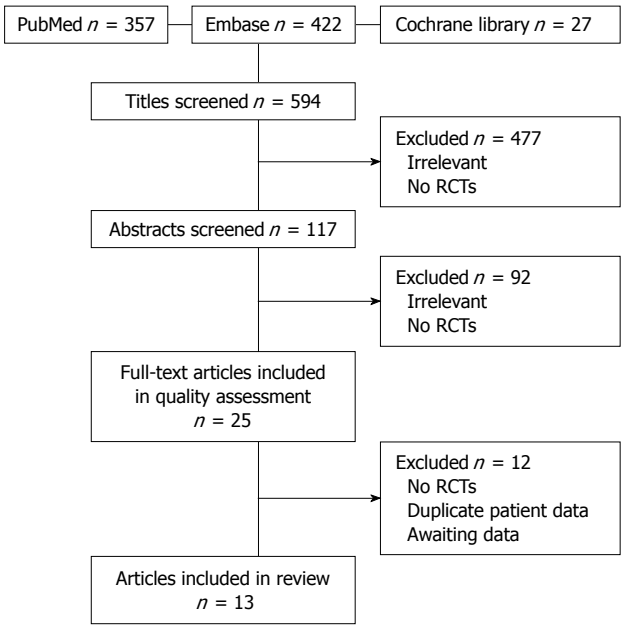

Figure 1 Flow chart article inclusion. RCTs: Randomized clinical trials.

related quality of life. It contains 36 items and covers symptoms, physical, emotional and social functioning[15].

LITERATURE SEARCH

A total of 594 potentially relevant titles were identified from the initial literature search in the aforementioned electronic databases. After scanning of all titles by both reviewers independently, 117 abstracts were selected to be reviewed for inclusion criteria. Hereafter, 25 full-text articles remained for assessment of inclusion criteria and of methodological quality. After this assessment, 12 articles were excluded for the following reasons: four articles for being non-randomized studies[16-19]; three for presenting data on similar patients[20-22]; three for reporting on ongoing trials, i.e. not presenting data[23-25]; one for not presenting quality of life data[26]; and one could not be translated from Russian[27]. A total of 13 full-text articles remained for final analysis and data extraction. These articles reported on the results of nine different RCTs[1,2,4,28-33]. The long-term results of four of the nine included RCTs were presented in separate papers; therefore, 13 articles were included[34-37]. Details of the search are shown in Figure 1.

RISK OF BIAS IN INCLUDED TRIALS

The methodological quality of the nine included trials is summarized in Figure 2. In general, overall study quality was good. All studies were properly randomized and in one[31], concealment of allocation was unclear. Patients were blinded for the approach in one of nine studies, and in none of the studies were the personnel (i.e. the surgeons) blinded. In most studies, it was unclear if the outcome assessor was blinded; only one study stated adequate blinding of the outcome assessor. Eight out of nine studies were analyzed according to the intention-to-treat

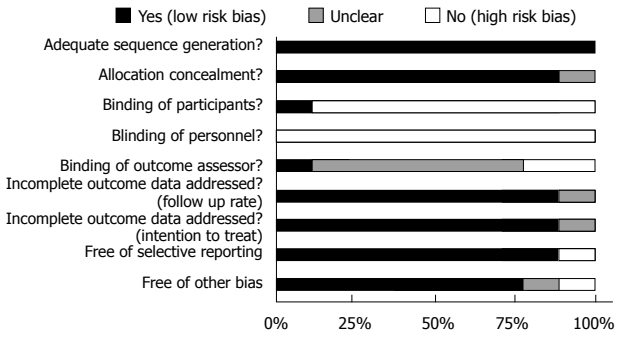

Figure 2 Assessment of risk of bias of the nine included trials.

principle; in one this was unclear. All predefined outcome parameters were reported in eight trials, and thus, free of selective reporting. Seven studies were free of other bias: baseline characteristics of the patients were comparable and treatment was similar apart from the intervention.

DESCRIPTION OF TRIALS

An overview of the included trials is given in Table 1. A total of 2263 patients (laparoscopic surgery, n = 1257; open surgery, n = 1006) were included in nine trials. Six trials reported on patients with colon or colorectal cancer and three reported on patients with diverticulitis, Crohn's disease and UC or FAP. Quality of life was a primary outcome measure in five of the trials. The following validated questionnaires were used for measuring quality of life: EORTC-C30 (4 times), SF-36 (4 times), EORTC-C38 (2 times), GIQLI (2 times) and EQ-5D, QLI, SDS and Global Quality of Life, which were all used once.

QUALITY OF LIFE

An outline of the results is shown in Table 2. The studies were heterogeneous in terms of variation in diseases treated, outcome measures, and timing of measurements. Hence, meta-analysis was not feasible. Preoperative quality of life was measured in eight of the nine studies. In all but one of these, preoperative quality of life was similar between the groups. The study of Janson *et al*[28] reported a significantly better quality of life in one of the five scales of the EQ-5D ("usual activities") for the group which was about to undergo open surgery (P = 0.006). Postoperative follow-up in the different studies ranged from 2 d to 6.7 years. Except for Weeks *et al*[30], all studies started measuring quality of life at least 1 wk after surgery.

King *et al*[33,34], Guillou *et al*[4], Jayne *et al*[35], Maartense *et al*[32], Eshuis *et al*[36], Maartense *et al*[2] and Polle[37] showed no significant differences in postoperative quality of life following open or laparoscopic colorectal surgery on short-term (1-12 wk) or long-term (3 mo to 6.7 year) follow-up.

Five studies, Janson *et al*[28], Braga *et al*[29], Weeks *et al*[30], Schwenk *et al*[31] and Klarenbeek *et al*[1], did find a significant difference in postoperative quality of life in favor of laparoscopic surgery. Janson *et al*[28] showed a significant

Table 1 Overview of included trials

Author	Trial	QoL 1˚ or 2˚ endpoint	No. of patients		Conversion rate (%)	Patients	Surgery	QoL measures	Timing of measures	
			Lap	Open					Pre operative	Post operative
Janson *et al*[28]	Color	Primary	130	155	17.7	Colon cancer	Colon resection	EORTC-C30, EQ-5D	Yes	2, 4 and 12 wk
King *et al*[33,34]		Secondary	41	19	0.0	Colorectal cancer	Colorectal resection	EORTC-C30 and C38	Yes	2 and 6 wk
		Secondary	41[1]	19[1]						3, 6 and 12 mo
Guillou *et al*[4], Jayne *et al*[35][1]	Classic	Secondary	526	268	29.0	Colorectal cancer	Colorectal resection	EORTC-C30 and C38	Yes	2 and 12 wk
		Secondary	696[2]							6, 18 and 36 mo
Braga *et al*[29]	Consort	Secondary	190	201	4.2	Colorectal cancer	Colorectal resection	SF-36	No	1, 2 and 4 yr
Weeks *et al*[30]	Cost	Primary	228	221	25.7	Colon cancer	Colon resection	QLI, SDS, Global QoL	Yes	2 d, 2 and 8 wk
Schwenk *et al*[31]		Primary	30	30	-	Colorectal cancer	Colorectal resection	EORTC-C30	Yes	1, 4 and 12 wk
Klarenbeek *et al*[1]	Sigma	Secondary	52	52	19.2	Diverticulitis	Sigmoid resection	SF-36	Yes	6 wk
Maartense *et al*[32], Eshuis *et al*[36][1]		Primary	30	30	10.0	Crohn's disease	Ileocolic resection	SF-36, GIQLI	Yes	1, 2, 4 and 12 wk
		Secondary	29[1]	26[1]						6.7 yr[3]
Maartense *et al*[2], Polle *et al*[37][1]		Primary	30	30	0.0	UC and FAP	RP & IPAA	SF-36, GIQLI	Yes	1, 2, 4 and 12 wk
		Secondary	26[1]	27[1]						1 yr[3]

[1]Long-term follow-up of same study population; [2]Number of patients included in long term follow-up, data not specified for laparoscopic or open surgery; [3]Median; -: No data available; RP & IPAA: Restorative proctocolectomy with ileo pouch anal anastomosis; QoL: Quality of life; UC: Ulcerative colitis; EORTC: European Organization for Research and Treatment of Cancer; SDS: Symptom Distress Scale; QLI: Quality of Life Index; GIQLI: Gastro Intestinal Quality of Life Index.

Table 2 Outline of results

Timing QoL measure		Pre operative	2 d	1-2 wk	4-8 wk	12 wk	6 mo	1 yr	1.5-2 yr	3-6.7 yr
Author	QoL measure									
Janson *et al*[28]	EORTC-C30	NS	-	LAP (2/15)	LAP (1/15)	NS	-	-	-	-
	EQ-5D	OPEN (1/5)	-	NS	NS	NS	-	-	-	-
King *et al*[33,34]	EORTC-C30	NS	-	NS	NS	NS	NS	NS	-	-
	EORTC-C38	NS	-	NS	NS	NS	NS	NS	-	-
Guillou *et al*[4],	EORTC-C30	NS[1]	-	NS[1]	-	NS[1]	NS[1]		NS[1]	NS[1]
Jayne *et al*[35]	EORTC-C38	NS[1]	-	NS[1]	-	NS[1]	NS[1]		NS[1]	NS[1]
Braga *et al*[29]	SF-36	-	-	-	-	-	-	LAP (2/3)	LAP (1/3)	NS
Weeks *et al*[30]	QLI	NS	-	NS	NS	-	-	-	-	-
	SDS	NS	NS	NS	NS	-	-	-	-	-
	Global QOL	NS	-	LAP	NS	-	-	-	-	-
Schwenk *et al*[31]	EORTC-C30	NS	-	LAP (9/15)	LAP (3/15)	NS	-	-	-	-
Klarenbeek *et al*[1]	SF-36	NS	-	-	LAP (4/8)	-	-	-	-	-
Maartense *et al*[32],	SF-36	NS	-	NS	NS	NS	-	-	-	NS
Eshuis *et al*[36]	GIQLI	NS	-	NS	NS	NS	-	-	-	NS
Maartense *et al*[2],	SF-36	NS	-	NS	NS	NS	-	NS	-	-
Polle *et al*[37]	GIQLI	NS	-	NS	NS	NS	-	NS	-	-

LAP: Significantly in favor of laparoscopic group; OPEN: Significantly in favor of open group; (1/5): In 1 out of 5 subscales; NS: No significant difference between laparoscopic and open surgery ($P > 0.05$); [1]$P > 0.01$; -: No data available; EORTC: European Organization for Research and Treatment of Cancer; SDS: Symptom Distress Scale; QLI: Quality of Life Index; QoL: Quality of life; GIQLI: Gastro Intestinal Quality of Life Index; SF-36: Short Form-36.

difference in favor of laparoscopic surgery in two ("social function" and "role function") and one ("social function") of 15 subscales of the EORTC-C30 questionnaire at 2 and 4 wk, respectively, following surgery. The authors also calculated the effect size (Cohen's) of these subscales: the effect size of "role function" was 0.51 (moderate) and the effect sizes of social function were 0.42 (low) and 0.38 (low) at 2 and 4 wk, respectively. In the same study, there was no difference between the open and laparoscopic group as measured with EQ-5D.

Braga *et al*[29] have measured quality of life at 1, 2 and 4 years postoperatively. Only three subscales ("general health", "physical functioning" and "social functioning") of the SF-36 were used for analysis. Two of three subscales ("physical functioning" and "social functioning") scored significantly better in the laparoscopic group at 1

year after surgery; scores on one subscale ("social functioning") were still significantly better at 2 years postoperatively, and no significant difference was found at 4 years following surgery.

Weeks *et al*[30] have reported no difference between the groups measured with the SDS at 2 d postoperatively. At 2 wk after surgery, the authors reported a significantly better outcome for the laparoscopic group on the Global QoL questionnaire; at the same time point, scores on the QLI and SDS were similar for both groups. At 8 wk postoperatively, no significant differences were found.

After 1 wk, Schwenk *et al*[31] found a significant difference in favor of laparoscopy as measured with the EORTC-C30 questionnaire. These differences were shown on four of five functional scales ("physical, emotional, social, and cognitive function"), on "global quality of life" and on four of nine symptom or single-item scales ("fatigue", "pain", "dyspnea" or "appetite loss"). After 4 wk, two of the five functional scales ("social and cognitive function") and "global quality of life" remained significantly better in the laparoscopic group. After 12 wk, quality of life scores were similar.

Klarenbeek *et al*[1] performed one quality of life measurement after 6 wk and reported a difference in four ("pain", "social functioning", "role limitations due to physical health" and "role limitations due to emotional problems") of eight dimensions of the SF-36.

CONCLUSION

This systematic review showed no substantial differences in quality of life, as measured at 2 d to several years postoperatively, between laparoscopic and open surgical procedures for colorectal disorders. In only five of the nine trials found, quality of life after laparoscopic colorectal surgery appeared slightly but significantly better during short-term follow-up compared to that with open colorectal surgery. However, this was not considered clinically relevant, because the observed differences were merely found in certain subscales at few and differing time intervals.

The clinical relevance of significant differences in quality of life is debatable. Osoba *et al*[38] have studied the outcomes of the EORTC-C30 by comparing changes in C-30 scores to a subjective significance questionnaire (SSQ). The SSQ asked patients to rate their own changes in physical, emotional and social functioning. These results were compared to the outcomes of the C-30, which resulted in a small change (5-10 points), moderate change (10-20 points) and large change (> 20 points). These results imply that statistical significance does not necessarily correlate with clinical relevance, which was illustrated in the trial of Janson *et al*[28]. In that study, a low and moderate effect size was calculated for significant differences in quality of life outcomes in the EORTC-C30. They also stated that, due to the large number of subscales analyzed in multiple tests at different assessment points in time, the finding of false-positive results is likely to occur. Hence, the relatively small differences found in this review on sets of subscales were not considered to be clinically relevant findings.

Several studies have shown that laparoscopic surgery results in less perioperative blood loss, less inflammatory response[39], and smaller incisions. Obviously, laparoscopic surgery is associated with less perioperative trauma to the abdominal wall compared to that with open surgery. Therefore, differences in quality of life are expected to be more prominent in the first week after surgery. Unfortunately, in this review no conclusions could be drawn about that period, because almost all included studies started measuring quality of life after a minimum of 1 wk. This is a possible explanation for the rare differences that we found in quality of life, which is corroborated by the fact that nearly all of the reported differences in quality of life disappeared over time. If quality of life was indeed influenced by the surgical technique, another explanation for the marginal differences we found could be that in four of the nine trials included, quality of life was not a primary outcome measure, which possibly led to an underpowered quality of life analysis. Finally, quality of life is determined by many other postoperative factors, even if baseline characteristics are similar at the time of preoperative assessment. For example, the course of the disease differs per patient and may subsequently affect quality of life.

Results from this systematic review are in accordance with recent literature. Dowson *et al*[10] have shown no significant quality of life advantages after a laparoscopic approach compared to open surgery, but also stated that there was a lack of good quality data. The authors did state that there was a possible trend of improved quality of life after laparoscopic surgery. In a Cochrane systematic review on short-term benefits for laparoscopic colorectal surgery, Schwenk *et al*[40] found that quality of life might be improved in the early postoperative course. The authors, however, were not able to present a clear conclusion due to the low methodological quality of the studies that they included. In addition to the earlier review, the present systematic review included sufficient high-level evidence to state that there was no clinical relevant difference in quality of life on short- or long-term follow-up, measured 1 wk to 6.7 years postoperatively.

A limitation of this review is the clinical heterogeneity among the included studies. Virtually every study used different quality of life instruments and did not present exact data. Furthermore, the recruited patients were treated for a range of different disorders. Therefore, it was impossible to recalculate the statistical analyses or to perform a meaningful meta-analysis. Future randomized trials that compare open with laparoscopic surgery are needed[41], and should be well-designed, sufficiently powered, and focus on quality of life; in particular, shortly after the operation, i.e. within 1 wk, in which period, most of the differences are likely to occur.

In conclusion, based on presently available high-level evidence, this systematic review showed no clinically relevant differences in postoperative quality of life between laparoscopic and open colorectal surgery.

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REVIEW

Breastfeeding and chronic HBV infection: Clinical and social implications

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Abstract

Mother-to-child transmission of hepatitis B virus (HBV) is among the most important causes of chronic HBV infection and is the commonest mode of transmission worldwide. Currently, the presence of HBsAg, HBeAg and HBV DNA in breast milk is confirmed. Several studies have reported that breastfeeding carries no additional risk that might lead to vertical transmission. Beyond some limitations, the surveys have not demonstrated any differences in HBV transmission rate regarding feeding practices in early childhood. Promotion of breastfeeding is substantial, especially for low-income individuals and regions with uncertain, unfeasible, and unsafe water supplies. Lactoferrin, minimal inflammation or activation within the infant gut during exclusive breastfeeding, and nonspecific biological molecules in the milk are identified as major factors of breast-milk defense. This review discusses preemptive antiviral therapy during pregnancy and lactation. Long-term follow up of breast-milk HBV concentrations and correlation with serum viral load; nucleos(t)ide analogue concentrations in breast milk in HBV-positive mothers in the setting of chronic HBV infection; safety of antiviral therapy during pregnancy and lactation; and the difference in viral load in the milk in exclusive or non-exclusive breastfeeding are still open questions. The paper reviews the current data and outlines the course of further investigation into this often underestimated issue.

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Key words: Breastfeeding; Lamivudine; Tenofovir; Hepatitis B virus; Chronic hepatitis B

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VERTICAL TRANSMISSION OF HEPATITIS B VIRUS

It is estimated that almost 50% of the cases of chronic hepatitis B virus (HBV) infection result from vertical transmission or are acquired in early childhood, especially in endemic areas[1]. The prevalence of chronic HBV infection in pregnant women reflects that in the general population[2]. About 5% of mothers worldwide are chronic HBV carriers[3]; ranging from 0.6% in low-endemic regions to > 20% in high-endemic areas in the Far East and Africa[4,5]. The prevalence of HBeAg in pregnant HBV-positive women exceeds 50% in some high-endemic regions[5].

Mother-to-child transmission (MTCT, or vertical transmission) of HBV is one of the most important causes of chronic HBV infection[6] and is the most common mode of transmission worldwide. Maternal screening programs and universal active and passive immunoprophylaxis of

newborn have reduced dramatically the HBV transmission rates by 95%[7]. However, vertical transmission is still responsible for a significant number of cases of chronic infection[8] and remains a serious problem. MTCT can occur prenatally, during delivery, or postpartum. HBV is found in amniotic fluid, breast milk, vaginal fluids, cord blood and infant gastric content[9,10].

A large number of vertically transmitted cases occur intrapartum due to possible transfusion of the mother's blood to the fetus during contractions, as a consequence of membrane rupture, and *via* direct contact of the fetus with infected secretions, blood or other fluids from the maternal genital tract[11]. The risk of intrauterine infection is relatively low because the fetus is protected from HBV by the placenta. However, reported vaccination failures imply *in utero* transmission especially in high-risk groups (highly viremic and/or HBeAg-positive mothers)[12,13]. Furthermore, the risk is related to the presence of HBV DNA in the placenta[14] and to maternal viremia[15], which supports an association between maternal HBV load during pregnancy and the risk of transmission to the baby.

Despite successful screening and vaccination programs, high maternal HBV DNA is correlated with perinatal transmission. Wiseman *et al*[16] have shown a transmission rate of 8.5% for infants born to mothers with virus levels > 8 $\log_{10}$ copies/mL and no transmission below this cut-off. We need additional studies to assess the potential risk reduction associated with treatment of high maternal viremia during pregnancy. A study from India[17] has found that maternal viral load > 1.5×10^5 copies/mL is associated with intrauterine transmission. This presumes that nucleos(t)ide therapy during the third trimester with lamivudine (greater experience; FDA Category C) or tenofovir and telbivudine (Category B) might be beneficial. All these data concern prevention of intrauterine and perinatal transmission in mothers with HBeAg-positive chronic hepatitis and/or high titers of HBV DNA.

Some questions arise regarding breastfeeding in cases of chronic hepatitis B. Firstly, most studies that have been concerned with vertical or early childhood transmission have not reported breastfeeding rates, or if they have, the data are still the subject of debate. Secondly, the nucleoside analogues are likely to pass into breast milk to some degree. We have not established a policy for what to do in the case of nucleos(t)ide prevention in mothers who chose to breast feed. Thirdly, we have to measure the risk of breast-milk transmission in low-income individuals and regions with uncertain, unacceptable, unfeasible, and unsafe water supplies, or replacement feeding.

Recent studies have demonstrated the short- and long-term benefits of breastfeeding both for mothers and children. Breastfeeding, especially if exclusive and prolonged, leads to significant reduction in hospitalization rates for gastroenteritis, respiratory infections, and sepsis in the early months of life[18]. The benefits for low-resource countries are even greater because breastfeeding is related to better infant and childhood survival[19]. Exclusive breastfeeding for 6 mo, followed by continued breastfeeding at least to 12 mo, could prevent 1 301 000 deaths or 13% of all child deaths under 5 years of age in a hypothetical year[20]. New findings have emerged for older children, which support the role of prolonged breastfeeding in their cognitive functions[21]. A gene related to neuronal growth that depends for its effects on breastfeeding has been found recently. Individuals who carry at least one copy of this gene have higher IQ scores, but only if they have been breast-fed[22].

For older children and adults, long-term effects most probably include a possible impact of breastfeeding on cholesterol levels, body mass index, obesity and type 2 diabetes[23-25].

The benefits for breastfeeding mothers have been well described. Shorter recovery and oxytocin-stimulated uterus contraction prevents anemia. Lactation-induced suppression of ovulation has contraceptive effect during the period of exclusive breastfeeding[26]. Lactating women seem to regain their pre-pregnant weight sooner, and in the long-term have reduced obesity risk. The risks of breast and ovarian cancer and osteoporosis are also decreased[27-29]. When considering breastfeeding, we cannot omit the positive emotions and close mother-to-infant contact.

Despite consistent global policies in support of breastfeeding for the general population and World Health Organization (WHO) recommendations, the debate regarding HBV-infected women is still open in some countries. Guo *et al*[30] recently have reported an embarrassing number of HBV-positive women who opted for bottle feeding because of concern about the risk of transmission of HBV *via* breast milk.

HBV IN BREAST MILK AND SAFETY OF BREASTFEEDING

WHO postulates that chronic HBV infection of the mother could not be an argument against breastfeeding. However, some researchers and many clinicians disapprove it based on the results of some studies that have investigated the content of viral markers in human milk. In the early 1970s, Linnemann *et al*[31] and Boxall *et al*[32] published data on HBsAg transmission in breast milk of chronically infected mothers. Later, other researchers found not only HBsAg, but also HBeAg and HBV DNA in breast milk. In addition, both colostral HBsAg and HBeAg titers correlate positively with the corresponding level in maternal blood[33]. HBV DNA was found in 81.25% of Chinese mothers if HBsAg and HBeAg were positive and in 45.24% if HBeAg was negative[34]. de Oliveira *et al*[35] have evaluated HBV DNA in first- and fourth day colostrum of 24 Brazilians before and after pasteurization and have not found a significant difference (75% *vs* 67%).

Several studies have reported that breastfeeding carries no additional risk that might lead to vertical transmission. Table 1 summarizes comparative surveys regarding the rate of MTCT according to breastfeeding (BF) or formula feeding (FF).

Although one could find limitations, these studies have failed to demonstrate differences in HBV transmission regarding feeding practices in early childhood. Even in cases of no active or passive prophylaxis, the risk of transmis-

Table 1 Safety of breastfeeding in case of chronic hepatitis B virus infection of mothers

Author	No. of infants	Population	Prophylaxis	Infected or failed seroconversion to antiHBs		*P*
				BF (%)	FF (%)	
Beasley *et al*[56]	147	USA, Taiwan (China)	No	53	60	NS
Tseng *et al*[57]	170	Hong Kong (China)	HBIG + Vx	7	6	NS
de Martino *et al*[58]	85	Italy	Vx	4.6	3.2	NS
Hill *et al*[59]	369	USA	HBIG + Vx	0	3	0.06

BF: Breastfeeding; FF: Formula feeding; HBIG: Hepatitis B immune globulin; NS: Nonsignificant.

sion from HBV-positive mothers to their breastfed infants is at least equal.

WHAT COULD BE THE POSSIBLE EXPLANATION?

Beyond HBV infection, many studies have shown considerable differences between breast-fed and formula-fed infants, in spite of the notable progress in the composition of infant formulas. Breast milk provides a number of bioactive proteins that have different physiological activities. Lactoferrin is a major human milk protein with bacteriostatic (*Enterobacter sakazaki*[36]) and bactericidal activity (*Vibrio cholerae*[37]). It has antiviral activity against hepatitis C virus, adenovirus, cytomegalovirus, herpes simplex virus, rotavirus, adenovirus and human immunodeficiency virus (HIV)[38,39]. Recently, it has been found that lactoferrin and iron- and zinc-saturated lactoferrin significantly inhibit the amplification of HBV DNA in a dose-dependent manner in HBV-infected HepG2 cells[40].

There have not been sufficient studies that can even partially explain the possible effect of breastfeeding on eventual prevention of MTCT of HBV. However, there have been some discussions about the benefits of exclusive breastfeeding for HIV transmission. Inflammation or activation within the infant gut as a result of introduction of foreign antigens, contaminants and pathogens could activate probable mechanisms that facilitate viral transmission[41,42]. Small changes in the rate of suckling or non-exclusive breastfeeding could be associated with milk stasis and breast engorgement. If not reversed in a short time, the epithelial permeability might increase (leaky tight junctions)[43]. This phenomenon allows more efficient paracellular transfer of HIV and increased HIV RNA in breast milk[44,45]. Thus, irregular or non-exclusive breastfeeding might contribute to increased infectivity of human milk. It remains unclear if the situation with HBV is similar and could be a subject of future research. This issue is of considerable importance for high-prevalence regions with low social standards where promotion of exclusive breastfeeding is vital for child morbidity and mortality.

Research into probable HBV transmission mechanisms via breastfeeding is still open. The majority of studies have not measured maternal viral load quantitatively. Furthermore, it is difficult to draw any conclusion on the speculative correlation between the rate of MTCT and the duration of breastfeeding. The studies listed in Table 1 do not include randomly assigned cohorts, thus providing possible bias if some high-risk women decided to bottle-feed. Even the most recent study of Hill has evaluated a heterogeneous breastfeeding group and does not provide data for HBV DNA in mothers and infants (see the table for reference).

Finally, the American Academy of Pediatrics has recommended that HBV infection should not be considered as a contraindication to breastfeeding of infants who receive the approved hepatitis B immune globulin (HBIG) and HBV vaccine[46].

CONTROVERSIES IN NUCLEOS(T)IDE PROPHYLAXIS OF MTCT

Preemptive antiviral therapy[47] during pregnancy reflects an attempt to prevent intrauterine and perinatal transmission of HBV to newborn infants. However, some controversies remain, namely, when to start the therapy and for how long. The period immediately after birth is a time of treatment uncertainty in mothers who choose to breast-feed, because nucleoside analogues are likely to pass into the breast milk to some degree, and it is probably unwise to expose the child in this manner[47]. The pharmacokinetics of lamivudine are similar in patients with HIV or HBV and healthy volunteers. The absolute bioavailability of the drug is approximately 82% and 68% in adults and children, respectively. In pregnant women, concentrations in maternal serum, amniotic fluid, umbilical cord and neonatal serum are comparable, which indicates that the drug diffuses freely across the placenta[48]. Breast-milk to plasma concentration ratio has been determined at 2.96 for lamivudine, measured with high-performance liquid chromatography and tandem mass spectrometry[49]. Lamivudine has not been studied in HIV-negative nursing mothers who are being treated for hepatitis B infection, but the low doses used would not be expected to cause any serious adverse effects in breastfed infants. A recent study of maternal antiretroviral treatment from gestational week 34 to 6 mo postpartum has measured infant lamivudine concentrations at biologically significant concentrations. The median concentrations were 67 μg/L at delivery, 32 μg/L at week 2, 24 μg/L at week 6, 20 μg/L at week 14, and not measurable (< 16 μg/L) at week 24 postpartum[50]. Another study[51] has shown that neutropenia was 15.9% in the HAART-exposed infants at 1 mo of age compared to 3.7% in an unexposed group. Hematological toxicity was

transient and asymptomatic. The authors did not observe any difference in haematological and hepatic toxicity between breastfed and formula-fed infants from 2 to 6 mo postpartum[51]. Supported by the studies concerned with HIV transmission, we might reconsider stopping the preemptive antiviral therapy during BF if there is a high risk of HBV hepatitis flare. We also have to keep in mind the possible selection of resistant strains in case of suboptimal dosage in infected infants and the risk of adverse events.

After tenofovir had been studied in humans, it was classified by the US FDA in group B, thus presenting another treatment option during pregnancy. There are still no data to elucidate its transfer and concentration in human milk. An animal study with two nursing rhesus macaques has found that tenofovir is transferred to breast milk, but the peak concentrations were approximately 2%-4% of those detected in serum, with milk area under curve values being approximately 20% of the serum values[52]. For breastfeeding mothers who are taking the orally bioavailable prodrug tenofovir disoproxil fumarate, breast milk is expected to contain almost exclusively the parental compound tenofovir. It exhibits low oral bioavailability in animals and is expected to show low oral bioavailability after ingestion by nursing animals[53]. The small amounts of tenofovir in the milk are also very unlikely to select for resistance in already infected infants, thus preserving future treatment options[52]. To the best of our knowledge, a relevant study regarding drug levels in human breast milk and possible effects in breastfed infants has still not been published.

Breastfeeding seems not interfere with the immune response to the HBV vaccine. Wang *et al*[54] have followed up 230 infants for 1 year to assess the influence of breastfeeding on the efficacy of hepatitis B immunoprophylaxis. The rate of anti-HBs was 89.9% in the breastfed, compared to 73.2% in formula-fed infants among those that received HBV vaccine alone. In those who received a combination of HBV vaccine and HBIG, the anti-HBs levels were found to be similar (90.9% *vs* 90.3%).

When we compare inexpensive breastfeeding to formula feeding, we always have to remember the disturbing phenomenon of current industry practices. Bekelman *et al*[55] have confirmed the "sponsorship bias" in pharmaceutical research; their meta-analysis found industry sponsorship was associated with an OR of 3.6 (95% CI: 2.63-4.91) in favor of a pro-industry conclusion.

Finally, we encourage further studies to investigate urgently some hot topics in breastfeeding and HBV infection. These include the long-term follow-up of breastmilk HBV concentrations and correlation with serum viral load; the nucleos(t)ide analogues concentrations in breast milk in mothers with chronic HBV infection; the safety of tenofovir during pregnancy and lactation in chronic hepatitis B; and not least, the difference in viral load in the milk in exclusive and nonexclusive breastfeeding.

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ORIGINAL ARTICLE

Daily genetic profiling indicates JAK/STAT signaling promotes early hepatic stellate cell transdifferentiation

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Abstract

AIM: To identify signaling pathways and genes that initiate and commit hepatic stellate cells (HSCs) to transdifferentiation.

METHODS: Primary HSCs were isolated from male Sprague-Dawley rats and cultured on plastic for 0-10 d. Gene expression was assessed daily (quiescent to day 10 culture-activation) by real time polymerase chain reaction and data clustered using AMADA software. The significance of JAK/STAT signaling to HSC transdifferentiation was determined by treating cells with a JAK2 inhibitor.

RESULTS: Genetic cluster analyses, based on expression of these 21 genes, showed similar expression profiles on days 1-3, days 5 and 6, and days 7-10, while freshly isolated cells (day Q) and day 4 cells were genotypically distinct from any of the other days. Additionally, gene expression clustering revealed strong upregulation of interleukin-6, JAK2 and STAT3 mRNA in the early stages of activation. Inhibition of the JAK/STAT signaling pathway impeded the morphological transdifferentiation of HSCs which correlated with decreased mRNA expression of several profibrotic genes including collagens, α-SMA, PDGFR and TGFβR.

CONCLUSION: These data demonstrate unique clustered genetic profiles during the daily progression of HSC transdifferentiation and that JAK/STAT signaling may be critical in the early stages of transdifferentiation.

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Key words: Cluster analysis; Fibrosis; Genetic profile; Hepatic stellate cell; Interleukin-6

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INTRODUCTION

Hepatic stellate cells (HSCs) play an important role in the development of liver fibrosis. Following exposure to a fibrogenic stimulus (e.g. virus, toxins, alcohol), the quiescent HSC transdifferentiated into an activated myofibroblast-like cell. During this process the HSC undergoes morphological changes (i.e. stellate cell to a stretched-polygon morphology), becomes hypercontractile and increases expression of fibrillar collagens and cytokines[1]. Increased collagen deposition leads to accumulation of scar matrix,

the major cause of liver dysfunction during hepatic fibrosis[2]. The transdifferentiation process, while very difficult to monitor *in vivo*[3], can be seen and studied *in vitro*. HSCs *in vivo* undergo transdifferentiation when exposed to an altered microenvironment (e.g. increased type I collagen deposition as seen in fibrosis). This process can be mimicked *in vitro* by culturing these cells on a plastic substrate. Several groups have performed microarray analyses on both *in vitro* and *in vivo* HSC activation[4,5]; however, little is known about the daily genetic alterations that occur. To understand this complex process, it is necessary to know the sequential activation of key genes, as well as the rise and fall of expression levels. Therefore, based on known gene expression profiles of the quiescent and activated HSC, several genes were selected to follow the transdifferentiation process throughout.

HSCs are an important source of cytokines, and cytokine cross-talk is the main pattern of cellular communication in the injured liver. Specifically, continual wound healing perpetuated by HSC transdifferentiation is associated with increased interleukin-6 (IL-6) expression, an important cytokine involved in the acute phase response observed post liver injury[1]. IL-6 initially binds to specific receptor IL-6R (gp80) and subsequently two molecules of gp130 are recruited leading to activation of down-stream signaling. Classically, for induction of pro-inflammatory target genes, canonical JAK/STAT signaling is activated leading to increased inflammation as well as degradation of ECM[6]. Signaling pathways such as the MAP kinase (MAPK) pathway are also transduced with the activation of soluble IL-6R[7]. However, studies have shown that JAK/STAT signaling is the primary pathway for up-regulation of pro-inflammatory mediators/genes during acute phase response Ⅱ, the body's innate immune response provoked as a result of liver injury[7]. JAK/STAT downstream signaling affects expression of numerous genes including those involved in cellular proliferation and migration. Additionally, JAK/STAT signaling is associated with down-regulation of anti-apoptotic genes, including BCL-2 family proteins[8]. Stimulation of proliferative pathways (MAPK) and increased cellular differentiation by JAK/STAT signaling promotes the fibrotic response and leads to increased activation of HSCs[2]. Additionally, our lab has shown (unpublished data; Schrum lab) that JAK/STAT signaling increases collagen expression at both mRNA and protein levels supporting that this pathway is critical in modulating fibrosis.

To determine the daily genetic profile during normal transdifferentiation in HSCs, the expression of a mini-array of 21 genes (including members of the IL-6 JAK/STAT signaling pathway) across 10 d in culture was examined. Our results clearly demonstrate unique genetic profiles during different days of transdifferentiation and select days of activation showed similar patterns of gene expression. Results of the genetic and day cluster analyses suggest responsiveness of the cell to different signals will depend upon the temporal state of transdifferentiation. Inhibition of JAK/STAT signaling impeded the progression of HSC transdifferentiation as assessed morphologically and by gene expression. Thus, our data indicate that JAK/STAT signaling may play a key role in the initiation of HSC transdifferentiation and that the changes in gene expression during a precise time period within the activation phase may determine the response of the HSC during this process.

MATERIALS AND METHODS

HSC isolation and culture

Primary HSCs were isolated from male Sprague-Dawley retired breeder rats (> 600 g) (Charles River, Raleigh, NC, USA). *In situ* liver perfusion using a pronase (Roche Molecular Biochemicals; Chicago, IL)/type I collagenase (Sigma-Aldrich; St. Louis, MO, USA) digestion was performed followed by Optiprep (Axis-Shield; Oslo, Norway) density gradient centrifugation. Cells were recovered at approximately 95% purity based on autofluorescence and washed with Gey's Balanced Salt Solution (GBSS: 137 mmol/L NaCl, 2.7 mmol/L $NaHCO_3$, 5.0 mmol/L KCl, 1.5 mmol/L $CaCl_2$-$2H_2O$, 1.0 mmol/L $MgCl_2$-$6H_2O$, 0.7 mmol/L Na_2HPO_4, 0.2 mmol/L KH_2PO_4, 0.3 mmol/L $MgSO_4$-$7H_2O$, 5.5 mmol/L glucose, 25 mmol/L HEPES) and either used immediately (termed: freshly isolated, Q or quiescent) or cultured on plastic using Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mmol/L L-glutamine (Gibco), 100 units penicillin/mL, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO_2 humidified atmosphere at 37℃. Growth media was changed every 2 d unless otherwise noted. All animal procedures were performed under the guidelines set by the University of North Carolina at Charlotte Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Treatment of HSCs

HSCs were either harvested immediately (day Q or quiescent) or were grown in culture for the designated number of days (every 24 h from the time of plating is considered 1 d). Cells were treated continually with 100 μmol/L AG490, a JAK2 inhibitor (CalBiochem, San Diego, CA, USA). Media during treatments was changed every 24 h.

Isolation of RNA and quantitative polymerase chain reaction

Total RNA was isolated from HSCs using TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA). RNA isolated from Q through day 3 was then cleaned using RNEasy Clean-Up (Qiagen) following the manufacturer's recommendations. Total RNA was reverse-transcribed using Superscript Ⅱ reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's recommendations. Quantitative polymerase chain reaction (qPCR) was run at 94℃ for 15 s; 58℃ for 25 s; 72℃ for 20 s, and read for 5 s (Table 1). The reaction mixture consisted of 1 μL each of cDNA, forward and reverse primers at 5 nmol/L, 2 μL DEPC water, and 5 μL of SYBR Green Master Mix

Table 1 Primers used in this study

Gene	Forward	Reverse	Size (bp)	Read (℃)
β-actin	GAGCTATGAGCTGCCTGACG	GGATGTCAACGTCACACTTC	154	80
Collagen α1(I)	CACTGCAAGAACAGCGTAGC	ATGTCCATTCCGAATTCCTG	200	82
Collagen α2(I)	AAGGCATTCGAGGACACAAC	TTACCAACAGGCCCAAGTTC	296	84
Cyclin D1	TGGAAAGAAAGTGCGTTGTG	TGCAAATGGAACTGCTTCTG	291	82
Desmin	TACACCTGCGAGATTGATGC	ACATCCAAGGCCATCTTCAC	209	81
Focal adhesion kinase	TTACCCAGGTCAGGCATCTC	GGAATCGCTCTTCCTTTTCC	218	80
Glyceraldehyde 3 phosphate dehydrogenase	ATCCCGCTAACATACCCTGG	ACTGTGGTCATGAGCCCTTC	292	80
Glial acidic fibrillary protein	AGAAAACCGCATCACCATTC	TTGGGCCTAGCAAACAAGAC	264	81
Hypoxanthine-guanine phosphoribosyltransferase	CGCCAGCTTCCTCCTCAG	CCAGCAGGTCAGCAAAGAAC	288	80
Interleukin 6	CCCACCAGGAACGAAAGTC	TTTTCTGACAGTGCATCATCG	240	76
Interleukin 6 receptor/gp80	CAGGTGCCTTGCCAGTATTCT	AACTGACTTTGAGCCAACGA	228	79
Janus Kinase 2	GGTGCCCTAGGATTTTCTGG	CGACCAGCACTGTAGCACAC	284	79
Matrix metalloproteinase 13	CATACGAGCATCCATCCCGAGAC	GCATCTACTTTGTCGCCAATTCC	290	78
Platelet-derived growth factor receptor β	ATGCAGTGCAGACTGTGGTC	CCGTGGTCATTCACACTCAC	247	82
Peroxisome proliferator-activated receptor γ	CGAGGACATCCAAGACAACC	CCGTCTTCTTGATCACATGC	161	81
Ras homolog gene family, member A	CGGAATGATGAGCACACAAG	GCACCCCGACTTTTTCTTC	207	80
Smooth muscle α-actin	CATCAGGAACCTCGAGAAGC	TCGGATACTTCAGGGTCAGG	247	81
Signal transducer and activator of transcription 3	TATCTTGGCCCTTTGGAATG	GTGGGGATACCAGGATGTTG	284	80
Transforming growth factor β	ATGACATGAACCGACCCTTC	TGGTTGTAGAGGGCAAGGAC	283	82
Transforming growth factor β receptor 1	GCTCCTGGGTGTTTTGGAG	CCAGCTCCTTCACCCTACAG	288	80

(Qiagen). The primers listed in Table 1 were all designed for rat. cDNA concentration was used as a reference to normalize samples since the expression of housekeeping genes was modulated through days in culture. Data were reported as cross-point, the point at which the detectable level of SYBR green fluorescence was detected above the background. All experiments were performed a minimum of three times, as noted.

Microscopy

For microscopic images, cells were visualized with transmission light microscopy at 200 × magnification. A second exposure was taken under fluorescent light with a DAPI filter to image the fluorescent retinol esters within the HSC using an Olympus IX71 microscope (Olympus America, Inc.; Hamburg, Germany). The white light image was overlaid with the fluorescent image to produce the final image.

Statistical analysis

Data are presented as mean ± SE. One way repeated measures ANOVA was used for determination of statistical significance between the control and treatment groups using SigmaStat version 2.0. A P value of less than 0.05 was considered significant. AMADA 2.0.7 software was used to perform cluster analyses using Spearman correlation and average linkage[9].

RESULTS

Gene expression profile time-course

To generate a daily profile of gene expression, 21 genes were selected as representatives of cellular behavior exhibited by HSCs. They included standard housekeeping genes (β-actin, G3PDH, HPRT), markers of quiescence [GFAP, peroxisome proliferator activated receptor γ (PPARγ)], markers of activation (SMA, Desmin), matrix remodeling genes [col α1(I), col α2(I), MMP13], mitotic and migratory associated genes (CycD, FAK, RhoA, PDGFR), and profibrotic cytokines, including the IL-6 JAK/STAT signaling pathway (IL-6, IL6R, JAK2, SOCS3, STAT3, TGFβ, TGFβR). Total RNA was harvested at day Q and days 1-10 at exactly 24 h intervals (n = 4) and converted to cDNA for quantitative PCR analysis. These data were graphed as raw mRNA expression based on cycle number and subsequently normalized to total cDNA concentration. In order to examine relative fluctuations in gene expression, all genes were normalized to day Q (Figure 1A).

Since wide variations existed in mRNA expression of G3PDH, HPRT and β-actin based on total cDNA content (Figure 1A), a housekeeping gene was not used for normalization. For example, when IL-6 was normalized to a housekeeping gene (G3PDH, β-actin or HPRT), large variations in gene expression were observed (Figure 1B). Therefore, total cDNA concentration was used for normalization to quantitate daily changes in gene expression.

Due to the extensive amount of data generated from the mini-array, AMADA software was used to detect significant relationships in gene expression patterns. Genes with similar expression patterns over 10 d of culture were clustered together (Figure 2A). Genes incorporated in the same bracket have a more comparable expression pattern than those outside that bracket. The degree of correlation decreases with the distance between bifurcations[10]. Further examination identified that SMA and collagen α1(I) and α2(I) were similarly regulated, while FAK, TGFβR and desmin displayed a similar yet not identical regulation as they were positioned on adjacent brackets. Conversely, GFAP, IL-6, JAK2, MMP13 and SOCS3 had distinctly different expression patterns as they were separated by multiple bifurcations. Additionally, PPARγ demonstrated the least amount of relatedness to any other gene. Interestingly, a majority of IL-6 signaling pathway constituents

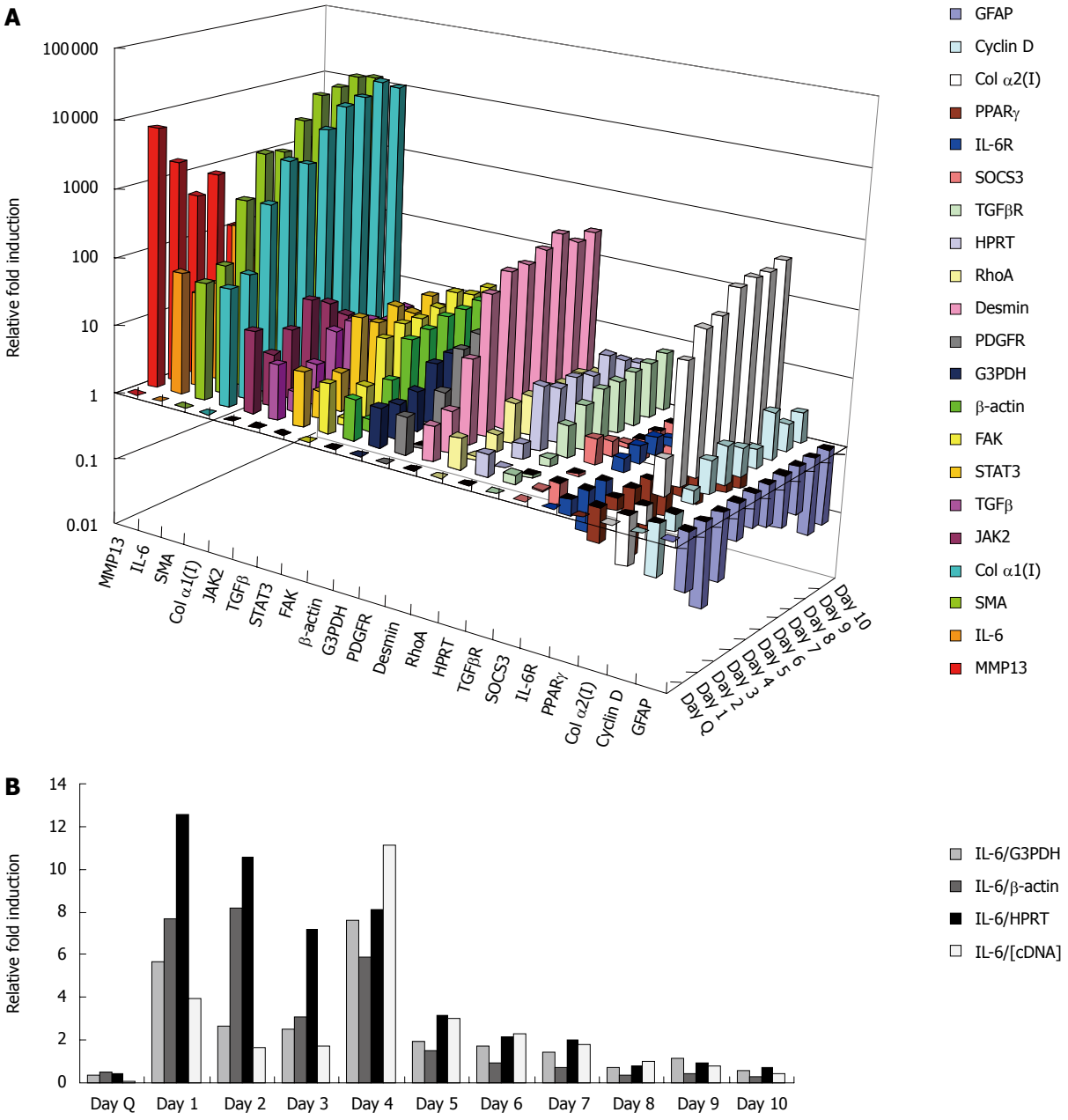

Figure 1 Gene expression profile time course. A: Mini-array of 21 genes across 10 d in culture as analyzed by real time polymerase chain reaction. Results are averages from three separate experiments. Quiescent time point was set to 1 for all genes; B: Classic housekeeping markers (G3PDH, β-actin and HPRT) fluctuated over days in culture activation, thus they served as unreliable normalization markers. IL-6: Interleukin 6; IL-6R: Interleukin 6 receptor; SOCS: Suppressors of cytokine signaling protein; TGF: Transforming growth factor; STAT: Signal transducers and activators of transcription; PPAR: Peroxisome proliferator activated receptor; FAK: Focal adhesion kinase; JAK: Janus kinase 2; SMA: Smooth muscle α-actin; MMP: Matrix metalloproteinase.

(IL-6, JAK2, SOCS3) were clustered together indicating a high degree of correlation in expression pattern, while STAT3 and IL-6R exhibited a divergent expression pattern from these three IL-6 signaling components.

Clustering software was utilized to demonstrate which days of culture-activation were most closely linked based on gene expression profile (Figure 2B). Day Q is distinct from all other days in culture. Days 1, 2 and 3 were closely linked, with day 1 having a more comparable gene expression profile to day 2 than day 3. Days 5 and 6 were also well-coupled as were days 7-10; however, as in all hierarchal clustering dendrograms, ordering within the bracket is arbitrary and thus does not contribute to the relatedness. Day 4, like day Q, exhibited a distinct expression profile that did not directly correspond with other days in culture indicating that these days may be important cellular transition points.

Markers of gene expression

After examining gene expression by mini-array, we further dissected distinct markers of quiescence (GFAP, PPARγ; Figure 3A), markers of early activation (MMP13, IL-6; Figure 3B), markers of late activation (SMA, Desmin, col α1(I), col α2(I); Figure 3C), profibrotic markers (TGFβ, TGFβR, MMP13, PDGFR; Figure 3D) and constituents of the IL-6 JAK/STAT signaling pathway (IL-6, IL6R,

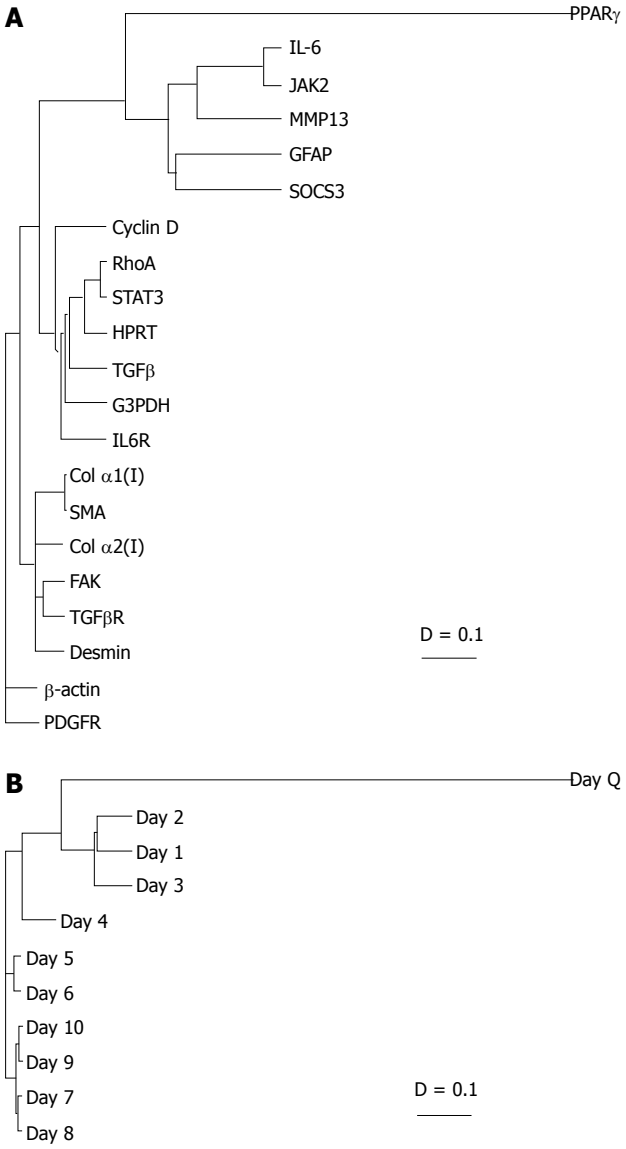

Figure 2 Clustered genetic and day analyses. A: The genes were grouped based on the degree of relatedness in their expression patterns over time using AMADA software. Distance relationships were calculated using Spearman rank correlation (D = 0-2). The shorter the distance between two genes, the more similar the expression profile over days in culture; B: The clustering tree demonstrated which days in culture were most coordinately regulated. As expected, day Q branches alone, while culture-activated HSCs are more closely regulated. Unexpectedly, day 4 also branched alone. IL-6: Interleukin 6; SOCS: Suppressors of cytokine signaling protein; TGF: Transforming growth factor; STAT: Signal transducers and activators of transcription; PPAR: Peroxisome proliferator activated receptor; FAK: Focal adhesion kinase; JAK: Janus kinase 2; SMA: Smooth muscle α-actin; MMP: Matrix metalloproteinase.

JAK2, SOCS3, STAT3; Figure 3E). Classic quiescent markers were clustered closely and decreased steadily over days in culture (Figure 3A), while markers of early activation increased sharply on day 1 and fell to basal levels over time (Figure 3B). Consistent with previous findings in our lab, we observed an approximate 10^3-fold induction in MMP13 mRNA and a 100-fold induction in IL-6 mRNA expression from day Q to day 1. Markers of HSC activation increased steadily for the first seven days of activation then plateaued (Figure 3C). PDGFR, TGFβ and TGFβR expression increased after day 2 and remained constant (Figure 3D). Notably, five genes in the IL-6 JAK/STAT signaling pathway were coordinately regulated during early HSC transdifferentiation (Figure 3E). Although differences in gene expression magnitude existed, the overarching pattern remained consistent. These data suggest that the IL-6 JAK/STAT signaling pathway may be linked to regulation of HSC transdifferentiation which warrants further investigation.

JAK/STAT pathway in early HSC transdifferentiation

Since gene expression analysis indicated early transient spikes in IL-6, JAK2 and STAT3 mRNA (Figure 3E), the contribution of this signaling pathway to the initiation of HSC transdifferentiation was examined. A specific JAK2 inhibitor, AG490, was used to block JAK/STAT signaling within the cell to determine if inhibition of this pathway could alter changes seen in transdifferentiation. Cells were exposed continually to AG490 over a five day period and assessed morphologically at days 1, 3 and 5. Culture-activated HSCs lose retinol esters and cytoplasmic processes, and proliferate vigorously. As indicated by black arrows in Figure 4, cytoplasmic processes were only evident at day 1 in control cells compared to day 3 and 5 which exhibited stretched-polygon morphology characteristic of the activated phenotype. However, HSCs treated with AG490 retained these cytoplasmic processes over five days in culture indicating an inhibition of the activated phenotype and suggesting the importance of the JAK/STAT signaling pathway in early HSC transdifferentiation.

AMADA software was again used to cluster days based on gene expression profiles of the AG490-treated cells (Figure 5). Cluster analysis indicated that the expression profile of the HSCs treated with the inhibitor on days 1, 3 and 5 shared a high degree of relatedness to day Q as opposed to later days in culture. Specific gene expression associated with HSC activation was also examined. Inhibiting the JAK/STAT pathway with AG490 clearly showed a significant decrease in expression of markers of activation (collagen, SMA, PDGFR and TGFβR) thereby impeding early HSC activation (Figure 6). Day 3 showed the most prominent reduction in gene expression compared to day 1 and day 5. Even though significant decreases in gene expression were observed on day 5, a less dramatic effect was seen compared to day 3 suggesting that JAK/STAT may be involved in early transdifferentiation and plays a lesser role during later stages.

DISCUSSION

The process of HSC transdifferentiation is key in understanding and eventually ameliorating liver fibrosis. During the course of HSC transdifferentiation there are several genes/proteins that are altered in expression. Genes involved with lipid/vitamin A regulation[11-13], such as PPARγ[14,15], are lost, while cytoskeletal genes are shifted, decreasing glial fibrillary acidic protein (GFAP) but increasing desmin and smooth muscle α-actin (SMA)[16-19]. Manipulation of PPARγ expression by adenoviral expression

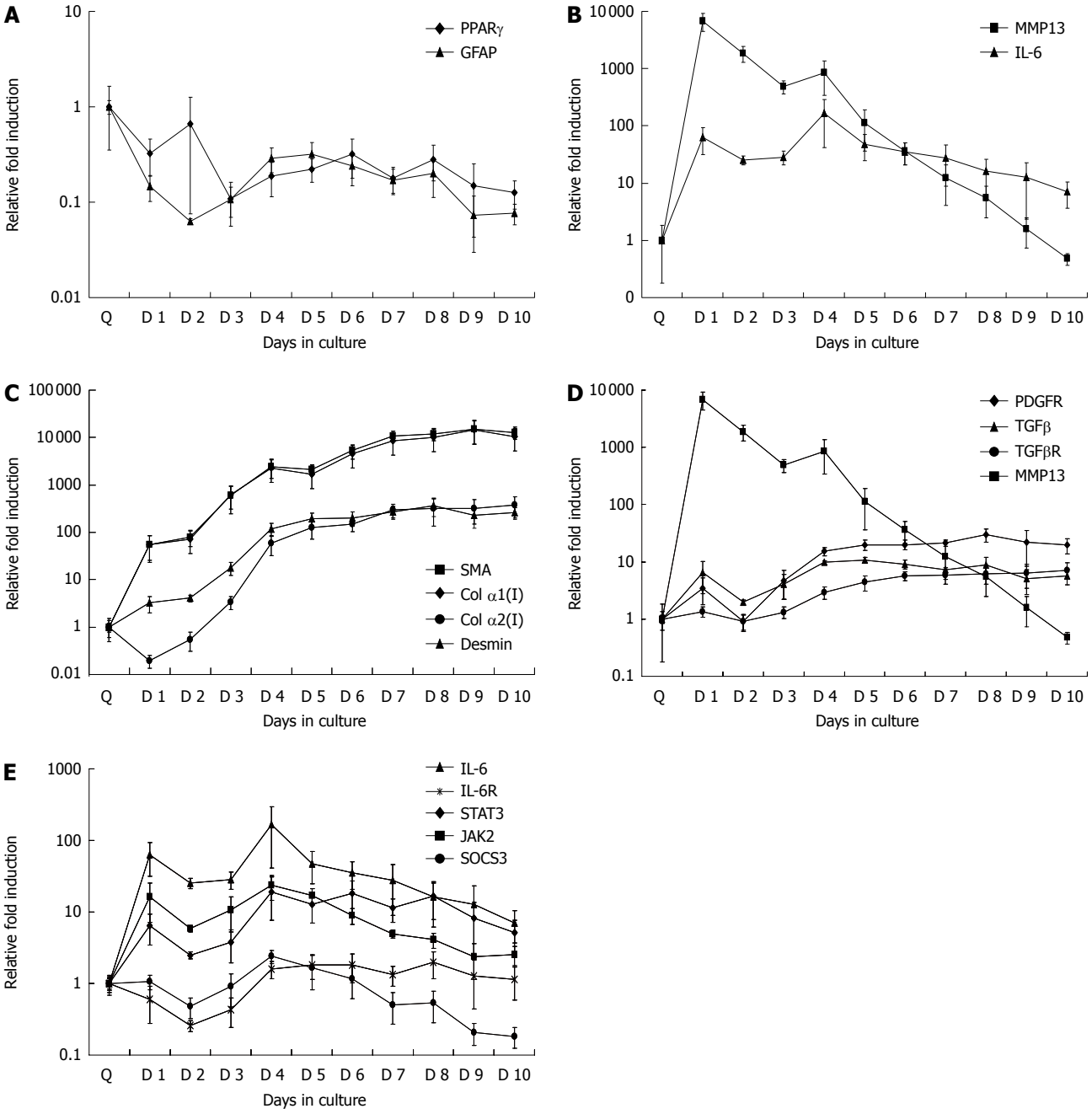

Figure 3 Gene expression markers during different stages of hepatic stellate cells activation. A: Markers of quiescence, early and late activation, fibrosis and the IL-6 signaling pathway (A-E, respectively) were examined over 10 d in culture. Real time polymerase chain reaction was used to analyze gene expression. Fold induction was relative to day Q expression levels. Data are presented as mean ± SE. IL-6: Interleukin 6; IL-6R: Interleukin 6 receptor; SOCS: Suppressors of cytokine signaling protein; TGF: Transforming growth factor; STAT: Signal transducers and activators of transcription; PPAR: Peroxisome proliferator activated receptor; JAK: Janus kinase 2; SMA: Smooth muscle α-actin; MMP: Matrix metalloproteinase.

in activated cells leads to a reversal of the activated phenotype (flattened polygonal morphology with prominent actin stress fibers) to the quiescent phenotype (retracted cytoplasm and appearance of processes)[20]. These phenotypic changes are also correlated with decreased expression of HSC activation marker genes such as collagen and TGFβ[14]. Extracellular matrix components change from normal basement matrix components, such as type IV collagen, to a fibrotic matrix, including type I collagen[21-23]. In all previous studies, quiescent HSCs or HSCs in early activation have been compared to activated cells. Although continuous changes throughout the transdifferentiation process have been observed, changes in gene expression/profiles have not been tracked in a daily manner defining which days show similar phenotype and which days serve as a transition. While individual components of HSC phenotypes have been identified, there has been no concerted attempt to demonstrate the interplay of relevant genes as they coordinate the transition from a quiescent to a fully activated phenotype.

The initial stimulus of the transdifferentiation process has not been identified; however, several cytokines includ-

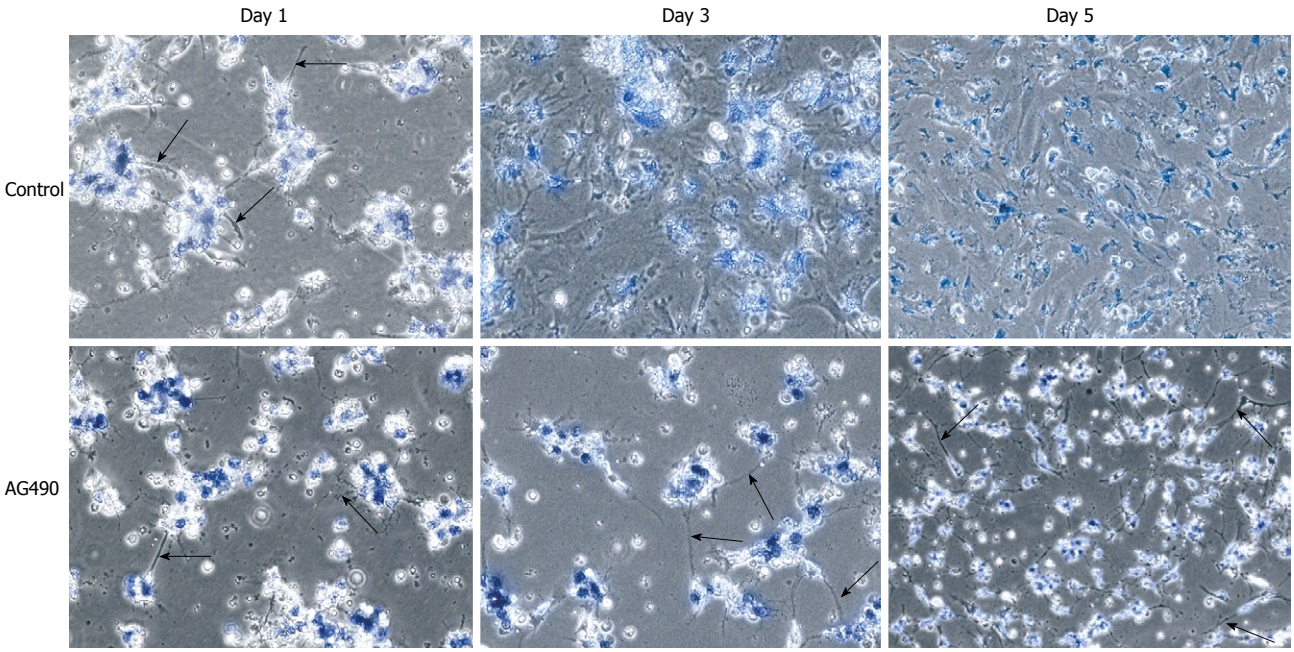

Figure 4 Inhibition of JAK/STAT signaling pathway: morphological alterations. Culture activated hepatic stellate cells were treated continually with AG490 throughout a five-day period and subsequently analyzed morphologically at days 1, 3 and 5. Cells treated with AG490 maintained their cytoplasmic projections (black arrows) throughout the five days of culture activation, whereas the control cells lost these processes as early as day 3.

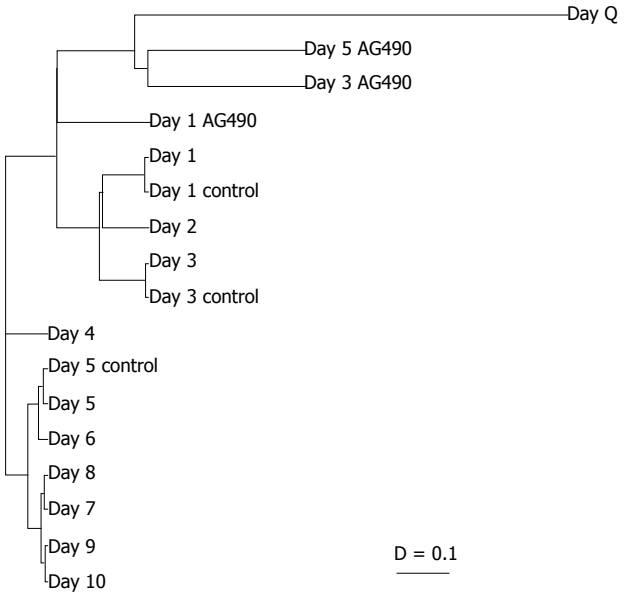

Figure 5 Inhibition of JAK/STAT signaling pathway: cluster analyses. AMADA software was used to detect any relationships between the days in culture treated with or without AG490 over a five-day period. Distance relationships were calculated using Spearman rank correlation (D = 0-2). The shorter the distance between two genes, the more similar the expression profile over days in culture. Data from the treated cells (days 1, 3 and 5) were incorporated with the previous data for days in culture (day Q-day 10) to demonstrate days of similarity based on gene expression. The clustering showed that the gene expression profile of HSCs treated with AG490 was highly related to the expression profile of the day Q hepatic stellate cells.

ing TGFβ, TNFα and IL-6 have been implicated[24]. Several labs have previously used microarray analysis to examine differential expression of genes in HSCs[4,25]. However, these studies examined two or three specific time points, rather than daily changes or gene relationships that could play a role in the initiation of transdifferentiation. In a study by De Minicis *et al*[4], HSCs cultured for 20 h represented the quiescent phenotype and day 5 culture-activated cells were considered fully activated. In another study, days 0, 4 and 7 were the selected populations[25]. Furthermore, other studies examined cells at days 0 and 15 along with a third time point where cells were cultured until they had been passaged six or seven times[26]. The aforementioned studies are not consistent with the days HSCs are considered to be quiescent (ranging from freshly isolated to day 1 or 2) or activated (ranging from days 5 to 15 of culture-activation). Varying degrees of quiescence and activation can lead to inconsistent results and misinterpreted data. None of these studies followed the transdifferentiation process on a daily basis. Therefore, these previous studies could not pinpoint transitions and steady-state profiles. Additionally, because of the multitude of time points, data generated in these studies are not comparable to each other. Therefore, for the first time, our study analyzed an array of 21 genes over consecutive days in culture to identify candidate signaling pathways and genes which peak in expression to initiate and commit the HSC to activation/transdifferentiation.

Using AMADA software, genotypic clustering was performed to profile genes that are modulated in quiescence, early and late activation, and profibrotic conditions. This analysis demonstrated that day Q was clearly divergent from all other days during transdifferentiation, while days 1-3 were significantly different from days 5-10 (Figure 2B). Furthermore, day 4 was genetically distinct from other days in culture, suggesting this may be the commitment point to the activated phenotype. Although no dramatic changes

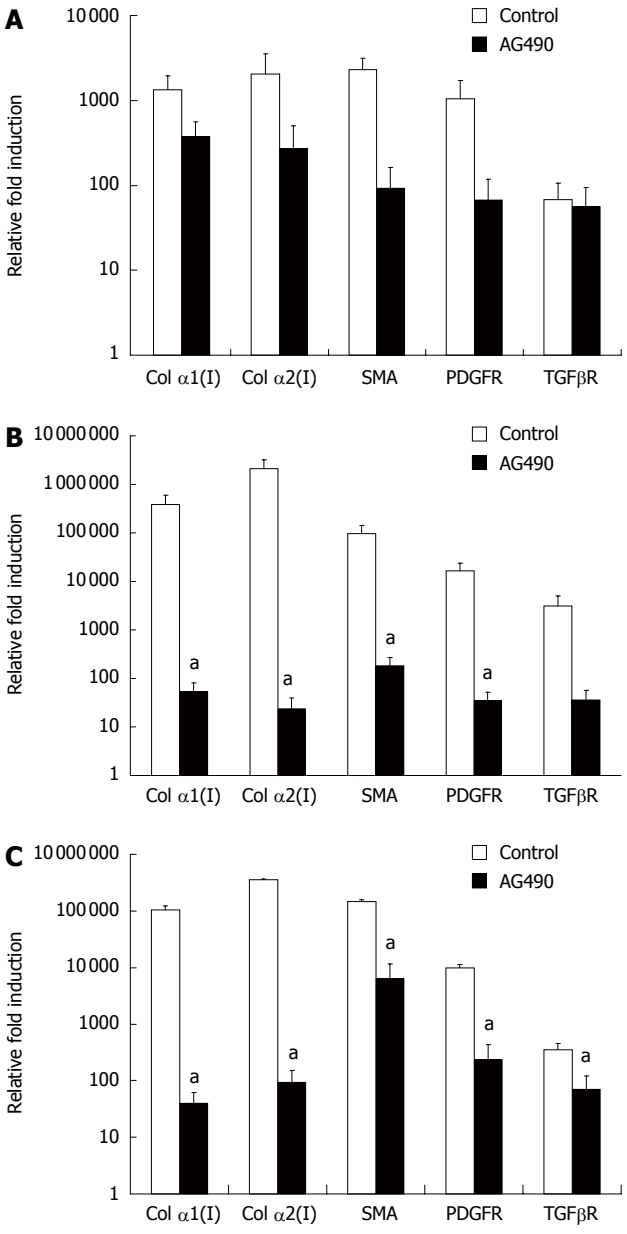

Figure 6 Inhibition of JAK/STAT signaling pathway: genetic alterations of fibrotic markers. mRNA expression of fibrotic genes from days 1, 3 and 5 (A-C, respectively) culture-activated hepatic stellate cells treated with or without AG490 were analyzed by real time polymerase chain reaction. Data were presented as mean ± SE. $^{a}P < 0.05$ compared to control.

in gene expression were seen at day 4, commitment to expression patterns was observed suggesting that day 4 is a critical point of transition (Figure 3). Additionally, components of the IL-6 JAK/STAT signaling pathway appeared to be crucial in the first 72 h of HSC transdifferentiation.

Our results also demonstrated that blocking the JAK/STAT signaling pathway inhibited phenotypic changes seen during activation as indicated by retention of cytoplasmic projections (Figure 4) and significantly decreased profibrotic gene expression (Figure 6). JAK/STAT signaling can be transduced by numerous factors/ligands including IL-6, leptin and interferon γ (IFN-γ) which are also associated with HSC activation[24,27].

IL-6 is upregulated in liver disease, with positive effects on liver regeneration and protection from hepatotoxins. IL-6 is an important cytokine in the regulation of immune and acute phase responses during bacterial infections or damage[28]. It can be synthesized by a variety of liver cells, including Kupffer cells (KCs), hepatocytes[29] and HSCs[30-33], as well as infiltrating lymphocytes, monocytes/macrophages, endothelial cells, smooth muscle cells and fibroblasts[28,34]. Conversely, IL-6 signaling can also be detrimental. In the case of liver fibrosis, it is profibrotic causing perpetual type I collagen stimulation by HSCs[28]. Thus, fibrogenic effects of autocrine and paracrine IL-6 signaling and the specific contribution of each on initiation of HSC activation and perpetuation warrants further investigation.

The profibrotic hormone, leptin, binds to OB-Rl transducing its signal through the JAK/STAT complex. Culture-activated but not quiescent HSCs express leptin[35] resulting in significant increases in α2(I) collagen mRNA[36] and suppression of PPARγ[37]. Inhibition of JAK2 with AG490 impeded leptin signaling thereby ameliorating the fibrotic response[38]. In contrast, IFNγ is considered to be anti-fibrotic and also signals through the JAK/STAT pathway. The profibrotic cytokine, TGFβ, increases ECM deposition from activated HSCs contributing to disruption of liver architecture in fibrosis. IFN-γ has been shown to abrogate the activation of TGFβ and diminish the excessive wound healing response[39]. While it is clear that JAK/STAT signaling is important in the fibrotic response of HSCs, the specific ligand initiating transdifferentiation is yet to be elucidated. Numerous studies have demonstrated that signaling through the MAP kinase (MAPK) pathway leads to increased HSC proliferation promoting the fibrotic response[2,7]. However, JAK/STAT signaling is the primary pathway for up-regulation of pro-inflammatory mediators/genes during liver injury[7], and no studies to date have examined the significance of JAK/STAT signaling during early HSC transdifferentiation.

We describe here that freshly isolated HSCs are distinct from all days of culture-activation and that these cells should not be equated with or used interchangeably with early days in culture. Based on our daily genetic profile assessment, the day of culture-activation used could significantly impact studies examining different cellular processes including transdifferentiation, proliferation, gene expression and migration of HSCs. It would be interesting to determine if other fibrogenic stimuli such as alcohol or acetaldehyde could also initiate HSC activation through JAK/STAT, particularly given that commitment to the activated phenotype perpetuates the fibrotic response. Overall, these data demonstrate that the daily changing genetic profile of the HSC results in differentiation into a unique phenotype rendering the cell sensitive to ligands which signal through JAK/STAT. Since inhibition of the JAK/STAT signaling pathway impedes the progression of HSC transdifferentiation/activation, this may serve as a potential therapeutic target to inhibit or slow the development of liver fibrosis.

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COMMENTS

Background

Chronic liver disease is among the top ten disease related causes of death in the US (CDC, National Center for Health Statistics, 2007). One of the primary forms of chronic liver disease is liver fibrosis, which is mediated by a number of factors including viral infection, genetic disease, and/or xenobiotic-induced damage. The hepatic stellate cell (HSC) plays a crucial role in the development and progression of liver disease to fibrosis.

Research frontiers

In the normal liver the HSC resides in a quiescent state; however, upon hepatic injury, the HSC transdifferentiated (activates) into a myofibroblast-like cell characterized by increased proliferation, extracellular matrix production and cell survival. However, the signaling molecules responsible for this activation process have not been fully elucidated. In this study the authors demonstrate that the JAK/STAT signaling pathway is critical for early HSC activation.

Innovations and breakthroughs

Numerous studies have shown that several profibrogenic genes are transduced by the JAK/STAT signaling pathway. This is the first study showing that JAK/STAT inhibition impedes initiation of HSC activation. Additionally, this study showed through a daily genetic profile assessment that the day of culture-activation used can significantly impact studies examining different cellular processes including transdifferentiation, proliferation, gene expression and migration of HSCs.

Applications

Understanding factors that regulate HSC activation is crucial for the development of therapeutic interventions for the treatment of liver fibrosis.

Terminology

JAK/STAT signaling communicates extracellular information to the cell's nucleus initiating target gene transcription influencing the fibrotic status. In order to signal through the JAK/STAT pathway, a cell membrane receptor, JAK and STAT are required. Transdifferentiation of the HSC is a transformation process in which the cell changes from one type to another. This process is critical for the development of liver fibrosis. Culture-activation refers to the routine practice of growing HSCs on plastic dishes in the lab to mimic the transdifferentiation process that occurs in the liver.

Peer review

This is a very well structured piece of basic research. It adds to the growing literature on HSC mechanisms leading to fibrogenesis. It is a well written manuscript, especially the discussion section is interesting. It includes comprehensive literature relevant to discussion.

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ORIGINAL ARTICLE

Increased TGF-β3 in primary biliary cirrhosis: An abnormality related to pathogenesis?

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Abstract

AIM: To investigate the transforming growth factor-β (TGF-β) isoforms in the peripheral and hepatic venous blood of primary biliary cirrhosis (PBC) patients.

METHODS: We examined TGF-β1, TGF-β2 and TGF-β3 (enzyme-linked immunosorbent assay), in 27 stage Ⅳ PBC patients (27 peripheral and 15 hepatic vein sera), 35 early (Ⅰ-Ⅱ) PBC and 60 healthy controls. As disease controls 28 hepatitis C virus (HCV) cirrhosis (28 peripheral and 17 hepatic vein serum), 44 chronic HCV hepatitis and 38 HCV-related hepatocellular carcinomas were included. We also tested liver tissue by immunohistochemistry to identify localization of TGF isoforms.

RESULTS: TGF-β1 was significantly decreased in all cirrhotics (PBC Ⅲ-Ⅳ: median 13.4 ng/mL; range, 7.4-26.2, HCV cirrhosis: 11.6 ng/mL; range, 5.0-33.8), compared to controls (30.9 ng/mL; range, 20.9-37.8). TGF-β2 was increased in viral cirrhosis but not in PBC and chronic hepatitis. TGF-β3 (47.2 pg/mL; range, 27.0-79.7 in healthy controls) was increased in early and late PBC (Ⅰ-Ⅱ: 94.3 pg/mL; range, 41.5-358.6; Ⅲ-Ⅳ: 152.8 pg/mL; range, 60.4-361.2; $P < 0.001$) and decreased in viral cirrhosis (37.4 pg/mL; range, 13.3-84.0; $P < 0.05$). Hepatic vein TGF-β levels were analogous to those in peripheral blood. Immunohistochemistry identified all isoforms in portal tract lymphocytes, sinusoidal cells and cholangiocytes. TGF-β3 was additionally overexpressed in hepatocytes in PBC patients.

CONCLUSION: The serum profile of TGF-β isoforms is different in cirrhotics. Increased TGF-β3 is characteristic of PBC. These findings may be related to the immunological abnormalities of PBC.

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Key words: Transforming growth factor-β; Primary biliary cirrhosis; Liver fibrosis; Cirrhosis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune disease characterized by lymphocytic infiltration of the portal tracts and selective destruction of intrahepatic small bile ducts with progressive fibrosis and finally cirrhosis[1]. T-regulatory cells (T-reg) may play an important role[2]. Transforming growth factor-β (TGF-β) is a pleomorphic cytokine regulating many cellular functions including proliferation, differentiation, migration and survival[3,4]. Three members of the TGF-β family have been identified with TGF-β1 being predominant in the immune system[5]. Many aspects of TGF-β functions may be related to the pathogenesis of PBC.

T-reg downregulate immune responses and there is a reciprocal relationship between T-reg and the recently described Th17 cells[6]. TGF-β induces both the differentiation[7,8] and maintenance[9] of T-reg Fox P3-positive cells, while in conjunction with the pro-inflammatory cytokine interleukin-6 (IL-6) induces the generation of the proinflammatory Th17 cells[10-12].

Evidence from transgenic mice indicate that impaired TGF-β signaling is involved in the pathogenesis of PBC, through deregulation of T-reg cells[13]. TGF-β is also involved in the fibrotic process. TGF-β stimulates the synthesis of laminin, collagen Ⅳ and entactin by liver endothelial cells[14] and stellate cells, but myofibroblasts are not responsive to TGF-β[15]. Liver fibrosis is reduced by 50%-70% after TGF-β blockade[16].

Despite experimental evidence for the involvement of TGF-β in the pathogenesis of PBC, data in humans is scarce.

We therefore studied serum and liver tissue TGF-β isoforms in patients with PBC in comparison with normal controls and patients with other chronic liver diseases.

MATERIALS AND METHODS

Patients

Informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital. Diagnosis and fibrosis staging in all patients was verified by liver biopsy. Patients with early PBC (35 stage Ⅰ-Ⅱ) and late PBC (27 stage Ⅲ-Ⅳ, according to Ludwig) were diagnosed on the basis of the histology plus the presence of antimitochondrial antibodies (AMA) and M2 antibodies by enzyme-linked immunosorbent assay (ELISA) and increased alkaline phosphatase. Patient demographics are presented in Table 1. Hepatitis C virus (HCV) patients were used as the disease control group (28 HCV cirrhosis, 44 chronic HCV hepatitis, genotype 1, and 38 HCV cirrhosis-associated hepatocellular carcinoma (HCC). All had positive anti-HCV tests (Abbott) and a positive qualitative and quantitative determination of HCV RNA. Sixty age-matched normal controls were included (from blood donors, staff and visitors of the unit). Blood samples were collected from the hepatic vein of 15 cirrhotic PBC patients and 17 HCV cirrhotics during measurement of intrahepatic wedge pressure. All sera were stored at -80℃ until assayed.

Table 1 Basic characteristics of patients

	PBC	Chronic hepatitis	HCC	Viral cirrhosis
No. of patients	62	44	38	28
Mean age	69.1 ± 8.8	53 ± 11	62 ± 8.3	64 ± 10.3
Mayo score (mean)	5.19 ± 1.44			
Gender (F/M)	56/6	36/8	13/25	16/12
Bilirubin (mg/dL)	1.5 ± 0.6	1.2 ± 0.4	1.4 ± 0.5	1.8 ± 0.5
Albumin (g/dL)	4.2 ± 1.0	4.5 ± 0.8	3.6 ± 0.4	3.0 ± 0.9
Alk phos (UI/L) (normal < 125)	185 ± 36	110 ± 15	152 ± 28	138 ± 18
γGt (UI/L) (normal < 50)	98 ± 25	39 ± 18	65 ± 23	57 ± 13
ALT (UI/L)	62 ± 13	92 ± 36	71 ± 11	66 ± 17
AST (UI/L)	55 ± 17	86 ± 32	65 ± 21	59 ± 12
IgM (mg/dL)	428 ± 52	165 ± 32	138 ± 28	139 ± 47
Histological stage				
Ludwig Ⅰ-Ⅱ	35			
Ludwig Ⅲ-Ⅳ	27			
Ishak 1-3		30		
Ishak 4-5		14		

PBC: Primary biliary cirrhosis; HCC: Hepatocellular carcinoma; Alk phos: Alkaline phosphatase; γGt: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ig: Immunoglobulin.

Immunohistochemistry was performed on liver tissue from 15 PBC patients (7 stage Ⅰ-Ⅱ and 8 stage Ⅳ), 10 patients with chronic hepatitis C and 10 patients with HCV-related cirrhosis.

Methods

ELISAs: TGF-β1, TGF-β2 and TGF-β3 were measured with commercially available ELISAs (DuoSet® ELISA Development System, human TGF-β1, human TGF-β2, human TGF-β3, R&D Systems Abingdon, UK), according to the manufacturer's instructions.

Immunocytochemistry: TGF-β isoform expressions were studied in paraffin sections by the alkaline phosphatase method or by immunofluorescence using monoclonal antibodies.

For the alkaline phosphatase method the DAKO APAAP Mouse Real™ Detection System, was used according to the manufacturer's instructions.

Incubation with the primary antibody (Mouse anti-human TGF-β1, TGF-β2 and TGF-β3 R&D Systems diluted 1:10 with the DAKO Rear™Antibody diluent) was done overnight at room temperature, followed by incubation with primary antibody enhancer for 25 min and with the secondary alkaline phosphatase conjugated antibody for 30 min. A-naphthol phosphate and Fast Red were used to demonstrate the presence of TGF-β isoforms.

For immunofluorescence, antigen retrieval was achieved by incubation with citrate buffer (1.8 mmol/L citric acid and 8.2 mmol/L sodium citrate) for 2 h at 37℃.

After blocking with phosphate-buffered saline containing 0.2% TritonX-100, 2 mmol/L $MgCl_2$ and 1% gela-

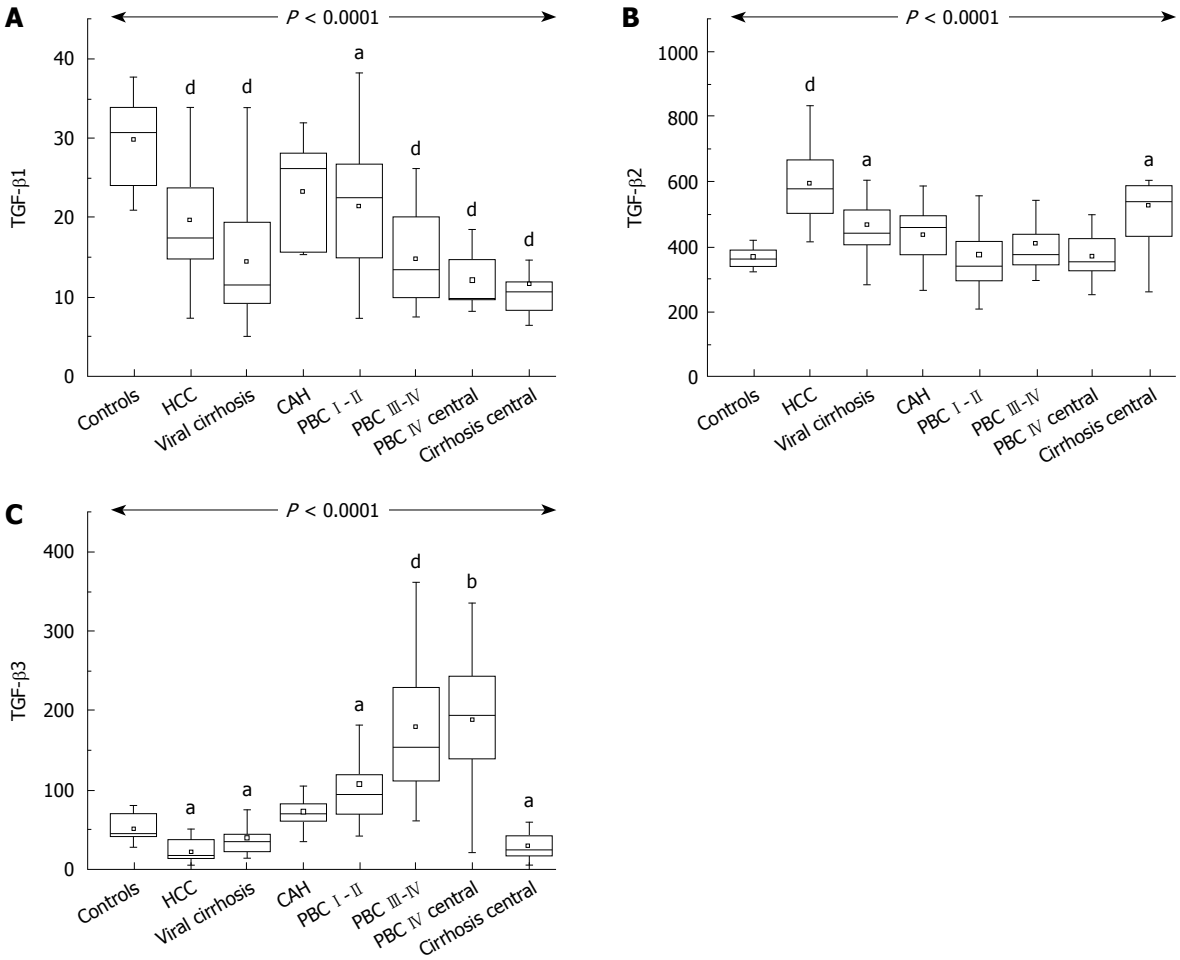

Figure 1 Transforming growth factor-β1 (A), transforming growth factor-β2 (B) and transforming growth factor-β3 (C). A: Box plots showing the interquartile range (box), median (horizontal line) and range (vertical lines) of the serum levels of transforming growth factor-β1 (TGF-β1) in normal controls, patients with hepatocellular carcinoma (HCC), viral cirrhosis, chronic active hepatitis C (CAH), primary biliary cirrhosis (PBC) stages Ⅰ-Ⅱ, PBC stages Ⅲ-Ⅳ, in hepatic vein blood from PBC and viral cirrhosis patients (central). B, C: Box plots showing the interquartile range (box), median (horizontal line) and range (vertical lines) in the peripheral and hepatic vein serum levels of TGF-β2 and TGF-β3 of the same group of patients. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$.

tin from cold water fish skin (Sigma-Aldrich, Germany) for 10 min, sections were incubated overnight at 4℃ with mouse anti-human TGF-β1 (R&D Systems, UK, dilution 1/10) in blocking buffer. Sections were washed with blocking buffer and incubated with Alexa fluor 488 F(ab')$_2$ fragment of goat anti-mouse IgG (H+L) (Invitrogen, UK, dilution 1/1000) for 1 h at room temperature.

For detection of TGF-β2 and TGF-β3 (R&D Systems, UK, dilution 1/20) we followed the same procedure, except for an incubation time of 2 h.

Negative controls for both alkaline phosphatase and immunofluorescence were run by omitting the primary antibody.

Statistical analysis

The results of serum TGF-β1, TGF-β2 and TGF-β3 were not normally distributed according to Bartlett's test. The Kruskal-Wallis non-parametric analysis of variance was used for comparisons among all groups, with Dunn's procedure for multiple comparisons. The Kolmogorov and Smirnov test was used to assess Gaussian distributions All tests were performed with the SPSS version 15 statistical package. $P < 0.05$ was considered significant. All values are expressed as median and range.

RESULTS

TGF-β1

Overall there was a significant difference in TGF-β1 among different groups ($P < 0.0001$). Patients with cirrhosis, whether viral or PBC, had lower values compared to normal controls (HCV cirrhosis 11.6 ng/mL; range, 5.0-33, PBC stages Ⅲ-Ⅳ 13.4 ng/mL; range, 7.4-26.2 ng/mL *vs* 30.9 ng/mL; range, 20.9-37.8 for healthy controls). Early PBC patients also had lower levels than controls (22.5 ng/mL; range, 7.2-38.3). TGF-β1 levels were also low in the blood from hepatic veins (Figure 1A).

TGF-β2

There was also a significant difference in TGF-β2 among groups ($P < 0.0001$). However, patients with viral cirrhosis (442.4 pg/mL; range, 282.8-967.0) had higher levels than controls (370.5 pg/mL; range, 235.0-495.7) while HCC cirrhotics had the highest values. By contrast, patients with

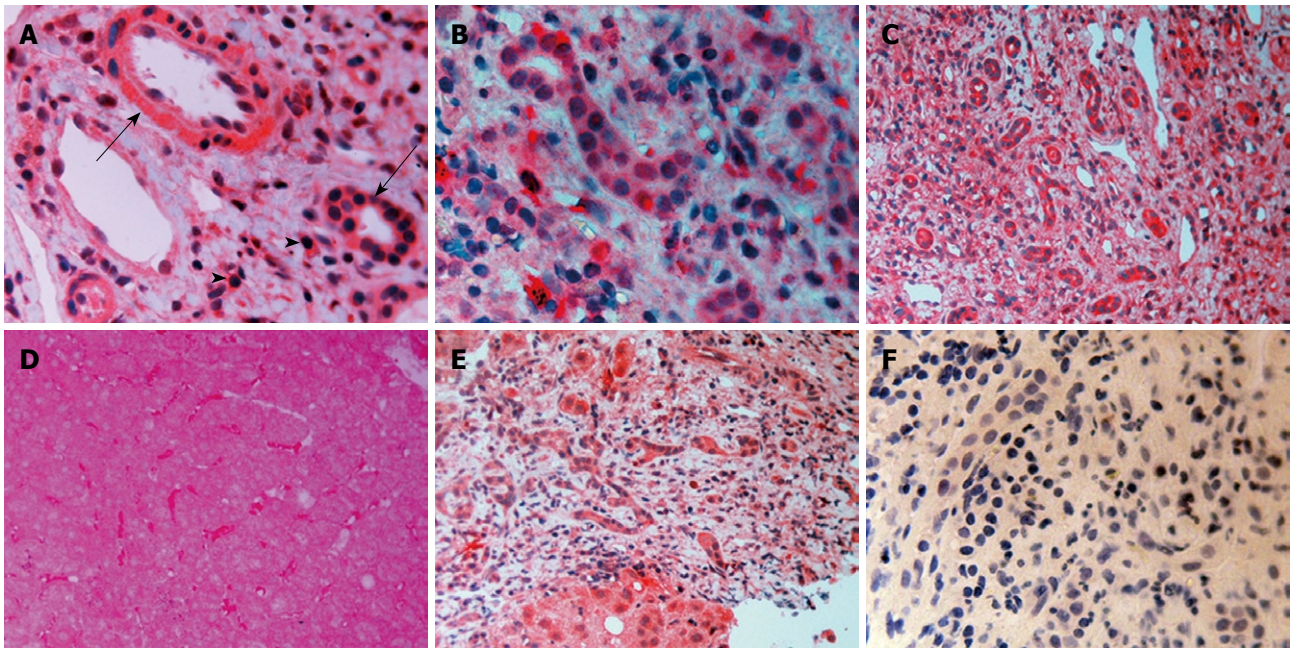

Figure 2 Immunocytochemistry for transforming growth factor-β1, transforming growth factor-β2 and transforming growth factor-β3. A: Transforming growth factor-β1 (TGF-β1) in viral cirrhosis. Positive cholangiocytes (arrows), cells in the hepatic arterial wall and many positive lymphocytes (arrowheads); B: Primary biliary cirrhosis (PBC) stage Ⅱ, TGF-β2. Numerous positive cholangioles and positive mononuclear cells, probably lymphocytes; C: TGF-β3 in viral cirrhosis. Many positive hyperplastic cholangioles and positive lymphocytes; D: PBC stage Ⅰ, TGF-β3. Hepatocytes vaguely stained. Strong staining of sinusoidal cells; E: PBC stage Ⅳ, TGF-β3. Positive hepatocytes, cholangioles and mononuclear cells; F: PBC stage Ⅳ, negative control. Magnifications are A, B and F × 400; C-E, × 200.

either early PBC (349.2 pg/mL; range, 208.4-616.2) or late PBC (384.7 pg/mL; range, 298.9-543.8) had levels comparable with controls. This was also reflected in values from the hepatic veins (Figure 1B).

TGF-β3

Although the overall difference in TGF-β3 among groups was also highly significant ($P < 0.0001$) the profile was completely different. Patients with early (94.3 pg/mL; range, 41.5-358.6) and late PBC (152.8 pg/mL; range, 60.4-361.2; $P < 0.001$) had high values compared to controls (47.2 pg/mL; range, 27.0-79.7 in healthy controls), but viral cirrhotics (37.4 pg/mL; range, 13.3-84.0; $P < 0.05$) had statistically lower values compared to either controls ($P < 0.005$) or to both groups of PBC patients ($P < 0.05$ and $P < 0.01$, respectively). Levels were high in the hepatic vein from PBC patients and low in HCV cirrhotics (Figure 1C).

Immunohistochemistry

TGF-β1: There was TGF-β1 expression in bile duct epithelium and in numerous mononuclear cells in portal tracts in all disease groups. Endothelial cells of either the hepatic artery or the portal vein were positive (Figure 2A). Sinusoidal cells were also positive. Periportal hepatocytes showed a variable expression of TGF-β1 with either a diffuse cytoplasmic or a more globular pattern.

TGF-β2: A similar picture was also present. (Figure 2B). Globular deposition of TGF-β2 was evident in many hepatocytes. Some cholangioles were negative in PBC patients.

TGF-β3: Although the pattern was similar in all disease groups (Figure 2C-E), hepatocytes from patients with PBC showed a strong staining compared with the either chronic HCV hepatitis or HCV cirrhosis patients (Figure 3B). In addition, on immunofluorescence, positive lymphocytes infiltrating portal bile ductules demonstrated weaker rim fluorescence compared with other positive portal lymphocytes (Figure 3A).

DISCUSSION

We have demonstrated an increase in TGF-β3 in both peripheral blood and blood from the hepatic vein in patients with PBC. Interestingly, the increase in TGF-β3 was observed in early stage PBC, indicating that this is a disease-specific finding and not merely the result of the cirrhotic process. TGF-β1 was decreased in all cirrhotic patients, either HCV-related or PBC and this was also found in the blood of hepatic veins. TGF-β2 was the isoform that was increased in viral cirrhosis but not in PBC, a finding also verified in hepatic veins. In addition, we identified cholangiocytes as a source of liver TGF-β isoforms, in addition to portal lymphocytes and sinusoidal cells. Hepatocytes were also positive for TGF isoforms but it was TGF-β3 that was strongly expressed in hepatocytes from PBC patients.

There is strong experimental evidence that TGF-β is implicated in the pathogenesis of PBC, probably through deregulation of T-reg.

Transgenic mice with the dominant-negative TGF type Ⅱ receptor (dn TGF-βRⅡ), spontaneously develop

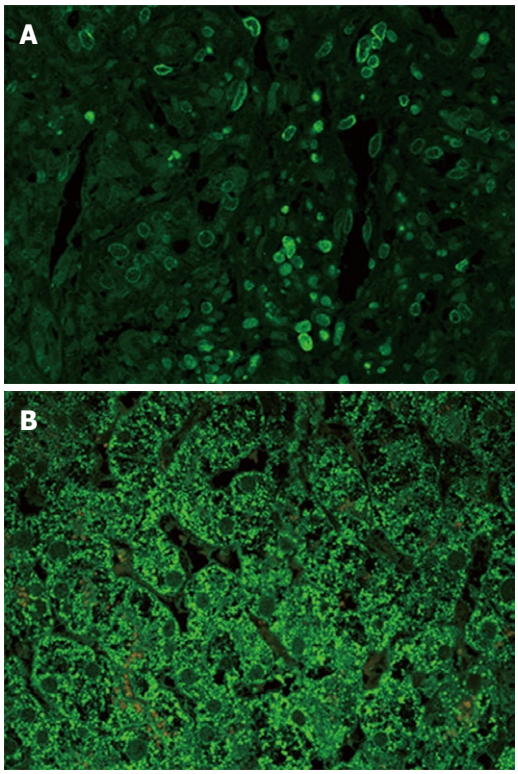

Figure 3 Immunofluorescence for transforming growth factor-β in PBC 3. A: Many positive mononuclear cells. Rim staining of varying intensity is evident. Some cells with low rim staining seem to infiltrate a bile ductule; B: Hepatocytes strongly stained in stage Ⅳ.

biliary disease resembling human PBC[13]. The normal liver contains only a few quiescent CD4 FoxP3 T-reg but it can rapidly recruit these cells when CD8 T blasts appear[17].

In PBC liver, destruction of biliary cells is mediated by liver infiltrating T-cells, especially CD8 cytotoxic cells which are highly enriched in PBC livers[18]. However in the CD25-negative mouse model, lack of CD8 cells attenuates but does not abolish bile ductular destruction, indicating that an additional mechanism of destruction is also present in PBC[19].

TGF-βRⅡ is also directly implicated in liver fibrosis[20].

Mice deficient in the IL-2 receptor (CD25) demonstrate a PBC-like lesion in the liver[21]. A child with a complete deficiency of the α subunit of the IL-2 receptor in peripheral CD25 lymphocytes developed a PBC-like disease[22], indicating that animal models may be relevant in human disease. Similarly, experimental evidence that T-reg are reduced in a model of PBC[23] have been verified in human disease where decreased numbers of peripheral and liver T-reg were found in PBC patients and most importantly in the peripheral blood of their relatives[24].

A substantial number of FoxP3 T-reg are negative for CD25[25]. It seems therefore that the lymphocytes positive for TGF-β histochemically identified in our study are either CD25-FoxP3 T-reg or other conventional T-cells capable of producing TGF-β isoforms[4,26].

Peripheral blood levels of TGF-β have not been adequately studied in PBC. Circulating TGF-β has been reported to reflect the severity of PBC and is reduced after 2 years of treatment with ursodeoxycholic acid[27].

Although the 3 isoforms of TGF-β are considered to have similar functions, there is evidence that this might not be so. Loss of TGF-β1 is compatible with postnatal survival, but the liver and other tissues are infiltrated with mononuclear cells, while loss of TGF-β2 and TGF-β3 leads to perinatal death[28,29].

Hepatic stellate and Kupffer cells are reported to be the main sources of TGF-β[30]. However the origin of TGF-β isoforms is not well established. Studies from experimental animals indicate that in the normal state Kupffer cells express all 3 isoforms[31]. Endothelial cells express mostly TGF-β1 and lower levels of TGF-β2 and TGF-β3 while stellate cells express very little TGF-β, and hepatocytes have no constitutional expression[32]. After a fibrogenic injury all forms of TGF-β increase in stellate cells probably through stimulation by retinoic acid produced by hydrolysis of retinol esters during activation of stellate cells[33]. Induced T-reg secrete TGF-β[34]. TGF-β from other sources such as dendritic cells may also be important[35]. Therefore analysis of peripheral levels is difficult due to the many potential sources of TGF-β that may reach the circulation. In particular, the cellular source of TGF-β required for T-reg maintenance in PBC or other liver disease is not clear.

Our histochemical findings indicate that portal lymphocytes are sources of TGF-β isoforms in both PBC and chronic HCV and so are sinusoidal cells. As already mentioned, lymphocytes are probably T-reg although detailed studies are required. We identified a novel source of TGF-β isoforms as intrahepatic bile ductules were strongly positive for TGF-β. It is conceivable that the increase in TGF-β3 observed in PBC may result from an overproduction by intrahepatic cholangiocytes.

Clearance of TGF-β is complex. It may be sequestered by α2 macroglobulin[36], undergo renal clearance or be taken up by hepatocytes[37]. This might explain the positive globules observed within hepatocytes in our study, at least for TGF-β1 and TGF-β2. However, hepatocytes from patients with PBC particularly from stage Ⅳ were strongly positive for TGF-β3 compared with the other 2 isoforms (Figure 3), a finding which is unlikely to be due to increased uptake. Hepatocytes therefore are an additional source for the increased levels of TGF-β3 in patients with PBC.

Levels in the hepatic vein blood followed the same pattern as in peripheral blood both for TGF-β3 and TGF-β2, confirming the liver as the source of TGF-β isoform differences found in our study.

In our study, TGF-β2 and TGF-β3 circulate in pg quantities as compared to ng for TGF-β1. In fibroblast cultures, TGF-β3 was proved to be 3-5 times more potent than TGF-β1[38]. TGF-β3 is also significantly more potent than the other 2 isoforms in stimulation of neovascularization[29]. The *in vitro* inhibitory activity of TGF-β3 on an epithelial cell line is in the range of 10-50 pg/mL[39] while transformation and proliferation of rat fibroblasts is

achieved at a concentration of 100 pg/mL. Therefore, the levels we found are biologically relevant.

Based on previous reports and on the findings of the present study, we suggest a hypothetical model for the pathogenesis of PBC based on a dual mechanism for bile duct destruction. Our findings may provide a link to explain the reported immunological abnormalities in PBC.

Differences in TGF-β isoforms may lead to a deranged balance between T-reg and Th17 cells. It is TGF-β1 that modulates FoxP3 expression and the regulatory activity of CD4 cells[40]. The presence of increased local levels of TGF-β3 (and the concomitant relative lack of TGF-β1) in conjunction with increased levels of IL-6 may shift the balance towards an increased activity of the proinflammatory Th17 cells adding to the CD8 cytotoxic lymphocytes destructive mechanism, while at the same time it leading to functionally defective T-reg.

Although plasma levels of IL-6 are decreased in PBC[41], monocytes from PBC patients secrete more IL-1 and IL-6 after *in vitro* challenge with Toll-like receptor ligands[42], and increased expression of IL-6 has been described in the liver of PBC patients[43]. The increased presence of TGF-β3 instead of TGF-β1 could be a further mechanism that favors Th17 activity since one can postulate that TGF-β3 could either be associated with functionally defective T-reg or could directly favor the increase of Th17. Further evidence for such a hypothesis is provided by a recent report that mice with mutation of the gene encoding the FoxP3 transcriptional factor developed AMA positivity and features resembling PBC with increased levels of IL-17 and IL-23, cytokines associated with Th17 cells[44]. There is also recent evidence that Th17 cells are implicated in the pathogenesis of PBC as they are increased in the portal tracts of PBC patients[45].

As we found cholangiocytes to be a major source of TGF-β isoforms in the liver, one can further postulate that these cells may be the origin of the aberrant production of TGF-β isoforms in PBC. This requires further clarification.

Recent experimental evidence adds further support on the proposal for a crucial role of TGF-β3 in the pathogenesis of PBC. TGF-β3 was reported to decrease collagen synthesis and tissue inhibitor of metalloproteinases-1 expression, and increase matrix metalloproteinase-9 in CCl_4-induced liver fibrosis[46]. This finding could offer a potential explanation for the delayed development of fibrosis and cirrhosis in PBC compared with other chronic liver diseases.

In our study we also found significantly increased levels of TGF-β2 in HCV-associated cirrhosis with the highest values found in cases with HCC in accordance with previous reports of increased TGF-β expression in human HCC[47,48]. Interestingly, T-reg are increased in numbers and correlate with disease progression and survival in patients with HCC[49].

In summary, we have demonstrated that the serum profile of the 3 TGF-β isoforms is different in the disease groups studied. An increase in TGF-β3 is a characteristic of PBC irrespective of stage and therefore it may be pathogenetically important. Results from the hepatic vein samples indicate that the liver seems to be the source of the TGF-β abnormalities. We also propose that these findings may be the causative link leading to T-reg and/or Th17 deregulations reported in PBC.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is an autoimmune disease characterized by mononuclear infiltration of the portal tracts and destruction of intrahepatic small bile ducts, fibrosis and finally cirrhosis. transforming growth factor-β (TGF-β) is a cytokine regulating many cellular functions including proliferation, differentiation, migration and survival of hepatic stellate cells. Three members of the TGF-β family have been identified with TGF-β1 being the most studied. Many aspects of TGF-β functions may be related to the pathogenesis of PBC as it regulates both immune responses and fibrosis in the liver.

Research frontiers

The role of the 3 different isoforms of TGF-β in chronic liver disease has not been investigated. Different isoforms may be related to the pathogenesis of different chronic liver diseases. With this purpose in mind, the authors aimed to investigate the levels these isoforms in different chronic liver diseases both in peripheral and in hepatic vein blood samples in order to identify the pattern of their increase in these liver diseases.

Innovations and breakthroughs

Although TGF-β1 was significantly decreased in all cirrhotics compared to controls. TGF-β2 was increased in viral cirrhosis but not in PBC and chronic hepatitis. Interestingly TGF-β3 was increased in both early and late PBC and decreased in viral cirrhosis with the levels of this marker in the hepatic vein being analogous to the peripheral blood. TGF-β3 was also overexpressed in the hepatocytes of PBC patients. This is the first report of differences in the expression of TGF-β isoforms in a disease characterized by progressive fibrosis.

Applications

Increased TGF-β3 levels found in PBC might be genetically controlled and can induce immunological responses and profibrotic processes during disease progression. The findings of this study may provide support of further research in the field that will further elucidate the role of the TGF-β isoforms during liver disease progression and could lead to novel therapeutic approaches in PBC. Furthermore, the findings of this study suggest that TGF-β isoforms may also be regulated differently in other diseases characterized by autoimmune and fibrotic phenomena.

Terminology

TGF-β is a protein that regulates immune responses and fibrotic progression during liver diseases. Although the 3 isoforms of TFG-β have been known for many years the question whether they have distinct roles in different liver diseases has not been studied.

Peer review

This paper finds differences in TGF-β isotypes production in PBC. Until now we do not have a good answer for the role of TGF-β isoforms, then we can not approach the pathological reason why TGF-β3 is increased in PBC directly. The findings of this paper will indicate the new mechanism of PBC.

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BRIEF ARTICLE

Hepatic lipogranulomas in patients with chronic liver disease: Association with hepatitis C and fatty liver disease

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Abstract

AIM: To study the significance and clinical implication of hepatic lipogranuloma in chronic liver diseases, including fatty liver disease and hepatitis C.

METHODS: A total of 376 sequential, archival liver biopsy specimens were reviewed. Lipogranuloma, steatosis and steato-fibrosis were evaluated with combined hematoxylin and eosin and Masson's trichrome staining.

RESULTS: Fifty-eight (15.4%) patients had lipogranuloma, including 46 patients with hepatitis C, 14 patients with fatty liver disease, and 5 patients with other diseases. Hepatic lipogranuloma was more frequently seen in patients with hepatitis C (21%) and fatty liver disease (18%), and its incidence was significantly higher than that in control group ($P < 0.0002$ and $P < 0.007$, respectively). In addition, 39 out of the 58 patients with lipogranuloma were associated with steatosis and/or steato-fibrosis. Of the 18 lipogranuloma patients with clinical information available for review, 15 (83%) had risk factors associated with fatty liver disease, such as alcohol use, obesity, hyperlipidemia, and diabetes mellitus. Although the incidence of these risk factors was greater in patients with lipogranuloma than in control group (60%), it did not reach statistical significance.

CONCLUSION: Hepatic lipogranuloma is not limited to mineral oil use and commonly associated with hepatic steatosis, hepatitis C and fatty liver disease. With additional histological features of steato-fibrosis, lipogranuloma can also be used as a marker of prior hepatic steatosis.

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Key words: Lipogranuloma; Hepatitis C; Fatty liver disease; Steatosis; Steato-fibrosis

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INTRODUCTION

Chronic hepatitis C and fatty liver disease are the most common chronic liver diseases in the Western world[1-4]. Hepatitis C affects approximately 2.7 million individuals in the United States and up to 20% of them will eventu-

ally progress to cirrhosis[1,2]. It is well known that hepatitis C, particularly genotype 3, is associated with hepatic steatosis. On the other hand, it is estimated that up to two thirds of obese adults and nearly 50% of obese children can develop fatty liver disease[2]. A significant proportion of those patients can have concurrent hepatitis C and fatty liver disease. Hepatic steatosis can cause insulin resistance, decreased response to antiviral therapy, accelerated fibrosis, and cirrhosis[2,5].

Clinically, no effective laboratory test can evaluate hepatic steatosis, and liver biopsy remains the gold standard. However, one of the dilemmas in patients with suspected fatty liver disease is the occasional appearance of "non-fatty" liver biopsy specimens, particularly when cirrhosis is well established[6]. This deceptive histology can lead to erroneous diagnosis of so-called cryptogenic cirrhosis, most of which are now recognized as a "burnt-out" fatty liver disease[6,7]. Therefore, recognition of fatty liver disease, particularly in the absence of steatosis, is important for correct interpretation and management of patients with chronic liver disease.

Lipogranuloma, comprised of aggregates of lipid-containing histiocytes, is observed in liver biopsy specimens at an estimated frequency of 2.4%-5%[8,9]. Early investigations including lipid histochemistry analysis suggested that lipogranuloma is due to a reaction to absorbed saturated hydrocarbons, like mineral oil used widely in food, laxatives or oily vehicle for medication[10-13]. However, it has been shown that lipogranuloma is associated with hepatic steatosis[6,9,14,15]. The ruptured fat from lipid-containing hepatocytes can be taken up by macrophages and form lipogranuloma[9]. Lipid and its oxidative products can also stimulate inflammatory response, release cytokines, activate stellate cells and cause fibrosis[16-18].

Here, we reviewed 376 sequential liver biopsy patients to investigate the potential association of lipogranuloma with fatty liver disease and chronic hepatitis C.

MATERIALS AND METHODS

All liver biopsy patients were retrieved from the archives in Department of Pathology, Beth Israel Medical Center of the Albert Einstein College of Medicine. Both routine hematoxylin and eosin (H/E) and Masson's trichrome stained slides were reviewed. All hepatitis B and C patients were confirmed clinically by serology or molecular/PCR tests for viral genome. Viral hepatic necroinflammation (activity) and fibrous scarring (stage) were evaluated according to the modified Ishak system[19].

Hepatic steatosis was evaluated based on the HE-stained slides, semi-quantitated as mild (< 1/3), moderate (1/3 to 2/3) and severe (> 2/3)[20], and further evaluated for the presence of steatohepatitis (hepatocyte ballooning, inflammatory cells, and Mallory's hyaline) and steatofibrosis (pericellular, perivenular, and/or portal fibrosis).

Hepatitis C-associated steatosis is most often mild and presents in a non-zonal distribution. When steatosis is more than that seen in patients with no complication of hepatitis C, and/or primary distribution in zone 3 with some features of pericellular and/or perivenular fibrosis, then a separate diagnosis of concurrent fatty liver disease is rendered, recognizing that hepatitis C itself may be a potential cause of insulin resistance and metabolic syndrome[21-23].

In the absence of significant steatosis (> 5%), identification of pericellular or perivenular fibrosis on the trichrome-stained slides suggests a tentative diagnosis of resolving fatty liver disease.

Lipogranuloma, comprised of loose aggregates of lipid-containing macrophages, lymphocytes, and a few poorly developed epithelioid cells, was evaluated on both H&E and trichrome-stained slides. Morphologically, lipogranuloma is different from the foam cell aggregates resulting from scavenge of dying hepatocytes. The later usually contains PASD-positive foamy cytoplasm without lipid vacuoles. They were also further classified according to their location: portal/periportal, perivenular, parenchymal, or their combination. Of the 58 patients with lipogranuloma, 18 had clinical chart information available for review for the presence of known risk factors associated with fatty liver disease such as history of alcohol use, diabetes mellitus, hyperlipidemia, and obesity. The control group was consisted of forty sequentially retrieved cases out of the total 376 patients that were absent of lipogranuloma on liver biopsy, but had clinical chart information available for review of risk factors for fatty liver disease.

Fisher exact test was used for statistical analyses. A two-tailed *P* value less than 0.05 was considered statistically significant.

RESULTS

Clinical information

Liver biopsy specimens from 376 patients were studied. The average age of patients was 48.8 years (range 19-85 years) and the male to female ratio was 1.5:1. Diagnosis was established as chronic hepatitis C in 217 (58%) patients, fatty liver disease in 80 (21%) patients, chronic hepatitis B in 56 (15%) patients, autoimmune hepatitis (AIH) in 13 (3.5%) patients, primary biliary cirrhosis (PBC) in 11 (3%) patients, and other diseases in 47 (13%) patients including nonspecific changes in 14, malignancy in 10, no pathological alteration in 6, resolving hepatitis in 5, methotrexate-related changes in 3, hemosiderosis in 3, cholestasis in 2, necrotizing granuloma in 1, sarcoid granuloma in 1, hepatic adenoma in 1, and bile duct paucity with unknown cause in 1 (Table 1). Of the 217 patients with chronic hepatitis C, 38 (17.5%) had histologically and clinically concurrent fatty liver disease. Of the 40 patients in control group, 60%, 12.5%, 12.5%, and 10% were diagnosed as hepatitis C, FLD, hepatitis B, and other diseases, respectively.

Lipogranuloma in liver

Lipogranuloma was identified in 58(15.4%) out of the 376 liver biopsy specimens with HE and Masson's trichrome staining. The average age of these patients was 52.6 years (range 22-70 years) and the male to female ratio was 2.6:1. Of the 58 patients with lipogranuloma, 46 were diagnosed as chronic hepatitis C (21% of total hepatitis C

Table 1 Distribution and incidence of lipogranuloma in 376 sequential liver biopsy specimens *n* (%)

Disease	Patients[1]	Lipogranuloma patients	*P*
HCV (all)	217 (58)	46 (21)	0.0002
HCV alone	160 (43)	36 (22.5)	0.0001
HCV + FLD	38 (10)	7 (18)	0.0200
HCV + other	19 (5)	3 (16)	0.1000
FLD (all)	80 (21)	14 (18)	0.0070
FLD alone	24 (6.4)	5 (21)	0.0240
FLD + HCV	38 (10)	7 (18)	0.0200
FLD + other	18 (5)	2 (11)	0.2600
Non-HCV/FLD	116 (31)	5 (4)	
HBV	56 (15)	2 (3.6)	
PBC	11 (3)	1 (9)	
AIH	13 (3.5)	1 (7.7)	
Other	47 (13)	3 (6)	

[1]Some patients had more than one disease. *P*: Fisher's exact test, two-tailed value *vs* non-HCV/FLD. HCV: Hepatitis C virus; FLD: Fatty liver disease; HBV: Hepatitis B virus; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis.

patients), 14 as fatty liver disease (18% of total fatty liver disease patients, including 7 with concurrent hepatitis C), 2 as hepatitis B (1 with concurrent fatty liver disease), 1 as PBC, 1 as AIH with concurrent fatty liver disease, and 3 as other diseases (1 with lipofuscinosis, 1 with non-specific change, and 1 with no pathological alteration). The incidence of lipogranuloma was significantly higher in patients with hepatitis C and fatty liver disease than in controls ($P < 0.0002$ and $P < 0.007$, respectively). However, the difference was statistically less significant in patients with fatty liver disease when those with concurrent hepatitis C were excluded ($P = 0.024$) (Table 1).

Approximate one third of the patients had only single lipogranuloma, while the remaining patients had multiple lesions. The distribution of lipogranulomas varied from portal (zone 1) to central area (zone 3), including portal/periportal in 24 patients, perivenular in 16 patients, both portal and perivenular in 16 patients, and both portal and parenchymal distribution in 2 patients. In the 14 lipogranuloma patients with the diagnosis of fatty liver disease, the distribution of lipogranuloma was similar (portal in 5, perivenular in 3, and combination in 6) (Figure 1), indicating that lipogranuloma distribution does not correlate with any disease category.

Lipogranuloma in patients with fatty liver disease

The typical patterns of mild to moderate fibrosis associated with fatty liver disease were sclerosis of central veins and/or delicate thin fibrous bands distributed in perivenular and acinus zone 3. Of the 58 lipogranuloma patients, 12 had perivenular/pericellular fibrosis only, 14 had steatosis only, 14 had both steatosis and fibrosis, and 18 had neither steatosis nor fibrosis. Overall, 39 (67%) out of the 58 lipogranuloma patients were associated with either steatosis or steato-fibrosis. However, most lipogranuloma-containing biopsy specimens showed either steatosis (13%, 32 out of 237 patients) or mild steatosis (26%, 19 out of the 73 patients) and moderate to severe steatosis (12%, 8 out of the 66 patients).

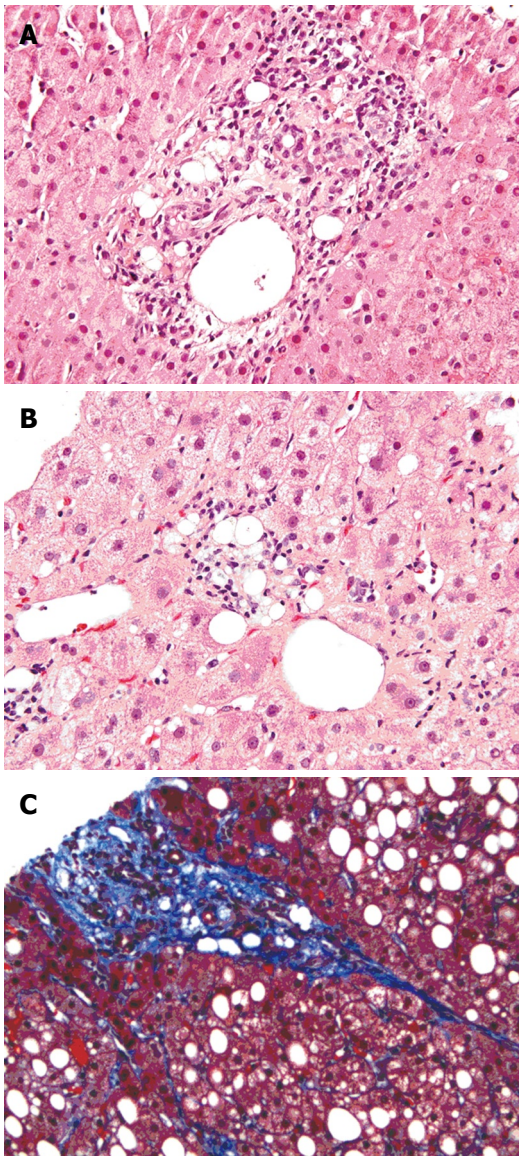

Figure 1 Liver biopsy showing lipogranuloma with or without significant steatosis or steato-fibrosis. A and B: HE-stained slides showing lipogranuloma in the portal and perivenular area (original magnification × 400); C: Masson's trichrome-stained slides showing portal lipogranuloma associated with delicate fibrous bands and steatosis (original magnification × 200).

In order to further investigate the relationship between lipogranuloma and fatty liver disease, we reviewed the patients' clinical information for history of risk factors for fatty liver disease, including alcohol drinking, obesity, diabetes mellitus, or hyperlipidemia. Of the 18 patients with chart information available for review, 15 (83%) had at least one of the risk factors associated with fatty liver disease (Table 2). However, 24 (60%) of the 40 patients in control group also had clinical risk factors for fatty liver disease, which did not reach the statistical significance.

DISCUSSION

Earlier studies suggested that lipogranuloma is an inci-

Table 2 Clinical risk factors for fatty liver disease in 15 patients with lipogranuloma

Patient	Diagnosis	Alcohol hx	Diabetes mellitus	Hyper-lipidemia	Obesity
1	Cirrhosis, 4/4	Y	N	N	N
2	HCV, 3/4	N	N	Y	N
3	HBV, 1/4	N	N	Y	N
4	HCV, 2/4	Y	N	N	N
5	HCV, 2/4	Y	N	Y	Y
6	HCV, 1/4	?	N	?	Y
7	HCV, 2/4	Y	N	N	N
8	HCV, 2/4	Y	N	N	N
9	HCV, 1/4, iron 2/4	Y	?	?	N
10	HCV, FLD, 1/4	Y	N	N	N
11	HCV, 1/4	Y	N	N	Y
12	Resolving FLD	N	N	N	Y
13	AIH	N	N	N	Y
14	FLD	N	N	Y	Y
15	HCV, 1/4	N	N	Y	Y
Total	15	8	0	5	7

HCV: Hepatitis C virus; FLD: Fatty liver disease; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis.

dental finding with no particular clinical significance[10,13]. Histochemical analysis of lipid extracts from non-fatty liver and food showed that lipogranuloma in non-fatty liver is likely caused by a reaction to the ingested, absorbed, and saturated hydrocarbons similar to mineral oil[10]. It has been reported that lipogranuloma can be found in 48% of livers without obvious association with steatosis, suggesting that lipogranuloma is probably related to mineral oil with no clinical significance[10,13].

However, ultrastructural examination of severe fatty liver biopsy specimens suggested that lipogranuloma is also probably formed from fat droplets[12]. A more recent study also indicated that lipogranuloma may be associated with fatty liver disease, particularly in alcoholic patients[15]. When lipid release overwhelms the ability of lipid absorption, the excess fat can be taken up by macrophages and form lipogranuloma[9]. Recent studies suggested that lipid release from ruptured hepatocytes can cause lipid peroxidation, mitochondrial dysfunction and inflammatory injury to hepatocytes and venules, releasing inflammatory cytokines, such as interleukin-6 and tumor necrosis factor, thus further activating stellate cells and ultimately causing fibrosis[16-18].

Over the last two decades, hepatitis C and fatty liver disease have become the most common chronic liver diseases in the United States and our sample shows a similar demographic profile which consists of 58% of hepatitis C patients and 21% of fatty liver disease patients. The frequency of lipogranuloma (15.4%) in our study was significantly higher than that of previously reported (2.4%-5%). In our study, lipogranuloma was most often identified in biopsy specimens from patients with hepatitis C (21%) and/or fatty liver disease (18%), while the prevalence remained similar to historical levels in hepatitis B and other diseases, suggesting that an increased incidence of lipogranulomas is related to fat accumulation seen in patients with hepatitis C and fatty liver disease. Lipogranuloma in patients with pure fatty liver disease, exclusive of concurrent hepatitis C, was statistically less significant ($P = 0.024$), suggesting that lipogranuloma is more closely related to chronic hepatitis C than to fatty liver disease.

It is well known that hepatitis C, particularly genotype 3, is associated with steatosis. Hepatic steatosis is found in 50%-80% of individuals with hepatitis C[2,5]. Meanwhile, 5%-18% of hepatitis C patients have concurrent fatty liver disease on biopsy[5]. Hepatitis C-associated steatosis, usually mild and located in a random distribution, is also recognized. However, hepatitis C-associated steatosis may directly cause insulin resistance and metabolic syndrome with patterns of steatosis typical for overt fatty liver disease[19,21,23]. The fact that there are more lipogranulomas seen in patients with hepatitis C than in those with fatty liver disease suggests that there may be factors other than steatosis that are associated with lipogranuloma formation. One may consider that the unique combination in hepatitis C with increased hepatocyte turnover from chronic viral infection and concomitant hepatocyte steatosis, may lead to increased lipid release from injured or dead steatotic hepatocytes, thus promoting lipogranuloma formation. Other unknown factors that may also play a significant role in lipogranuloma formation remain to be elucidated.

The previously reported mineral oil-related lipogranuloma is mainly formed around the portal tract, presumably due to lipid taken up by macrophages immediately upon entry into the liver *via* the portal vein after absorption from the gastrointestinal tract[10,13]. In our study, lipogranuloma was more evenly distributed from portal to central area, and 40 out of the 58 lipogranuloma patients were accompanied with steatosis, steato-fibrosis, or both, suggesting that lipogranuloma seen in the current study is more likely associated with steatosis from hepatitis C or fatty liver disease rather than with mineral oil ingestion or laxative use. More importantly, most of these lipogranulomas are also associated with other histological features of fatty liver disease. Review of the patients' chart information, when possible, also indicated that 15 (83%) of the 18 patients with lipogranuloma had at least one of the risk factors for fatty liver disease, including alcohol drinking, obesity, diabetes mellitus, or hyperlipidemia, which was higher than that in controls (60%), although no statistical insignificance was reached.

Our data, therefore, suggest that lipogranuloma is potentially associated with hepatic steatosis. We hypothesize that lipogranuloma is resulted from hepatocellular lipid accumulation (either from direct hepatitis C viral infection or through insulin resistance and metabolic syndrome arising from that virus, or from alcohol use, obesity, diabetes mellitus, and/or dyslipidemia syndrome), necroinflammatory activity due to virus or steatohepatitis, release of lipid from injured hepatocytes, uptake of released lipid by macrophages, and finally formation of lipogranulomas. The presence of such lesions in the absence of current steatosis (some of which also show persistent steato-fibrosis) suggests that lipogranulomas, particularly those away from the portal tract, may be indicative of prior hepatic steatosis and most probably of resolved or resolving fatty liver disease (in the absence of hepatitis C).

It is well recognized that many of those liver diseases previously labeled as cryptogenic cirrhosis are actually a burnt-out fatty liver disease[1]. Therefore, persistence of lipogranuloma in otherwise non-fatty liver disease, particularly in the absence of steatosis, is important for unmasking the nature of past, more active liver disease and facilitating correct interpretation and management of patients.

In conclusion, while mineral oil may play a role in some lipogranulomas as reported, most of the lipogranulomas in current liver disease patients are related to hepatic steatosis. Lipogranuloma can be used as a surrogate for prior, more active hepatic steatosis possibly related to hepatitis C. The otherwise normal liver biopsy specimen containing lipogranuloma (with or without mild, subtle steato-fibrosis) perhaps should not be considered "normal", but rather as an indicator of past, now resolving fatty liver disease, a diagnostic finding that is not without possible clinical relevance. Whether the presence of lipogranuloma also has prognostic importance in patients with chronic hepatitis C remains to be explored in follow-up analysis.

COMMENTS

Background

Fatty liver disease and hepatitis C have been the most common chronic liver diseases in the United States and both are associated with steatosis in the liver. Hepatic lipogranuloma has been previously regarded as mineral oil-related, incidental findings and its role in hepatic steatosis is rarely discussed. It will be interesting and potentially clinically significant to elucidate lipogranuloma, which may also be more significantly related to hepatic steatosis rather than to mineral oil laxative use.

Research frontiers

Fatty liver disease has become endemic in the United States. Clinically, patients with suspend fatty liver disease may have "non-fatty" appearance on liver biopsy and the disease of these patients may progress without proper treatment. Some of these patients may eventually develop so called "cryptogenic cirrhosis". Therefore, it will be very interesting and clinically important to study the relationship between hepatic lipogranuloma, fatty liver disease and hepatitis C.

Innovations and breakthroughs

This study reviewed the sequential liver biopsies and the association of lipogranuloma with chronic liver diseases, demonstrating that lipogranuloma is more commonly seen in patients with hepatitis C and fatty liver disease. Lipogranuloma associated with other features, such as steato-fibrosis, can be used as a potential marker of prior fatty liver disease.

Applications

By showing the potential association of lipogranulomas with hepatitis C and fatty liver disease, the authors can identify under recognized fatty liver disease.

Terminology

Lipogranuloma is due to histiocytic reaction to lipid aggregate. Steato-fibrosis is typically seen in around the hepatocytes and central venules more commonly seen in fatty liver disease.

Peer review

This manuscript presents some interesting data based on an observation in a large series of liver biopsies. The authors tried to link the morphologic phenotype to hepatic steatosis and hepatitis C, which may contribute to a better understanding of lipogranuloma formation.

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BRIEF ARTICLE

Retransplantation for graft failure in chronic hepatitis C infection: A good use of a scarce resource?

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Abstract

AIM: To investigate the outcome of patients with hepatitis C virus (HCV) infection undergoing liver retransplantation.

METHODS: Using the UK National Registry, patients undergoing liver transplantation for HCV-related liver disease were identified. Data on patient and graft characteristics, as well as transplant and graft survival were collected to determine the outcome of HCV patients undergoing retransplantation and in order to identify factors associated with transplant survival.

RESULTS: Between March 1994 and December 2007, 944 adult patients were transplanted for HCV-related liver disease. At the end of follow-up, 617 of these patients were alive. In total, 194 (21%) patients had first graft failure and of these, 80 underwent liver retransplantation, including 34 patients where the first graft failed due to recurrent disease. For those transplanted for HCV-related disease, the 5-year graft survival in those retransplanted for recurrent HCV was 45% [95% confidence interval (CI): 24%-64%] compared with 80% (95% CI: 62%-90%) for those retransplanted for other indications (P = 0.01, log-rank test); the 5-year transplant survival after retransplantation was 43% (95% CI: 23%-62%) and 46% (95% CI: 31%-60%), respectively (P = 0.8, log-rank test). In univariate analysis of all patients retransplanted, no factor analyzed was significantly associated with transplant survival.

CONCLUSION: Outcomes for retransplantation in patients with HCV infection approach agreed criteria for minimum transplant benefit. These data support selective liver retransplantation in patients with HCV infection.

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Key words: Hepatitis C; Liver; Recurrence; Retransplantation; Outcome

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INTRODUCTION

End-stage liver disease due to chronic hepatitis C virus (HCV) is the most common indication for liver trans-

plantation in Europe and the United States[1,2]. Recurrent viral infection of the allograft is near universal. Ninety percent of all patients transplanted with HCV have evidence of HCV in the allograft within 6 mo[3] and at least 20% of patients transplanted for HCV infection will develop cirrhosis of their allograft[1,4,5]. Allograft cirrhosis is associated with recurrence of disease before 6 mo post-transplantation[6], severe necroinflammation and confluent necrosis at the time of first histological diagnosis, pre-transplant viral load and episodes of treated rejection[7]. Cirrhosis is associated with high rates of graft loss to recurrent disease[8]. Recurrent HCV infection in the setting of immunosuppression rarely follows the more aggressive course of recurrent cholestatic hepatitis which may cause early graft failure.

Current data show that the prognosis for patients with cirrhosis and graft failure from recurrent HCV in their grafts is poor, with only 41% surviving 1 year after the development of decompensation[5]. Antiviral therapy is largely ineffective in this setting[9] and retransplantation is the only therapeutic option. Consideration of retransplantation in the setting of graft failure due to recurrent HCV infection is therefore an increasing clinical problem and results in increasing pressure on an already limited resource. Retransplantation, however, is associated with a worse clinical outcome than for first grafts regardless of the indication[10]. There have been several studies addressing the issue of retransplantation in patients with HCV and results from these studies have yielded discordant results: these are summarized in Table 1[10-19]. Initial studies reported an increased mortality following retransplantation in patients with HCV infection, frequently in the first months after retransplantation[11,13,18]. These studies were all retrospective and largely database-dependent. Indeed the United Network for Organ Sharing (UNOS) data has been re-evaluated on 2 occasions with the predictors of poor outcomes changing with time: the most recent analysis showing no difference between outcomes for patients with and without HCV infection undergoing retransplantation[14]. That said, there have been particular factors [including model for end-stage liver disease (MELD) score and donor age] and subgroups (i.e. those retransplanted between 90 d and 1 year after first liver transplant) identified which increased the risk of retransplant failure. There may be differences amongst those transplanted for HCV infection whose grafts fail due to recurrent disease and those whose grafts fail for other reasons although this, too, is controversial[10,20]. Overall, there is no consensus on the role of retransplantation for recurrent HCV infection.

In recent years in the United Kingdom (UK) the number of transplants performed has fallen whilst the number of patients waiting has risen by over 90%[20]. Because of the disparity between the numbers of donor organs and potential recipients, rationing must occur and selection criteria for transplant and retransplant are in place in transplant programs around the world. In the UK a meeting of health care professionals, patient representatives, ethicists and the public agreed an expected transplant survival probability of at least 50% at 5 years following liver transplantation as a benchmark of minimum expected survival following liver transplantation[21].

Liver retransplantation in patients with HCV infection is controversial and has been inconsistently associated with transplant survival rates of less than 50% at 5 years. The aim of this current study was therefore to determine overall outcomes after retransplantation in patients with HCV infection in the UK. In addition, we sought to determine whether failure of the first graft due to recurrent HCV-related disease had a significant effect on survival after retransplantation, and whether we could identify factors which predict a poor outcome.

MATERIALS AND METHODS

Data were obtained from the National Transplant database. This database is maintained by the Organ Donation and Transplantation Directorate of NHS Blood and Transplant on behalf of transplant services in the UK and the Republic of Ireland. All 944 patients aged 17 years and older undergoing first elective liver transplants for HCV-related liver disease in the UK between 1 March 1994 and 31 December 2007 were included. Patients who were recorded as HCV-positive at time of registration and/or time of transplantation but who did not have an indication for transplantation recorded as HCV were excluded from the analyses. Also excluded were patients receiving grafts from non-heart-beating donors and patients with intestinal failure-related liver disease. Details of the indication were categorized into 4 groups: HCV alone, HCV and alcohol-related liver disease (ALD), HCV and hepatocellular carcinoma (HCC), and HCV and ALD and HCC. In addition, patients undergoing liver retransplantation for reasons other than HCV-related liver disease were identified to act as a comparison group.

Patient, graft and transplant survival were obtained for the 4 HCV groups using the Kaplan Meier estimation method. Survival was compared using the log-rank test. Patient survival was measured from the time of first liver transplant to patient death. Graft survival was defined as time from liver transplant (or retransplant) to graft failure (patient deaths with a functioning graft were censored). Transplant survival was defined as time from transplant (or retransplant) to patient death or graft failure whichever was the earlier (deaths with a functioning graft were included as events).

Graft and transplant survival for the 80 retransplanted patients were obtained using the Kaplan-Meier method. Univariate Cox regression models were used to identify factors associated with liver transplant survival after retransplantation, where the first transplant failed due to disease recurrence. Factors considered were donor age, blood group, gender, height and weight, and recipient age, blood group, gender, height and weight, HCV group for first graft, MELD score[22] at time of second graft, year of

Table 1 Comparison of peer-reviewed studies on retransplantation outcome in patients with hepatitis C virus infection

Author	Design	Number with HCV infection	Outcome	Predictors
Database studies				
Rosen *et al*[11]	UNOS database	HCV total 357	54% 5-yr survival	HCV associated with mortality (HR 1.3)
Watt *et al*[12]	UNOS database	HCV total 899	45% 5-yr survival	MELD > 25
Petellier *et al*[13]	SRTR database	HCV total 464	NA	HCV associated with mortality (HR 1.3)
Ghabril *et al*[14]	UNOS database	HCV total 1034	rOLT 90 d-1 yr: 44% 3-yr survival rOLT > 1 yr: 59% 3-yr survival	MELD > 25 Donor > 60 Donor black WIT > 75 min
Rowe *et al*, current study	UK Transplant database	34 recurrent HCV 46 HCV	Recurrent HCV rOLT 5-yr graft survival 45% HCV rOLT other reasons HCV 5-yr graft survival 80%	None
Center-led studies				
Roayaie *et al*[15]	Retrospective single center	HCV total 42	52% alive at 6 mo	Donor < 60 PT < 16 s
Neff *et al*[16]	Retrospective single center	HCV total 22	50% 1-yr survival	Normal physical activity
Carmiel-Haggai *et al*[17]	Retrospective single center	47 recurrent HCV 62 HCV	37% 1-yr mortality	Donor > 60 Cirrhosis FCH
McCashland *et al*[18]	Prospective multicenter	HCV total 43	3-yr survival 49%	MELD not predictive
Bahra *et al*[19]	Retrospective single center	18 recurrent HCV 11 HCV	Recurrent HCV rOLT 5-yr survival 59% HCV rOLT other reasons 5-yr survival 84%	MELD > 25 Bilirubin > 15 mg/dL
Ghabril *et al*[10]	Retrospective single center	25 recurrent HCV 48 HCV	63% 3-yr survival	Donor age > 60 Recipient > 60

UNOS: United Network for Organ Sharing; SRTR: Scientific registry of transplant recipients; HCV: Hepatitis C virus; rOLT: Retransplantation; MELD: Model for end-stage liver disease; PT: Prothrombin time; FCH: Fibrosing cholestatic hepatitis; WIT: Warm ischemia time.

first graft, year of second graft, cold ischemia time, hospitalization status, time from first graft to second graft and donor organ appearance. Missing values for donor height, donor weight, recipient height and weight, MELD score and cold ischemia time were replaced with the median value, and missing values for hospitalization status and donor organ appearance were replaced with the modal value.

RESULTS

Patient characteristics

Of the 944 patients transplanted for HCV-related liver disease during the study period, the majority (n = 521, 55%) were transplanted for HCV alone and for HCV with HCC (n = 261, 28%). In total, 698 (74%) patients were alive at the end of the study period. The 5-year patient survival estimate for patients transplanted for HCV alone was 70% [95% confidence interval (CI): 65%-74%] and for patients transplanted for HCV with HCC the 5-year patient survival was 63% (95% CI: 55%-69%).

Of those transplanted, 617 (65%) first grafts were still functioning at the end of the study period, 133 (14%) patients died with a functioning graft and 194 (21%) grafts failed. In total, the reason for graft failure was indicated as recurrent HCV in 77 patients, and other causes, most commonly acute vascular occlusion and chronic or ductopenic rejection, in 117 patients. Where the first graft failed, 89 patients (45.9% of those with graft failure) were relisted and 80 (41.2%) were retransplanted. Of the 80 patients retransplanted, the reason for failure of the first graft was recorded as recurrent disease in 26 cases, and other reasons in 54 cases. Further analysis of the other reasons for graft failure, as recorded in free text by transplant centers, indicated that graft failure of 8 additional patients was considered to be due to disease recurrence giving a total of 34 patients retransplanted for recurrent disease and 46 patients retransplanted for other reasons. There was no difference in the proportion of patients retransplanted where the first graft failed due to recurrent disease or due to other reasons (χ^2 test, P = 0.76).

Outcome after liver retransplantation

The clinical information of those patients who underwent retransplantation after first liver transplant for HCV-related liver disease is given in Table 2. The groups were similar in demographic data and severity of liver disease at the time of retransplantation. However, those patients retransplanted for recurrent HCV were less likely to be hospitalized at the time of retransplantation. Those retransplanted for reasons other than recurrent HCV also had a shorter interval between first and second liver transplant and the majority of those patients were retransplanted for acute vascular occlusion (n = 17, 37.0%) and primary graft non-function (n = 11, 23.9%). These 28 patients were transplanted as an emergency and these indications contributed largely to the shortened interval between first and second graft and the increased likelihood of hospitalization seen in this group. The remainder of the patients retransplanted for reasons other than recurrent disease were retransplanted for late vascular complications (6 patients),

Table 2 Characteristics of those patients undergoing retransplatation (mean ± SD) *n* (%)

Characteristic	First graft failed due to		*P*-value
	Recurrent disease	Other reasons	
n	34	46	
Recipient age (yr)	50.0 ± 8.16	53.1 ± 8.14	0.090
Recipient blood group			
O	11 (32.3)	18 (39.1)	0.800
A	16 (47.1)	18 (39.1)	
B	4 (11.8)	7 (15.2)	
AB	3 (8.8)	3 (6.5)	
Recipient gender, male	27 (79.4)	33 (71.7)	0.400
Recipient height (cm)	173.3 ± 9.72	169.6 ± 9.94	0.090
Recipient weight (kg)	81.0 ± 13.93	77.6 ± 17.48	0.400
HCV group for first graft			
HCV alone	25 (73.5)	24 (52.2)	
HCV + HCC	3 (8.8)	16 (34.8)	0.030
HCV + ALD	6 (17.6)	6 (13.0)	
MELD score	19.9 ± 5.08	22.1 ± 10.79	0.280
Year of first graft			
1994-2000	23 (67.6)	22 (47.8)	0.080
2001-2007	11 (32.4)	24 (52.2)	
Year of second graft			
1994-1998	7 (20.6)	14 (30.4)	0.060
1999-2003	13 (38.2)	24 (52.2)	
2004-2007	14 (41.2)	8 (17.4)	
Cold ischemia time (h)	11.9 ± 2.61	9.7 ± 3.29	0.020
Hospitalization status, Outpatient	19 (55.9)	4 (8.7)	< 0.001
Time from first graft to second graft (yr)	2.8 ± 2.17	0.38 ± 1.03	< 0.001
Donor organ appearance, normal	28 (82.4)	40 (87.0)	0.600

SD: Standard deviation; HCC: Hepatocellular carcinoma; ALD: Alcoholic liver disease.

biliary complications (3 patients), chronic or ductopenic rejection (3 patients), or other reasons (4 patients).

Overall graft survival for patients first transplanted for HCV and subsequently retransplanted for recurrent HCV was lower than for those retransplanted for other reasons, as shown in Figure 1A. Five-year graft survival was estimated as 45% (95% CI: 24%-64%) for the recurrent disease group, compared with 80% (95% CI: 62%-90%) for those retransplanted for other reasons (P = 0.01). However, there was no difference in overall transplant survival between these 2 groups, as shown in Figure 1B. Five-year transplant survival in those with recurrent disease was 43% (95% CI: 23%-62%) and 46% (95% CI: 31%-60%) in those retransplanted for other reasons (P = 0.8). The difference between graft and transplant survival between these 2 groups was related to deaths occurring with a functioning graft: one death in the patients retransplanted for recurrent disease and 17 deaths in the patients retransplanted for other reasons. The cause of death in these patients was largely multiple organ failure and infective complications.

Following retransplantation for recurrent HCV, the total number of grafts lost was 16 (47%). The most frequent causes of graft loss were acute vascular occlusion (n = 4), recurrent disease (n = 3) and chronic rejection (n = 2).

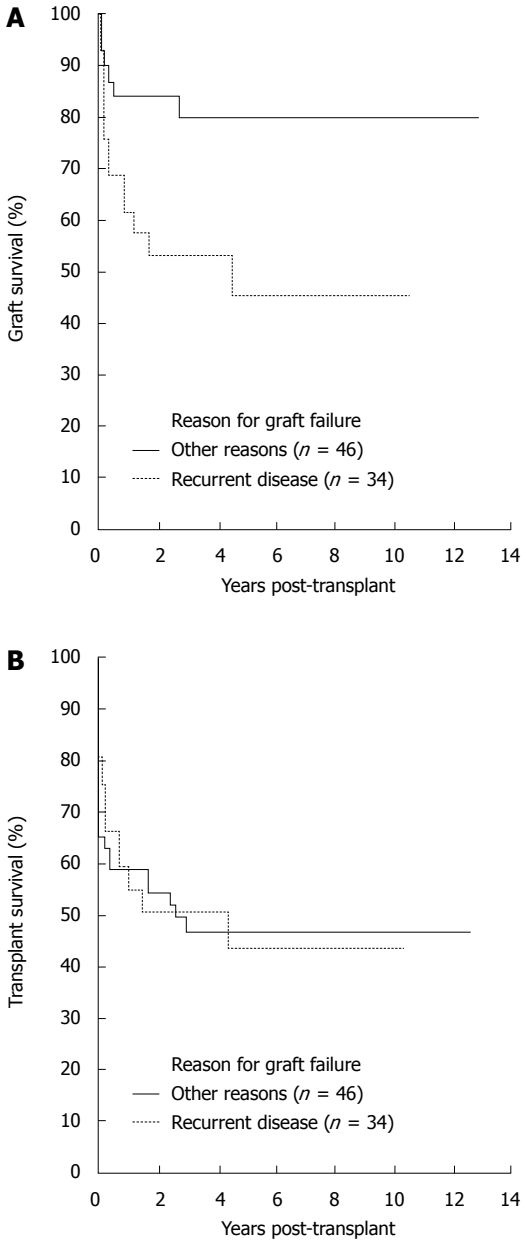

Figure 1 Graft survival is reduced in patients undergoing liver retransplantation for recurrent hepatitis C virus related disease. Kaplan-Meier survival curves were generated for A: Graft survival after second liver transplant in hepatitis C virus (HCV)-infected patients; B: Transplant survival after second liver transplant in HCV-infected patients. Graft or transplant survival is indicated for those retransplanted for recurrent HCV-related liver disease (dotted line) and for those retransplanted for reasons other than HCV-related liver disease.

Factors predicting outcome after liver retransplantation

Several factors including pre-transplant disease severity and donor factors have previously been identified as predictors of poor outcome after liver retransplantation in patients with HCV infection. These factors, together with other factors considered to impact on retransplant outcome including the timing of liver retransplantation after first liver transplant, were considered in univariate Cox regression analyses in those patients retransplanted for HCV-related liver disease. None of the factors considered in these analyses, including donor age and MELD score at the time of retransplantation, was found to be associated

Table 3 Predictive factors for transplant survival following liver retransplantation for patients with hepatitis C virus infection

Variable	Category	*n*	Hazard ratio (95% CI)	*P* value
Recurrent disease	No	46	1	
	Yes	34	0.92 (0.49-1.73)	0.8
Donor age (yr)	Linear	80	1.00 (0.98-1.03)	0.7
Donor blood group	O	43	1	
	A, B, AB	37	0.91 (0.49-1.70)	0.8
Donor gender	Male	37	1	
	Female	43	1.23 (0.66-2.29)	0.5
Donor height (cm)	Linear	80	0.98 (0.95-1.01)	0.3
Donor weight	Linear	80	0.98 (0.96-1.01)	0.2
Recipient age (yr)	Linear	80	0.99 (0.96-1.03)	0.6
Recipient blood group	O	29	1	
	A, B, AB	51	1.00 (0.52-1.91)	0.99
Recipient gender	Male	60	1	
	Female	20	1.38 (0.70-2.72)	0.4
Recipient height (cm)	Linear	80	0.98 (0.95-1.01)	0.1
Recipient weight (kg)	Linear	80	0.99 (0.98-1.02)	0.6
HCV group for first graft	HCV alone	49	1	
	HCV + HCC	19	2.15 (1.07-4.30)	0.03
	HCV + ALD	12	1.13 (0.46-2.81)	0.8
MELD score	Linear	80	1.00 (0.96-1.05)	0.9
Year of first graft	Linear	80	1.04 (0.89-1.21)	0.6
Year of second graft	Linear	80	0.96 (0.87-1.06)	0.47
Cold ischemia time (h)	Linear	80	0.99 (0.92-1.06)	0.74
Hospitalization status	Outpatient	24	1	
	Inpatient	56	1.36 (0.66-2.78)	0.4
Time from first to second graft (yr)	Linear	80	1.04 (0.89-1.21)	0.6
Donor organ appearance	Normal	68	1	
	Abnormal	12	1.04 (0.44-2.47)	0.9

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; MELD: Model for end-stage liver disease; ALD: Alcoholic liver disease.

with transplant survival after retransplantation in patients with HCV, as shown in Table 3.

DISCUSSION

For those patients developing graft failure, and in particular from recurrent HCV-related liver disease, the outlook is poor and there is no alternative to consideration of liver retransplantation. The outcome of patients retransplanted with chronic HCV infection is relatively poor, and, in the context of organ shortage, use of scarce resources for this indication is, therefore, controversial. As the number of patients being transplanted for HCV is increasing, so is the number of patients with graft failure due to recurrent HCV infection. Previous studies are conflicting, with some suggesting such a poor outcome in these patients that use of a liver graft cannot be justified[16,23]. More recent reports however have stated that patients should not be denied retransplantation merely based on a diagnosis of chronic HCV infection[10,14,18]. Furthermore, repeated analyses of the UNOS database initially demonstrated that HCV infection had a negative impact on survival after liver retransplantation but later studies did not support this finding[14] suggesting that, with increased experience, transplant centers may have improved outcomes for patients with graft failure and HCV infection. It should be noted that there is inconsistent reporting of confidence intervals of survival rates in these studies and these findings have contributed to the controversy regarding the utility of retransplantation in HCV infection.

The shortage of donor organs has focused attention on the optimal use of liver allografts. A consensus meeting of UK surgeons and physicians, health care professionals, ethicists, patients and the public agreed a minimum transplant survival of 50% at 5 years[21]. This is in keeping with the statement from the International Liver Transplantation Society Expert Panel who suggested a 1-year survival rate of at least 60% should be anticipated before listing a patient for retransplantation with recurrent HCV-related disease[24]. This guidance allows for the best use of a scarce resource, and also gives much needed transparency to transplant programs. Importantly, this will however disadvantage some patients who would otherwise have gained some benefit from transplantation.

In this study, representing a highly selected patient population, the 5-year transplant survival for patients with chronic HCV infection who were retransplanted was less than 50%, regardless of the cause of failure of the first graft, although the confidence intervals reported here were wide due to the relatively small numbers of patients included. However, in patients retransplanted for recurrent HCV, 5-year graft survival was 45%. In contrast, the 5-year graft survival was 80% in those transplanted for reasons other than recurrence. This discrepancy between graft and transplant survival in patients retransplanted for reasons other than recurrent HCV is likely to be explained in part by the short interval between first and second liver transplant. Many of these patients are, by definition, not fully recovered from the first transplant, and the proportion of patients dying from multiple organ failure and infective complications likely reflect this. This important observation indicates that, of the patients retransplanted for reasons other than HCV disease recurrence, those who survive the early weeks after transplantation have a relatively good long-term outlook.

In total, 5% of all patients retransplanted with HCV infection lost their second graft to recurrent disease, including 3 of 34 (8.8%) patients retransplanted for recurrent HCV-related disease. This is a clinically relevant proportion of these patients and this calls into question findings of previous studies addressing this. Since transplant failure following retransplantation is greater than after first transplant regardless of indication, factors that further impact on transplant survival after retransplantation are thus important. The timing of recurrent disease in the first liver transplant has been suggested to impact on the prevalence of recurrent disease in the second liver transplant in this group[25], although this too has been questioned[17]. In this study a large proportion of patients were retransplanted between 1 and 3 years after the first liver transplant. The small numbers of patients transplanted at times other than this has meant that we have been unable to reliably investigate this matter.

Several studies have investigated factors associated with

graft loss in HCV infection following retransplantation. These have determined negative predictors of mortality in the recipient, including MELD score greater than 25, elevated serum creatinine and bilirubin, and prolonged prothrombin time[7,14]. In addition, donor age greater than 60 years has consistently been associated with negative outcomes in individuals retransplanted with HCV infection. Analyses of these factors in this study did not show any association with transplant survival. This is likely to be related, at least in part, to the relatively low numbers of patients retransplanted with HCV infection, the heterogeneity of this group as well as the skill of the surgeon in matching donor and recipient. At present, in the UK, there are national guidelines for selection of patients but the allocation of donor livers is done on a center-specific basis with the surgeon determining the most appropriate recipient for a liver donated from a heartbeating or non-heartbeating donor. Similarly, there are center-specific protocols regarding the management of recurrent HCV infection after liver transplantation. In this study, the details of these protocols and whether patients being considered for liver retransplantation had received antiviral therapy were not recorded. However, widespread use of combination antiviral therapy has only recently entered clinical practice and although we saw no clear effect of year of first or second liver transplant on transplant outcome, it is likely too soon to assess what effect this strategy may have.

In this national study of transplant outcome after retransplantation for HCV, we have shown that transplant survival rates fall below those normally accepted for survival after transplant, regardless of whether retransplantation was related to HCV recurrence or not. Furthermore, we have not been able to demonstrate any factors associated with increased, or indeed decreased, transplant failure. Although numbers are relatively small, this series is one of the largest conducted.

Whilst there is much work focused on the early identification of individuals with rapid development of fibrosis after transplantation[26], and antiviral treatment outcomes may be improving[27], over the coming years many of those transplanted with chronic HCV infection will develop graft failure and the question of retransplantation will be raised. Previously published studies are conflicting and this study adds to the evidence base supporting selective retransplantation in the setting of chronic HCV infection. Clear and careful consideration must therefore be given when assessing these individuals for retransplantation.

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COMMENTS

Background

Hepatitis C virus (HCV) infection is common and is a leading indication for liver transplantation. Following transplantation, however, HCV infects the transplanted liver and disease recurrence is rapid and may lead to transplant failure. This inevitably leads to questions regarding the utility of repeat liver transplantation. It is not clear whether liver retransplantation is appropriate in patients with either recurrent HCV or with HCV who require liver retransplantation for other reasons.

Research frontiers

There is significant controversy as to whether patients with HCV infection should undergo liver retransplantation, and if so which patients would be most appropriate.

Innovations and breakthroughs

The authors demonstrate that those patients with recurrent HCV infection undergoing liver retransplantation fare worse, particularly with regard to graft survival, than those undergoing liver retransplantation for other reasons. However, at 5 years after liver retransplantation, transplant survival in both groups is at the limit of acceptability (50% survival at 5 years after transplant).

Applications

This study adds weight to the evidence supporting selective liver retransplantation in patients with HCV infection.

Terminology

Graft survival is defined as time from liver transplant (or retransplant) to graft failure. In this case a patient death with a functioning graft is not considered as an event. Transplant survival however is defined as time from transplant (or retransplant) to patient death or graft failure whichever was the earlier.

Peer review

This is a well-designed and structured manuscript addressing a controversial issue and is worth publication.

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BRIEF ARTICLE

Partially covered *vs* uncovered sphincterotome and post-endoscopic sphincterotomy bleeding

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Abstract

AIM: To prospectively compare partially covered *vs* uncovered sphincterotome use on post-endoscopic biliary sphincterotomy (ES) hemorrhage and other complications.

METHODS: All patients referred for therapeutic endoscopic retrograde cholangiopancreatography (ERCP) were randomly assigned to undergo ES either with a partially covered or an uncovered sphincterotome. Both patient and technical risk factors contributing to the development of post-ES bleeding were recorded and analyzed. The characteristics of bleeding was recorded during and after ES. Other complications were also compared.

RESULTS: Three-hundred and eighty-seven patients were recruited in this study; 194 patients underwent ES with a partially covered sphincterotome and 193 with conventional uncovered sphincterotome. No statistical difference was noted in the baseline characteristics and risk factors for post-ES induced hemorrhage between the 2 groups. No significant difference in the incidence and pattern of visible bleeding rates was found between the 2 groups (immediate bleeding in 24 patients with the partially covered sphincterotome *vs* 19 patients with the uncovered sphincterotome, $P = 0.418$). Delayed bleeding was observed in 2 patients with a partially covered sphincterotome and in 1 patient with an uncovered sphincterotome ($P = 0.62$). No statistical difference was noted in the rate of other complications.

CONCLUSION: The partially covered sphincterotome was not associated with a lower frequency of bleeding. Also, there was no difference in the incidence of other significant complications between the 2 types of sphincterotome.

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Key words: Sphincterotome; Endoscopic sphincterotomy; Hemorrhage; Complications

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INTRODUCTION

Endoscopic biliary sphincterotomy (ES) is a high-risk procedure with considerable possibility of complications[1]. Endoscopists with experience in ES are well aware that major problems can occur during the procedure, causing morbidity and occasionally death[2]. It is likely that the incidence of ES complications i.e. immediate (intraprocedural) and delayed bleeding (1%-10%)[2-6], pancreatitis (1%-3%)[4,7,8] or perforation (1%-2%)[4,7,8] can be reduced if cutting is more controlled. Apart from the skill and training of the endoscopist, definite risk factors that increase the risk of post-ES bleeding include coagulopathy, anticoagulation < 3 d after ES, cholangitis prior to ES and low endoscopic retrograde cholangiopancreatography (ERCP) case volume[9-11]. In a recent study[12] to investigate the efficacy of double injection of 50% dextrose plus epinephrine solution (1:10 000) in endoscopic hemostasis of post-ES bleeding, we found the "zipper-cut" phenomenon to be the sole significant risk factor for bleeding. In an effort to achieve a more controlled cutting and to avoid the "zipper-cut" phenomenon, a special sphincterotome has been manufactured having as its main characteristic a half-length of cutting wire insulated with polymeric plastic.

To date, there have been no studies comparing the 2 different types of sphincterotomes with regard to reduction in the incidence of post-ES bleeding or other complications. We hypothesized that, contrary to a conventional uncovered sphincterotome, the use of a partially covered sphincterotome, with the theoretical advantages of more controlled cutting and avoidance of the "zipper-cut" phenomenon, might reduce post-ES bleeding, one of the most frequent post-ES complications associated with morbidity[13]. Therefore, this prospective study was undertaken to compare partially covered *vs* uncovered sphincterotome use on post-ES hemorrhage and other complications.

MATERIALS AND METHODS

Study population

The study was carried out between September 2007 and September 2008. The patients enrolled came from our institutions or were referred to us from other hospitals of Northern and Central Greece for therapeutic ERCP. The study was approved by our institutions' Ethics Committees and each patient or his/her relatives gave written informed consent prior to ERCP. The following inclusion criteria were confirmed by the study coordinators: age > 18 years; no pregnancy; no evidence of significant cardiorespiratory or other medical conditions precluding participation (i.e. deemed able to tolerate routine sedation); and no known documented allergy to lidocaine anesthetic spray, meperidine, midazolam, propofol, or contrast agent. Patients were excluded using the following criteria: refusal to participate, failure to cannulate the common bile duct (CBD), and known gastroduodenal anatomic abnormalities (Billroth Ⅱ or Roux-en-Y operation and bariatric surgery). Patients undergoing needle-knife papillotomy were also excluded from the study. In contrast, patients undergoing needle-knife fistulotomy with extension of sphincterotomy with a sphincterotome were included in the study. All patients received a pre-procedural assessment which included a detailed medical and drug history, physical examination, complete blood count, serum liver biochemistry, coagulation studies, abdominal ultrasound or computed tomography and magnetic resonance imaging cholangiography. Specifically, demographic data recorded before ERCP included indications for ES and risk factors for post-ES bleeding as demonstrated in Table 1. In cases of prolonged prothrombin time (> 3 s from upper limit of normal) or platelet count < 70 000/mm^3 the patients received plasma or platelet transfusions, respectively, until normal prothrombin time and platelet count > 70 000/mm^3 were achieved. During ERCP, endoscopic evidence of periampullary diverticulum, length of ES, extension of previous ES and use of precut papillotomy (transpancreatic, needle-knife) or suprapapillary fistulotomy with extension of ES were also recorded.

Table 1 Risk factors for hemorrhage after endoscopic sphincterotomy[11]

Definite[1]	Maybe[2]	No[3]
Coagulopathy	Cirrhosis	ASA or NSAID
Anticoagulation < 3 d after ES	Dilated CBD	Ampullary tumor
Cholangitis prior ERCP	CBD stones	Longer length sphincterotomy
Bleeding during ES	Periampullary diverticulum	Extension of prior ES
Lower ERCP case volume	Precut sphincterotomy	

[1]Significant by multivariate analysis in most studies; [2]Significant by univariate analysis only in most studies; [3]Not significant by multivariate analysis. ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct; ES: Endoscopic sphincterotomy; ASA: Acetyl salicylic acid; NSAID: Nonsteroidal antiinflammatory drug.

Randomization and blinding process

Patients were randomized to undergo ES with either a partially covered sphincterotome (Clever-cut, Olympus, Tokyo, Japan; cost 220 euros) or an uncovered sphincterotome (Autotome, Microvasive, Boston Scientific, USA; cost 160 euros). The proximal half-length of the cutting wire of the partially covered sphincterotome is insulated (Figure 1). The insulation is bonded to the cutting wire and is made of polymeric plastic, which has good electrical insulating properties, is heat resistant and can be autoclaved. The insulated section is colored to minimize iris closure when using a videoscope and contrasts with the silver color of the cutting wire for easy visibility. The device is designed to take a 0.035 inch guidewire, but any wire of lesser diameter can be used. Contrast medium can

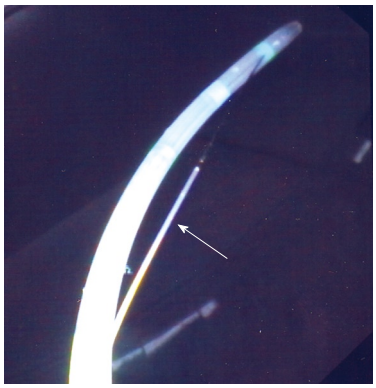

Figure 1 Partially covered sphincterotome with the polymeric plastic (arrow) covering half the length of the cutting wire.

be injected through the guidewire channel using a side arm valve with the guidewire in place. The proximal part of the wire is not used for cutting. Insulation is provided to avoid contact with the endoscope, but more importantly, to avoid contact with the medial wall of the duodenum anterior to the papilla, and to avoid inadvertent cutting of a low-lying duodenal fold or of the roof of a periampullary diverticulum. Proximal insulation also encourages the endoscopist to use only the distal portion of the wire for cutting, thereby maximizing current density, and minimizing the injury of the surrounding tissues even in exceptional difficult cases as reported by the manufacturer.

Assignments were prepared in a 1:1 proportion by an independent biostatistician using a computer-generated random numbers program. Allocation was concealed using an opaque envelope system. A trainee who was not participating in the assessment of the study outcomes carried out the randomization based on the assigned study number, which allowed allocation to remain concealed from the investigators, patients and research coordinators, who completed the assessment of the clinical outcomes. It was not possible for the endoscopists to be blinded to the study group allocation following randomization, as the 2 sphincterotomes can be readily distinguished based on their appearance.

Endoscopic intervention

All procedures were performed by 2 experienced endoscopists (PK, GP), without the participation of trainees. Procedures were performed with the patients in the oblique left lateral position using different types of Olympus duodenoscopy. The patients were given a topical throat spray with 10% lidocaine and sedated with intravenous midazolam and meperidine or propofol, all titrated according to the age and tolerance of each patient. Bowel relaxation was achieved with intravenous administration of hyoscine butylbromide or glucagon. The patients were given continuous nasal oxygen and their hemoglobin saturation and pulse rate were monitored with pulse oximetry. Cannulation of CBD was attempted initially with the sphincterotome, then with the assistance of a hydrophilic guidewire (Jagwire, Microvasive, Boston Scientific, USA) if cannulation by sphincterotome was unsuccessful. When the cannulation with both methods was unsuccessful, precut techniques (suprapapillary fistulotomy, transpancreatic and needle-knife sphincterotomy) were used. ES was performed by a controlled cut in a stepwise fashion using short pulses of current. The length of the ES was dependent upon the indication: small for stent placement or the maximum possible for choledocholithiasis. It is a policy in our units to perform ES by completely dividing the sphincter and by extending the incision to the maximum safe length in patients with choledocholithiasis. We believe this minimizes the risk of subsequent ampullary stenosis. All ESs were performed using an Olympus electrosurgical unit (PSD 30) and a blended current. The power output was set at 45-30 W. The device was periodically assessed for proper functioning, according to the protocol of our hospitals' biomedical services.

Study outcomes

The primary endpoint was the assessment of post-ES bleeding, according to the risk factors for hemorrhage after ES published by Freeman (Table 1)[11], with the addition of the "zipper-cut" phenomenon in the definite risk factors for post-ES bleeding on the basis of the findings of our previous study[12].

Postsphincterotomy bleeding was classified as immediate (intraprocedural) or delayed bleeding. Immediate bleeding was defined as: "none" if no blood was seen; "trickle" if blood was evident; "oozing" if a perceptible blood stream was present; and "pulsatile" if arterial bleeding was evident. Delayed bleeding was defined as hemorrhage not evident at the time of sphincterotomy but which presented subsequently as melena or hematemesis associated with a reduction in hemoglobin. The severity of the bleeding was graded as mild: clinical or endoscopic evidence of bleeding only without blood transfusion; moderate: endoscopic evidence of bleeding requiring endoscopic therapy and blood transfusion of 4 units or less; and severe: significant bleeding requiring surgery or radiographic embolization for control of bleeding or a total blood transfusion of 5 units or more.

The secondary outcome measures included any complication occurring secondary to the ERCP procedure. Post-ERCP pancreatitis was defined as new or exacerbated abdominal pain attributable to pancreatitis, together with a need for an unplanned hospitalization or an extension of a planned hospitalization and a serum amylase at least 3 times above the upper limit of normal at 24 h after the procedure. Perforation was defined as the presence of free air on any radiographic test. Cholangitis was defined as a fever of > 38℃ for more than 24 h that was thought to be due to biliary causes.

After the discharge from our endoscopic units, each patient's clinical status was monitored by a phone call; if the patient showed clinical symptoms and signs of bleeding, including hematemesis, melena, hematochezia or hypertension, an emergency endoscopic evaluation was carried out for further management.

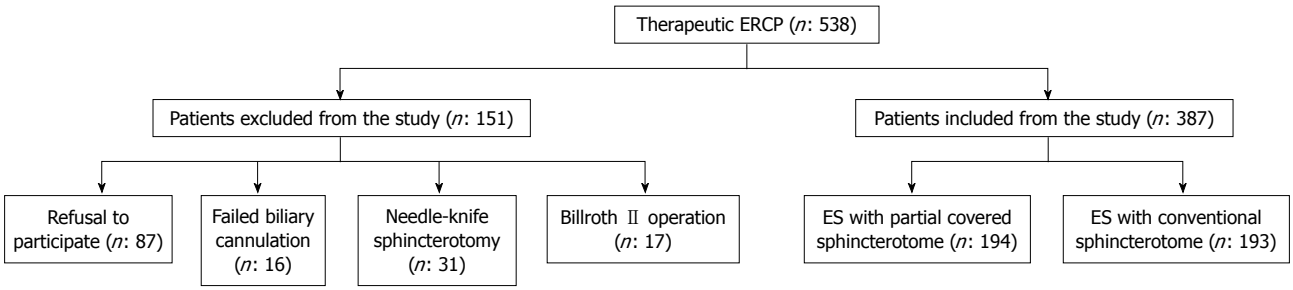

Figure 2 Flowchart showing all patients who underwent therapeutic endoscopic retrograde cholangiopancreatography during the study period. ERCP: Endoscopic retrograde cholangiopancreatography; ES: Endoscopic biliary sphincterotomy.

Table 2 Baseline characteristics of patients

	Partially covered sphincterotome (*n* = 194)	Uncovered sphincterotome (*n* = 193)	*P*
Sex (M/F)	85/109	89/104	0.640
Age (mean ± SD) (yr)	69.93 ± 13.85	70.71 ± 13.75	0.590
Indications for ES			0.304
Choledocholithiasis	143	136	0.382
Pancreatic malignancy	20	26	0.356
Biliary malignancy	11	8	0.473
Papillary cancer	7	6	0.771
SOD	8	5	0.392
Bile leak	2	3	0.657
Metastatic lymphadenopathy	1	4	0.187
Others	2	5	0.255
Associated comorbid disease			0.561
Coronary artery disease	11	7	0.329
Hypertension	31	42	0.160
Diabetes	22	24	0.768
Heart failure	21	24	0.647
COPD	9	10	0.823
Renal failure	4	6	0.527
Renal failure in hemodialysis	2	-	0.155
Others	53	61	0.390

ES: Endoscopic sphincterotomy; SOD: Sphincter of Oddi dysfunction; COPD: Chronic obstructive pulmonary disease.

Table 3 Risk factors for post-endoscopic sphincterotomy bleeding between the two groups

	Partially covered sphincterotome (*n* = 194)	Uncovered sphincterotome (*n* = 193)	*P*
Definite			
Anticoagulation < 3 d after ES	3	5	0.365
Bleeding during ES	24	19	0.408
Cholangitis prior to ERCP	26	21	0.425
"Zipper cut" phenomenon	4	3	0.497
Maybe			
Papillary cancer	7	6	0.771
Cirrhosis	5	3	0.359
Dilated CBD	72	74	0.865
CBD stone	143	136	0.382
Periampullary diverticulum	38	44	0.472
Precut (suprapapillary) sphincterotomy with extension	10	6	0.302

ES: Endoscopic sphincterotomy; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct.

Confounding variables

Clinical factors assessed to ensure equal distribution between study groups included demographic characteristics, indication for procedure, risk factors for post-ES bleeding and malfunction of any endoscopic equipment or sphincterotome.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS, version 13.0, Chicago, IL, USA). The continuous variable of age was examined with the Student *t*-test, whereas all categorical variables were analyzed with the χ^2 and Fisher's exact tests, as appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

During the study period 538 patients underwent therapeutic ERCP. Of these, 151 were excluded due to refusal to participate (87), previously performed needle-knife sphincterotomy (31), previous Billroth Ⅱ operation (17) and failed biliary biliary cannulation (16) (Figure 2).

A total of 387 patients were included in the study. Of these, 193 underwent ES using the uncovered sphincterotome and 194 patients underwent ES using the partially covered sphincterotome. Baseline characteristics of the patients and risk factors of post-ES hemorrhage were similar in both groups (Tables 2 and 3).

ES-visible hemorrhage was seen in 24 patients (12.37%) of the partially covered and in 19 patients (9.84%) of the uncovered group ($P = 0.418$). The endoscopic-visible bleeding patterns were: uncovered sphincterotome group [12 trickle (6.22%) and 7 oozing (3.63%)]; partially covered sphincterotome group [14 trickle (7.22%), 9 oozing (4.64%) and 1 pulsatile (0.52%)] (Table 4). Risk factors for post-ES hemorrhage in bleeders between the 2 groups were similar (Table 5). Three patients (2 in the partially covered with renal failure undergoing hemodialysis and 1 in the uncovered group) who underwent ES for choledocholithiasis and had no evidence of bleeding at the time of ES, presented with melena after 6, 7 and 9 d respectively. Bleeding from the ES site was confirmed at repeat endoscopy with raw red surfaces at the edges of the ES in 2 patients and trickling in one. The patient with trickling responded to injection treatment with 2 mL D_{50}+E solution. Hemostasis was

Table 4 Patterns of post-endoscopic sphincterotomy bleeding

	Partially covered sphincterotome (*n* = 26)	Uncovered sphincterotome (*n* = 20)	*P*
Immediate	24	19	0.418
Trickle	14	12	0.748
Ooze	9	7	0.965
Pulsatile	1	-	0.558
Delayed	2	1	0.620

Table 5 Risk factors for post-endoscopic sphincterotomy hemorrhage of patients presenting with immediate bleeding

	Partially covered sphincterotome (*n* = 24)	Uncovered sphincterotome (*n* = 19)	*P*
Definite			
Anticoagulation < 3 d after ES	2	3	0.373
Cholangitis prior to ERCP	7	5	0.883
"Zipper cut" phenomenon	1	1	1.000
Maybe			
Cirrhosis	1	-	0.565
Dilated CBD	12	14	0.106
CBD stone	21	16	0.590
Periampullary diverticulum	6	7	0.373
Precut (suprapapillary) sphincterotomy with extension	-	1	0.435

ES: Endoscopic sphincterotomy; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct.

successfully treated by spray irrigation of D50+E solution in all patients (26/26) with trickle and in 9 of 16 patients (56.25%) with oozing. Injection of D50+E solution was required in 7 patients (43.75%) with oozing who did not respond to solution irrigation. The spurting bleeding in 1 patient was treated with injection of 3 mL D50+E solution and electrocoagulation with the tip of a polypectomy of the bleeding vessel. The volume of D50+E solution injected ranged from 2 to 5 mL (median volume 3.1 mL). Overall successful hemostasis was achieved in all patients. None of the ES-induced bleeding episodes required transfusions or repeat endoscopy for hemostasis and therefore none was classified as a significant complication. None of the patients with ES-bleeding had a platelet count less than 100×10^9/L and an international normalized ratio greater than 1.5. No patient treated with D50+E injection developed pancreatitis or cardiac complications. In addition, the incidence of pancreatitis, perforation or other complications did not differ between the 2 groups (Table 6).

DISCUSSION

Short-term complications of ES, including bleeding, pancreatitis, and perforation can vary widely in different circumstances, and appear to be related principally to patient-related factors and the technical skill of the endoscopists[13,14]; both technical risk factors and patient risk factors contribute to the development of post-ES bleeding. The endoscopic technique is a major factor in complications, and this is in turn related to the case volume, and presumably the skill and training of the endoscopist. Endoscopists with adequate experience by performing large volumes of ERCP, learn to initiate cutting by using a short length of wire-tissue contact and reduce inefficient expenditure of energy by identifying optimal generator settings. After the first few millimeters of cutting, if a large length of cutting wire is in contact with papillary tissue, speed can be counterproductive, leading to rapid uncontrolled acceleration of cutting and increased risk of hemorrhage because the vessels are larger in the upper half of papilla[15,16]. Also, rapid cutting is related to less hemostasis because the wire is not in contact with tissue long enough to produce an effective zone of coagulative dissipation[17]. Another factor that increases the cutting rate may be the replacement of the liquid phase contact of the wire inside the bile duct with air as cutting progresses[18]. It is our belief, as well as that of others, that sphincterotomy is most safely and precisely achieved when only a short segment of cutting wire is in contact with the papilla. High current density is maintained over a very short segment of tissue allowing sphincterotomy to be performed under precise visual control and thereby minimizing total electrical energy requirement for sphincterotomy. To avoid an uncontrolled rapid cutting (the zipper effect) a sphincterotome has been introduced in the market with its main characteristic that half the length of the cutting wire is covered with polymeric plastic.

Table 6 Complications

	Partially covered sphincterotome (*n* = 194)	Uncovered sphincterotome (*n* = 193)	*P*
Pancreatitis	13	10	0.23
Mild	10	8	
Moderate	2	1	
Severe	1	1	
Perforation	1	-	0.50
Cholangitis	1	3	0.31
Bleeding	26	20	0.36
Mild	26	20	
Moderate	-	-	
Severe	-	-	
Basket impaction	1	-	0.50

To our knowledge, the present study is the first that compares a partially covered *vs* an uncovered sphincterotome on the incidence of post-ES-induced hemorrhage and other complications. With the exception of the significantly cheaper cost of the uncovered sphincterotome (160 euros *vs* 220 euros for the partially covered sphincterotome), we found no difference in the incidence, pattern (Table 4) and severity of post-ES bleeding between the 2 groups (Table 6). The risk factors for post-ES-induced hemorrhage of the bleeders of the 2 groups were comparable, presenting no statistical difference (Table 5). This can be explained by: performance of all procedures by 2 experienced endoscopists resulting in a controlled and

safe cut; type of cutting current (blended 40-30 W); and study design (prospective). It must be emphasized that, of all the risk factors for post-ES complications, the skill of the endoscopist who carries out the procedure is the most important one in determining the outcome of the procedure. Rabenstein *et al*[19] demonstrated that the case volume per year, rather than the total number or ERCPs performed, is one of the most important factors influencing the complication rate. A similar observation was made in the study of Freeman *et al*[4] in which endoscopists from 17 institutions who performed ES at least once per week had significantly better results compared with those at 12 centers who performed ES less than once per week. Salminen *et al*[10] also demonstrated that the rate of severe complications of ERCP is low in experienced hands at a high-volume center, comparing favorably to corresponding complication rates of multicenter series, which further supports the worth of centralizing ERCP procedures in high-volume advanced centers.

Two patients of the partially covered sphincterotome group with renal failure undergoing hemodialysis presented delayed post-ES hemorrhage (on the 7th and 11th day, respectively). Hemodialysis has not been previously recognized as a risk factor, possibly owing to the low proportion of hemodialysis patients referred for therapeutic ERCP. However, Nelson and Freeman, in an analysis of risk factors for major post-ES bleeding in 189 patients undergoing 191 ESs, found chronic hemodialysis to be the strongest predictor of major hemorrhage following ES (4/10)[20]. Renal failure and hemodialysis are associated with a number of hemostatic defects. Platelet function is altered in uremia and is only partially corrected by hemodialysis[21,22]. The routine use of anticoagulants during hemodialysis may further increase the risk of bleeding. Both patients in our study who presented mild delayed post-ES bleeding received heparin during the hemodialysis session prior to ES and low-dose heparin during dialysis after ES. We cannot determine whether bleeding resulted from underlying uremia or the systemic anticoagulation accompanying hemodialysis. In addition, the 3 patients with delayed post-ES bleeding did not present visible hemorrhage during ES, supporting the previous observation that the presence of oozing or trickling at the time of incision does not appear to be predictive of clinically significant delayed hemorrhage[12,17].

An interesting finding of our study was that immediate (trickling or oozing) or delayed post-ES hemorrhage did nor relate to the use of non-steroidal antiinflammatory drugs (NSAIDs) and clopidogrel. Equally, a case-control study provided controlled data suggesting that antiplatelet agents did not significantly increase the risk of clinically-important bleeding related to ES[23]. However, the low prevalence of these drugs in our study limits a definite conclusion on their elective use before ES. Guidelines from the American Society for Gastrointestinal Endoscopy published in 2002 concluded that aspirin and NSAIDs given in standard doses do not appear to increase the risk of significant post-ES bleeding[24]. Also, there is no consensus on whether it is necessary to discontinue NSAIDs and other antiplatelet agents, particularly in patients with other risk factors for post-ES bleeding.

Another interesting finding of our study was that immediate (oozing) post-ES hemorrhage responded to injection treatment with D_{50}+E solution, and overall successful hemostasis was achieved in all patients. The mechanism of action of E injection in post-ES bleeding is by exerting a local tamponade effect as well as localizing the E to cause constriction of the bleeding vessels and platelet aggregation to promote hemostasis. The addition of D_{50}, which has high viscosity, prolongs swelling and therefore both the tamponade and E effect.

In our study, we observed only 1 case of perforation in the partially covered sphincterotomy group, reflecting the experience of the endoscopists. The patient was treated conservatively with a good outcome. Duodenal perforation during ES often occurs during a rapid, poorly controlled cut of the sphincter beyond the boundaries of the intramural common bile duct. Clinically significant perforation occurs in less than 1% of ES although the risk for significant perforation may be as high as 8% in patients with small papilla or papillary stenosis[25,26]. Early diagnosis of duodenal perforation is essential for an optimum outcome, and subcutaneous emphysema may be a sensitive sign[27]. As in our case, others also reported that most duodenal perforations secondary to periampullary endoscopic interventions can be managed nonoperatively[28]. In addition, we found that endoclipping of duodenal perforation induced by ES is a safe, effective alternative to surgery treatment[29]. No statistical difference was noted in the rate of pancreatitis and cholangitis or other complications between the 2 groups (Table 6).

In conclusion, our study suggests no significant difference in post-ES hemorrhage and other complications between the partially covered and uncovered sphincterotome groups when ES is performed by experienced endoscopists. Because uncovered sphincterotome use appears to be cost-saving, further studies, which will include trainees or endoscopists with less experience, are needed to investigate the advertized theoretical beneficial role of the partially covered sphincterotome in post-ES complications.

COMMENTS

Background

Post-endoscopic biliary sphincterotomy (ES) bleeding is one of the most frequent post-ES complications associated with morbidity and rarely, with death. Uncontrolled cutting, known as the "zipper cut" phenomenon is the technical factor which contributes to the development of post-ES hemorrhage. In an effort to achieve a more controlled cutting and avoid the "zipper cut" phenomenon, a special sphincterotome has been manufactured having as its main characteristic half the length of the cutting wire insulated with a polymeric plastic.

Research frontiers

There are no studies comparing a partially covered *vs* uncovered sphincterotome with regard to reduction in the incidence of post-ES bleeding and other complications.

Innovations and breakthroughs

The present prospective randomized study investigated whether the use of a partially covered sphincterotome with the theoretical advantage of avoidance of the "zipper cut" phenomenon might reduce the incidence of post-ES hemorrhage. No significant differences in the incidence of intraprocedural and delayed post-ES

bleeding was found between the partially covered and uncovered sphincterotome group. In addition, no difference was noted in the rate of other complications.

Applications

When ES is performed by experienced endoscopists, using a partially covered or uncovered sphincterotome, no significant difference in post-ES bleeding and other complications are observed. However, further studies which will include trainees or endoscopists with less experience are needed to investigate the advertized theoretical beneficial role of the partially covered sphincterotome on post-ES complications.

Terminology

Zipper cut phenomenon is a rapidly poorly controlled cutting leading to post-ES hemorrhage; a partially covered sphincterotome, with half the length of the cutting wire insulated with a polymeric plastic, has an advertized advantage of avoidance of uncontrolled cutting during ES.

Peer review

The authors compared partially covered *vs* uncovered sphincterotome use on post-ES hemorrhage and other complications. They found that there was no significant difference between the 2 groups. The study is well designed and the data collected properly. Companies may claim that the partially covered sphincterotome has lower complications rate and they also encourage endoscopists to use it.

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BRIEF ARTICLE

Survival of geriatric patients after percutaneous endoscopic gastrostomy in Japan

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Abstract

AIM: To examine the long term survival of geriatric patients treated with percutaneous endoscopic gastrostomy (PEG) in Japan.

METHODS: We retrospectively included 46 Japanese community and tertiary hospitals to investigate 931 consecutive geriatric patients (≥ 65 years old) with swallowing difficulty and newly performed PEG between Jan 1st 2005 and Dec 31st 2008. We set death as an outcome and explored the associations among patient's characteristics at PEG using log-rank tests and Cox proportional hazard models.

RESULTS: Nine hundred and thirty one patients were followed up for a median of 468 d. A total of 502 deaths were observed (mortality 53%). However, 99%, 95%, 88%, 75% and 66% of 931 patients survived more than 7, 30, 60 d, a half year and one year, respectively. In addition, 50% and 25% of the patients survived 753 and 1647 d, respectively. Eight deaths were considered as PEG-related, and were associated with lower serum albumin levels (P = 0.002). On the other hand, among 28 surviving patients (6.5%), PEG was removed. In a multivariate hazard model, older age [hazard ratio (HR), 1.02; 95% confidence interval (CI), 1.00-1.03; P = 0.009], higher C-reactive protein (HR, 1.04; 95% CI: 1.01-1.07; P = 0.005), and higher blood urea nitrogen (HR, 1.01; 95% CI: 1.00-1.02; P = 0.003) were significant poor prognostic factors, whereas higher albumin (HR, 0.67; 95% CI: 0.52-0.85; P = 0.001), female gender (HR, 0.60; 95% CI: 0.48-0.75; P < 0.001) and no previous history of ischemic heart disease (HR, 0.69; 95% CI: 0.54-0.88, P = 0.003) were markedly better prognostic factors.

CONCLUSION: These results suggest that more than half of geriatric patients with PEG may survive longer than 2 years. The analysis elucidated prognostic factors.

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Key words: Percutaneous endoscopic gastrostomy; Enteral nutrition; Comorbidity; Survival; Risk factor

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) was initially developed as an enteral nutrition technique by Gaudere *et al*[1] and Ponsky *et al*[2] in 1980. Since then, PEG has been widely used for patients with swallowing difficulty, because of reduced laryngopharyngeal discomfort and a lower risk of aspiration pneumonia compared with a nasogastric tube[3,4]. However, PEG feeding was found to be associated with an absolute increase in risk of death compared with nasogastric feeding[5]. In spite of the evidence, PEG has been widely used especially for geriatric patients with the increasingly aging society. Despite the very large number of patients receiving this intervention, there is insufficient evidence to suggest that PEG is beneficial for patients with swallowing difficulty arising from dementia or other chronic diseases[6,7]. Therefore, we conducted retrospective studies in multiple community hospitals in Japan to examine the long-term survival of geriatric patients treated with PEG and to delineate the factors associated with their mortality.

MATERIALS AND METHODS

Study design and population

We conducted a retrospective cohort study of patients who underwent PEG between 2005 Jan 1st and 2008 Dec 31st at 46 community hospitals all over Japan, selected by the panel of 103 doctor-experts in PEG and the trustees of the PEG Doctors' Network as a non-profit organization. The study was approved by the institutional review board of each hospital. Doctors in charge of PEG in the selected hospitals were asked to examine 20 consecutive patients on whom a new PEG was performed, after excluding patients (1) whose age was ≤ 64 years old; (2) who had previously had a gastrectomy; (3) who had cancer considered to affect the patient's prognosis; and (4) who had gastrostomy performed for reasons other than nutritional support. The doctors were further asked to report the number of excluded cases as well as the number of patients who were considered as lost to follow-up.

Outcome measure

The primary outcome was set as death and the cut-off date was set at October 2009. In the case where the patient was alive, the doctor was further asked the status of the patient according to the following: (1) admission to this hospital, (2) admission in other hospital, (3) stay at nursing home, (4) stay at home, or (5) other. In case of loss to follow-up, the final date the patient was confirmed as alive was censured. Causes of death were selected from one from following: (1) primary diagnosis warranting PEG; (2) pneumonia; (3) cardiac failure; (4) cancer; (5) the PEG procedure; (6) other; and (7) unknown. The secondary endpoint was the removal of PEG because of improvement in the patient's condition.

Variables

The following data were collected: (1) age; (2) gender; (3) height; (4) weight; (5) body temperature; (6) white blood cell (WBC)/μL; (7) hematocrit (Ht): %; (8) hemoglobin (Hb): g/dL; (9) aspartate aminotransferase (AST): IU/L; (10) alanine aminotransferase (ALT): IU/L; (11) blood urea nitrogen (BUN): mg/dL; (12) serum creatinine (Cr): mg/dL; (13) serum albumin: g/dL; (14) C-reactive protein (CRP): mg/dL; (15) previous history of pneumonia or ischemic heart disease; (16) comorbidity of dementia, diabetes, serious malnutrition judged by the doctor in charge; (17) starvation period before having PEG: none, within 1 wk, within 1 mo, more than 1 mo; and (18) primary diagnosis or underlying disease warranting PEG, selected from one of the following: (Ⅰ) Neurological disease: (a) Parkinson's disease; (b) amyotrophic lateral sclerosis; (c) multiple system atrophy; or (d) other neurological disease; (Ⅱ) Cerebrovascular disease: (a) cerebral infarction; (b) cerebral hemorrhage; (c) subarachnoid hemorrhage; or (d) other cerebrovascular disease; (Ⅲ) Dementia: (a) severe; (b) mild; or (c) other type of dementia; and (Ⅳ) Other disease, at the time of the PEG procedure, obtained from medical charts.

Statistics analysis

The Student *t*-test and Mann-Whitney *U* test were performed for continuous variables with normal and not normal distribution, respectively. Overall survival curves were drawn using the Kaplan-Meier method and compared for each variable using log-rank tests. The Cox proportional hazard models were fitted for multivariate analysis using variables significant in the log-rank tests and other tests. Adjusted hazard ratios (AHR) and 95% confidence intervals (CI) were determined. All statistical analyses were performed using STATA 11.0 (STATA Corp., College Station, TX). $P < 0.05$ was considered statistically significant.

RESULTS

Of the 1168 patients who underwent PEG at the 46 se-

Table 1 Patients' characteristics stratified by survival (mean ± SD)

Variable	Total (n = 931)	Alive (n = 429)	Dead (n = 502)	P-value
Age (yr)	81.4 ± 7.8	80.0 ± 7.9	82.6 ± 7.6	< 0.0001[1]
Height (cm)	153.0 ± 10.4	152.4 ± 10.5	153.5 ± 10.2	0.20[1]
Weight (kg)	44.7 ± 10.7	44.9 ± 10.8	44.6 ± 10.6	0.79[1]
Body mass index (kg/m^2)	19.3 ± 3.7	19.5 ± 4.0	19.1 ± 3.6	0.22[1]
Body temperature (℃)	36.7 ± 0.5	36.7 ± 0.5	36.7 ± 0.5	0.82[1]
White blood cell (/μL) 25/50/75 percentile	5470/6770/8700	5500/6640/8570	5420/6800/8800	0.33[2]
C-reactive protein (mg/dL) 25/50/75 percentile	0.4/1.2/2.8	0.4/1.0/2.4	0.4/1.2/3.2	0.016[2]
Hematocrit (%) 25/50/75 percentile	29.8/33.5/37.1	30.9/34.2/37.7	29.2/32.8/36.6	0.0001[2]
Hemoglobin (g/dL) 25/50/75 percentile	9.8/11.0/12.3	10.1/11.3/12.6	9.5/10.8/12.1	0.0001[2]
Aspartate aminotransferase (IU/L) 25/50/75 percentile	18/24/35	18/23/31	18/24/39	0.019[2]
Alanine aminotransferase (IU/L) 25/50/75 percentile	12/19/33	12/19/29	11/20/40	0.22[2]
Blood urea nitrogen (mg/dL) 25/50/75 percentile	12.8/17.7/24.8	12.4/17.0/23.1	13.0/18.6/26.1	0.018[2]
Serum creatinine (mg/dL) 25/50/75 percentile	0.42/0.59/0.80	0.40/0.56/0.76	0.44/0.60/0.81	0.0096[2]
Albumin (g/dL) 25/50/75 percentile	2.6/3.0/3.3	2.7/3.0/3.4	2.6/2.9/3.2	0.0013[2]

[1]The Student t-test was applied because the distribution was considered as normal; [2]The Mann-Whitney test was applied because the distribution was considered as not normal.

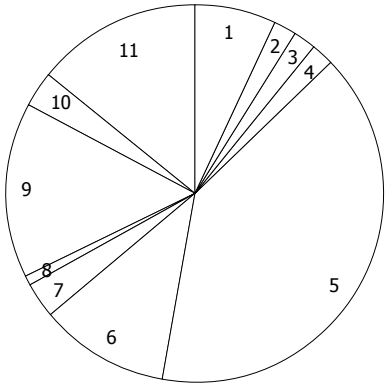

Figure 1 The distribution of primary diagnosis warranting percutaneous endoscopic gastrostomy. 1: Parkinson's disease (7%); 2: Amyotrophic lateral sclerosis (2%); 3: Multiple system atrophy (2%); 4: Other neurological disease (2%); 5: Cerebral infarction (40%); 6: Cerebral hemorrhage (11%); 7: Subarachnoid hemorrhage (3%); 8: Other cerebrovascular disease (1%); 9: Severe dementia (15%); 10: Mild dementia (3%); 11: Other type of dementia (14%).

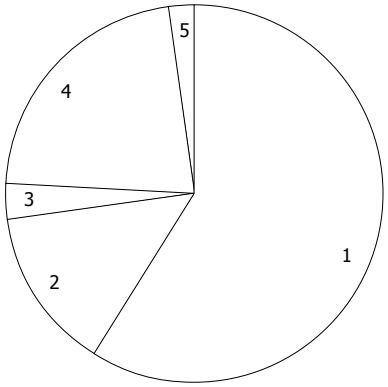

Figure 2 Causes of death classified by the doctors. 1: Pneumonia (59%); 2: Cardiac (14%); 3: Cancer (3%); 4: Other (22%); 5: Unknown (2%).

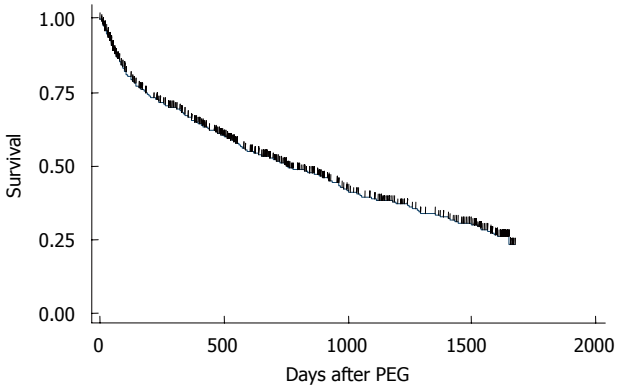

Figure 3 Kaplan-Meier survival curve of 931 patients. PEG: Percutaneous endoscopic gastrostomy.

lected hospitals, 237 were excluded from further analyses, having met at least one of the exclusion criteria, and thus 931 patients were followed and the median duration was 468 d, ranging from 1 to 1668 d. Among 931 patients, 122 patients were censored due to loss to follow-up at a median of 71 d, ranging from 6 to 1582 d. Mean age was 81.4 years old, ranging from 65 to 99 years. Females were predominant (54%). The distribution of primary diagnosis warranting PEG is shown in Figure 1, where cerebrovascular diseases accounted for more than half of the study population. In the previous history, pneumonia and ischemic heart disease were reported in 63% and 19% of patients, respectively. In addition, diabetes comorbidity was 18%. In total, 502 deaths were observed (mortality 53%), of which 10 deaths (2.0%) occurred within 7 d, 49 (9.8%) within 30 d, 105 (21%) within 60 d, 216 (43%) within 6 mo, and 287 (57%) within 1 year. Causes of death classified by the doctors are shown in Figure 2, and pneumonia was the major cause of death. Eight deaths were considered as PEG-related deaths, according to the reports of the doctors. On the other hand, among 28 surviving patients (6.5%), PEG was removed.

Continuous variables of demographic and laboratory data at PEG installation were first compared between alive and dead patients (Table 1). Age, CRP, AST, BUN

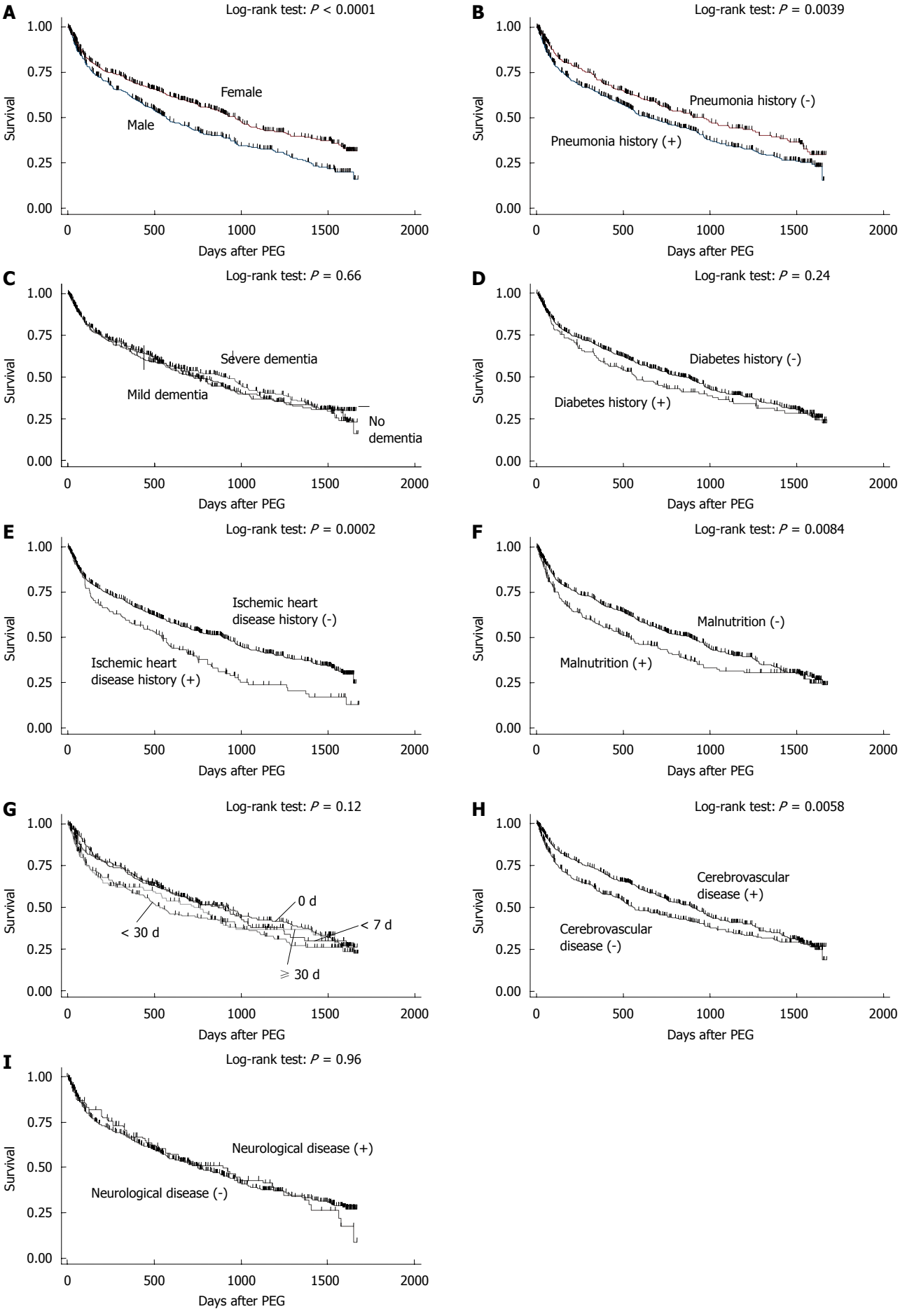
A
Log-rank test: P < 0.0001
Female
Male
B
Log-rank test: P = 0.0039
Pneumonia history (-)
Pneumonia history (+)
C
Log-rank test: P = 0.66
Severe dementia
Mild dementia
No dementia
D
Log-rank test: P = 0.24
Diabetes history (-)
Diabetes history (+)
E
Log-rank test: P = 0.0002
Ischemic heart disease history (-)
Ischemic heart disease history (+)
F
Log-rank test: P = 0.0084
Malnutrition (-)
Malnutrition (+)
G
Log-rank test: P = 0.12
0 d
< 7 d
< 30 d
≥ 30 d
H
Log-rank test: P = 0.0058
Cerebrovascular disease (+)
Cerebrovascular disease (-)
I
Log-rank test: P = 0.96
Neurological disease (+)
Neurological disease (-)
Survival
Days after PEG
0.00
0.25
0.50
0.75
1.00
0
500
1000
1500
2000

Figure 4 Kaplan-Meier survival curves compared. A: Gender; B: Previous history of pneumonia; C: Dementia; D: Previous history or comorbidity of diabetes; E: Previous history of ischemic heart disease; F: Presence of malnutrition as judged by the doctors; G: Starvation period before having percutaneous endoscopic gastrostomy: none, within 1 wk, within 30 d, more than 30 d; H: Cerebrovascular disease as an underlying disease; I: Neurological disease as an underlying disease. PEG: Percutaneous endoscopic gastrostomy.

Table 2 Cox proportional hazard models

Variable	Single variable analyses			Multivariate analysis		
	Crude HR	95% CI	*P*-value	AHR	95% CI	*P*-value
Age (yr)	1.03	1.01-1.04	< 0.001	1.02	1.00-1.03	0.009
C-reactive protein (mg/dL)	1.07	1.05-1.10	< 0.001	1.04	1.01-1.07	0.005
Hemoglobin (g/dL)	0.91	0.87-0.95	< 0.001	0.98	0.92-1.05	0.530
Alanine aminotransferase (IU/L)	1.01	1.00-1.01	< 0.001	1.00	0.10-1.00	0.170
Blood urea nitrogen (mg/dL)	1.01	1.01-1.02	< 0.001	1.01	1.00-1.02	0.003
Serum creatinine (mg/dL)	0.99	0.95-1.03	0.640	0.94	0.83-1.05	0.280
Albumin (g/dL)	0.52	0.43-0.63	< 0.001	0.67	0.52-0.85	0.001
Female	0.70	0.58-0.83	< 0.001	0.60	0.48-0.75	< 0.001
No history of pneumonia	0.76	0.63-0.92	0.004	0.99	0.79-1.24	0.950
No history of ischemic heart disease	0.66	0.53-0.82	< 0.001	0.69	0.54-0.88	0.003
No malnutrition	0.76	0.63-0.93	0.009	1.09	0.85-1.39	0.490
Cerebrovascular disease	0.78	0.66-0.93	0.006	0.83	0.67-1.02	0.080

Adjusted for all the variables listed in the table. HR: Hazard ratio; CI: Confidence interval; AHR: Adjusted hazard ratio.

and Cr were significantly higher in patients who died than in those who survived. In contrast, Ht, Hb and albumin were significantly higher in patients who survived than in patients who died. Regarding PEG-related death, albumin levels were significantly lower (Mann-Whitney *U* test: $P = 0.002$).

A Kaplan-Meier survival curve of the 931 patients was drawn (Figure 3): 99% survived more than 7 d; 95% survived more than 30 d; 88% survived more than 60 d; 75% survived more than 6 mo; 66% survived more than 1 year. Of the 931 patients, 50% and 25% survived 753 and 1647 d, respectively. Kaplan-Meier survival curves were then drawn for nominal or ordinal data (Figure 4). The prognosis of female patients was better than that of males (Figure 4A). Patients who had no history of pneumonia (Figure 4B) or ischemic heart disease (Figure 4E) survived longer than those with a history of the diseases. Patients who were considered as having malnutrition at the time of operation showed poorer survivals than those without malnutrition (Figure 4F). Patients whose comorbidity was cerebrovascular disease had a better outcome than patients with other comorbidities (Figure 4E). In contrast, the prognosis of patients with dementia (Figure 4C), diabetes (Figure 4D), or neurological disease (Figure 4I) were not different from those without the conditions. Duration of starvation before the PEG procedure had no significant impact on patient survival (Figure 4G).

Finally, using variables significant in the above analyses, Cox proportional hazard models were computed in single variable and multivariate analyses (Table 2). In single variable hazard models, patients with older age, and higher CRP, AST and BUN did show a significantly enhanced crude HR whereas higher Hb and albumin reduced the crude HR. In addition, no history of pneumonia or ischemic heart disease, no malnutrition, but a history of cerebrovascular disease significantly decreased crude HRs. However, in the multivariate hazard model, older age, higher CRP, higher BUN, lower albumin, male gender and a previous history of ischemic heart disease were significant risk factors of death after PEG insertion.

DISCUSSION

In this study, 502 deaths were observed (mortality 53%). However, 99%, 95%, 88%, 75% and 66% of 931 patients survived more than 7, 30, 60 d, 6 mo and 1 year, respectively. In addition, 50% and 25% of the patients survived 753 and 1647 d, respectively, suggesting that more than half of geriatric patients treated with PEG may survive more than 2 years. Although the situations of each study may not be the same, the overall survival rate of this study is superior[8-10] or equivalent to previous reports[11-13]. Eight deaths out of 931 in the study population were reported as PEG-related, a rate almost equal to that in previous studies[14,15]. Although urinary tract infection and previous aspiration were predictive factors for death at 1 wk after PEG[16] and CRP was found to be predictive of early mortality[17], we also found that a lower albumin level was a significant risk factor for PEG-related death, which was a novel finding.

We also attempted to delineate prognostic factors for geriatric patients treated with PEG. In single variable analyses, older age, higher CRP, lower Ht/Hb, higher AST, higher BUN, lower albumin as well as male gender, previous history of pneumonia or ischemic heart disease, malnutrition, and non-cerebrovascular disease as an underlying disease at the PEG procedure, were significantly associated with death. In a multivariate hazard model, older age, higher CRP and higher BUN were significant poor prognostic factors of death after PEG formation, where-

as higher albumin, female gender and no previous history of ischemic heart disease were markedly better prognostic factors. Thus, Hb, AST, previous history of pneumonia, malnutrition and cerebrovascular disease as an underlying disease were considered as confounders. Among prognostic factors significant in our study, older age, male sex, lower albumin levels and a previous history of pneumonia or aspiration were already recognized as poor prognostic factors[18-24]. In our single variable Cox hazard model, cerebrovascular disease as an underlying disease was a good prognostic factor, which was consistent with the previous evidence that patients with a previous diagnosis of stroke were more likely to be discharged home than others[25]. Thus, CRP, BUN and a previous history of ischemic heart disease were found as novel prognostic factors in our study.

The results of this study should be interpreted in the context of the study strengths and limitations. The study was performed in multiple community and tertiary hospitals spread over Japan, which enhanced its generalizability. To minimize selection bias, collaborating doctors were asked to choose 20 consecutive patients. The sample size was close to 1000 and the results of the Cox hazard models were considered relatively robust. On the other hand, because of the retrospective nature of the study, we could collect only basic clinical information that might result in recall bias in areas such as previous histories and diagnosis of underlying diseases.

In conclusion, these results suggest that more than half of geriatric patients treated with PEG may survive longer than 2 years in Japan. In the Cox proportional multivariate analysis, older age, higher CRP and higher BUN were significant poor prognostic factors of death after PEG, whereas higher albumin, female gender and no previous history of ischemic heart disease were markedly better prognostic factors.

COMMENTS

Background

Percutaneous endoscopic gastrostomy (PEG) has been widely used for patients with swallowing difficulty, because of reduced laryngopharyngeal discomfort and a lower risk of aspiration pneumonia compared with the nasogastric tube. However, PEG feeding was found to be associated with an absolute increase in risk of death compared with nasogastric feeding. In spite of the evidence, PEG has been widely used especially for geriatric patients with the increasingly aging society.

Research frontiers

Despite the very large number of patients receiving this intervention, there is insufficient evidence to suggest that PEG is beneficial for patients with swallowing difficulty due to dementia or other chronic diseases.

Innovations and breakthroughs

The patients were selected in a consecutive manner and followed up over a long time period.

Applications

In a Cox proportional multivariate analysis, older age, higher CRP and higher BUN were significant poor prognostic factors of death after PEG, whereas higher albumin, female gender and no previous history of ischemic heart disease were markedly better prognostic factors. The authors especially determined that a lower albumin level was a significant risk factor for PEG-related death, which was a novel finding.

Peer review

Good comprehensive effort, gives nice insight into PEG in elderly.

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BRIEF ARTICLE

HNF-4α determines hepatic differentiation of human mesenchymal stem cells from bone marrow

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Abstract

AIM: To investigate the differentiation status and key factors to facilitate hepatic differentiation of human bone-marrow-derived mesenchymal stem cells (MSCs).

METHODS: Human MSCs derived from bone marrow were induced into hepatocyte-like cells following a previously published protocol. The differentiation status of the hepatocyte-like cells was compared with various human hepatoma cell lines. Overexpression of hepatocyte nuclear factor (HNF)-4α was mediated by adenovirus infection of these hepatocyte-like cells. The expression of interesting genes was then examined by either reverse transcription-polymerase chain reaction (RT-PCR) or real-time RT-PCR methods.

RESULTS: Our results demonstrated that the differentiation status of hepatocyte-like cells induced from human MSCs was relatively similar to poorly differentiated human hepatoma cell lines. Interestingly, the HNF-4 isoform in induced MSCs and poorly differentiated human hepatoma cell lines was identified as HNF-4γ instead of HNF-4α. Overexpression of HNF-4α in induced MSCs significantly enhanced the expression level of hepatic-specific genes, liver-enriched transcription factors, and cytochrome P450 (P450) genes.

CONCLUSION: Overexpression of HNF-4α improves the hepatic differentiation of human MSCs from bone marrow and is a simple way of providing better cell sources for clinical applications.

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Key words: Bone marrow; Cytochrome P450 genes; Differentiation of hepatocyte; Hepatocyte nuclear factor 4; Human mesenchymal stem cells

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INTRODUCTION

The liver is an important organ and performs many biological functions, such as plasma protein synthesis, glucose and fatty acid metabolism, and detoxification. The standard treatment for irreversible liver failure has been focused on liver transplantation; however, the availability of donor tissues is limited and enables only 10% of candidate patients to receive transplants. Recently, hepatocyte transplantation or bioartificial livers have become promising alternatives for treatment[1,2], but the development of cell-based therapies is hindered by the propagation of hepatocytes and the maintenance of their hepatic functions *in vitro*. Therefore, it would be of great benefit to develop *in vitro* models of hepatocyte differentiation to facilitate future clinical applications.

Recently, success in driving stem cells into hepatic lineage has provided great hope for overcoming the limitation of cell sources for hepatocyte transplantation[3-6]. Previous studies have demonstrated that reservoirs of stem cells may reside in several types of adult and fetal tissues, including liver stem cells as a hepatic source and embryonic stem cells, bone marrow cells, and umbilical cord blood cells as a non-hepatic source. Most of these cells can be induced toward hepatic differentiation and to exhibit hepatic functions in both *in vivo* and *in vitro* systems[7-9]. Among them, the study of hepatic differentiation of bone marrow cells is most attractive because the autologous stem cells can be easily isolated, expanded extensively, and induced into hepatic differentiation for transplantation back into the patient. It has been reported that mesenchymal stem cells (MSCs) derived from bone marrow have the potential to differentiate into cells of mesodermal lineage, such as osteoblasts, chondrocytes, and adipocytes, as well as into various types of cells of other lineages, including neural and liver cells[10]. Furthermore, the hepatic differentiation of MSCs *in vivo* has been established in rat and mouse models[11-13]. These observations bring new hope for the possible application of cell-based therapy in severe liver diseases. However, the hepatic differentiation status of hepatocyte-like cells derived from stem cells is not sufficient for clinical use because the relatively low expression levels of drug metabolizing enzymes and their metabolic activities are not fully induced[14]. Therefore, it is important to develop a simple strategy for the efficient induction of hepatic differentiation.

The coordinated expression of various liver-specific genes is required for hepatic differentiation and the biological functions of adult liver. Previous studies have demonstrated that most hepatic gene expression is regulated primarily through the combinational action of several liver-enriched transcription factors[15-17]. Among them, hepatocyte nuclear factor (HNF)-4α plays a crucial role in the liver-specific phenotype through induction of various liver-specific functions[18]. Several studies have demonstrated that HNF-4α may act as a master gene in a transcription factor cascade that could drive hepatic differentiation[19,20]. These studies suggest that high expression of HNF-4α may be a simple strategy for the induction of hepatic differentiation and the functions of hepatocyte-like cells derived from stem cells in the cell culture system.

In this study, we demonstrated that human bone marrow MSCs can be differentiated into hepatocyte-like cells according to the procedure established by Lee *et al*[3]. Furthermore, overexpression of a single liver-enriched transcription factor, HNF-4α, could significantly improve the differentiation status of hepatocyte-like cells through activation of several target genes. Apparently, these more differentiated hepatocyte-like cells will provide a better cell source for future clinical applications and *in vitro* hepatotoxicity models for drug screening.

MATERIALS AND METHODS

Propagation of human bone marrow MSCs

Human bone marrow MSCs were isolated and characterized by Lee *et al*[3]. Briefly, human bone marrow was collected from healthy donors with informed consent and approved by the institutional review board of the Taipei Veterans General Hospital. Mononuclear cells were obtained by negative immunodepletion of CD3, CD14, CD19, CD38, CD66B, and glycophorin-A positive cells using a commercially available kit (RosetteSep, StemCell Technologies) according to the manufacturer's instructions, followed by Ficoll-Paque density-gradient centrifugation (1.077 g/cm^3), and plated in tissue culture plates in expansion medium. The expansion medium consisted of Iscove's modified Dulbecco's medium (IMDM) (Gibco) and 10% fetal bovine serum (Hyclone) supplemented with 10 ng/mL epidermal growth factor (EGF) (Becton Dickinson) and 10 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems). The adherent cells were amplified within the expansion medium until hepatic induction.

Hepatic differentiation of human MSCs

The hepatic induction of human bone marrow MSCs was performed according to the protocol developed by Lee *et al*[3]. In brief, 5th- to 13th-passage stem cells (at 1.0 to 1.3 × 10^4/cm^2) were serum deprived for 2 d in IMDM supplemented with 20 ng/mL EGF and 10 ng/mL bFGF prior to induction using a two-step protocol. Differentiation was induced by treating MSCs with step-1 differentiation medium, consisting of IMDM supplemented with 20 ng/mL hepatocyte growth factor (R&D Systems), 10 ng/mL bFGF and 0.61 g/L nicotinamide for 7 d, followed by treatment with step-2 maturation medium, consisting of IMDM supplemented with 20 ng/mL oncostatin M (R&D Systems), 1 μmol/L dexamethasone (Sigma), and 50 mg/mL ITS$^+$ premix (Becton Dickinson). Medium changes were performed twice weekly.

Cell culture

Human hepatoma cell lines HepG2[21], HA22T/VGH[22] and SK-Hep-1[23] were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal calf serum (Gibco) as described previously[24].

Table 1 Primer sets used for reverse transcription-polymerase chain reaction analysis

Gene	Forward primer	Reverse primer	Product size (bp)
Alb	TGCTTGAATGTGCTGATGACAGGG	AAGGCAAGTCAGGGCATCTCATC	161
AFP	TGCAGCCAAAGTGAAGAGGGAAGA	CATAGCGAGCAGCCCAAAGAAGAA	216
TAT	TGAGCAGTCTGTCCACTGCCT	ATGTGAATGAGGAGGATCTGAG	358
G6P	GCTGGAGTCCTGTCAGGCATTGC	TAGAGCTGAGGCGGAATGGGAG	350
TO	ATACAGAGACTTCAGGGAGC	TGGTTGGGTTCATCTTCGGTATC	299
HNF-1α	GTGTCTACAACTGGTTTGCC	TGTAGACACTGTCACTAAGG	251
HNF-3β	CACCACTACGCCTTCAACC	GGTAGTAGGAGGTATCTGCGG	235
HNF-4	CTGCTCGGAGCCACAAAGAGATCCATG	ATCATCTGCCACGTGATGCTCTGCA	371
C/EBPα	CAAGAAGTCGGTGGACAAGAAC	CCTCATCTTAGACGCACCAAGT	450
β2M	TGACGTGTGAACCATG	TGGAGACAGCACTCAAAG	242

The thermo-cycling parameters for β2M: 94℃ for 5 min/28 cycles of 94℃ for 30 s, 56℃ for 30 s, and 72℃ for 30 s/72℃ for 10 min; The thermo-cycling parameters for other genes: 94℃ for 5 min/38 cycles of 94℃ for 30 s, 56-58℃ for 30 s, and 72℃ for 30 s/72℃ for 10 min. Alb: Albumin; AFP: α-fetoprotein; TAT: Tyrosine-aminotransferase; TO: Tryptophan 2,3-dioxygenase; G6P: Glucose 6-phosphatase; β2M: β-2-microglobulin..

Table 2 Primer sets for real-time reverse transcription-polymerase chain reaction analysis

Gene	Forward primer	Reverse primer	Probe
HNF-1α	TGAGTCCGGGCTTCACAC	GGCTGCTGGAGGACACTG	42
HNF-3β	GCACCTGCAGATTCTGATTTT	GACTTCCCTGCAACAACAGC	25
HNF-4α	ATTGACAACCTGTTGCAGGA	CGTTGGTTCCCATATGTTCC	77
HNF-4γ	CTTGGTGGAATGGGCTAAA	AGCTCTCAACAGTGCCACCT	8
HNF-6	CCTGGAGCAAACTCAAATCC	TTCTTTCCTTTTGCATGCTG	88
C/EBPα	CAACACTTGTATCTGGCCTCTG	CGAGCAAAACCAAAACAAAAC	3
PPARα	GTCCCCGCAGATTCTACATT	GAAGCTGTCCTGGCTCAGAT	14
Alb	TGTTGCCAAGCTGCTGATA	CCTTCATCCCGAAGTTCATC	27
TAT	TGCTGAGCAGTCTATCCACT	CTGCTCACAGAACTCCTGGAT	67
G6P	GCTGCTCATTTTCCTCATCAA	TTCTGTAACAGCAATGCCTGA	67
CYP1A1	GACAGATCCCATCTGCCCTA	CAAACTTGTGTCTCTTGTTGTGC	80
CYP1A2	CAAGAAATGCTGTGTCTTCGTAA	AGAGGGGTCCTCCCACAG	59
CYP2A6	CAAAAAGGACACCAAGTTTCG	AGAGCCCAGCATAGGGTACA	69
CYP2B6	GAAGCTTTTATCCCCTTCTCCT	GCCATGGAGAAGTTCTGGAG	35
CYP2C8	AAGAAAAGTGACTACTTCATGCCTTT	CAAGTCCTTCTCCTGCACAA	18
CYP2C9	ATTGACCTTCTCCCCACCA	CAGGGAAATTAATATGGTTGTGC	43
CYP2C19	GTCCAGAGATACATCGACCTCA	AGTGAGGGAAGTTAATATGGTTGTG	43
CYP2D6	AGGAGGAGTCGGGCTTTCT	CGCTGGGATATGCAGGAG	56
CYP2E1	CAAGCCATTTTCCACAGGA	CAACAAAAGAAACAACTCCATGC	67
HPRT	AGGTTGCAGCGAGGAAAAA	AGCCACCCTGGAAGAAACTT	59

The thermo-cycling parameters for real-time reverse transcription-polymerase chain reaction: 95℃ for 10 min/50 cycles of 95℃ for 10 s, 60℃ for 30 s, and 72℃ for 1 s/40℃ for 30 s. Alb: Albumin; AFP: α-fetoprotein; TAT: Tyrosine-aminotransferase; G6P: Glucose 6-phosphatase; PPARα: Peroxisome proliferator-activated receptor α; HPRT: Hypoxanthine-guanine phosphoribosyltransferase.

Immunofluorescence

To stain for albumin, cells were fixed with 4% paraformaldehyde at 4℃, and permeabilized with 0.1% Triton X-100 for 15 min. The slides were incubated with mouse monoclonal antibodies against human albumin (Santa Cruz) (1:50 dilution) for 1 h, followed by fluorescein-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) (1:100 dilution) for 1 h. Hoechst stain was used for labeling DNA. Between incubations, samples were washed with phosphate-buffered saline (PBS).

Uptake of low-density lipoprotein

The Dil-Ac-low-density lipoprotein (LDL) staining kit was purchased from Biomedical Technologies and the assay was performed following the manufacturer's instructions.

Reverse transcription-polymerase chain reaction and real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from induced MSCs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA templates were obtained by reverse transcription of total RNA with oligo(dT) 18-mers and Superscript Ⅱ reverse transcriptase (Invitrogen). The products were then subjected to either reverse transcription-polymerase chain reaction (RT-PCR) analysis with the specific primer pairs and conditions[3] listed in Table 1 or quantitative real-time RT-PCR analysis with the specific primer pairs and Taqman probes listed in Table 2 (according to the instruction of the Assay Design Center, Universal ProbeLibrary System, Roche Applied Science).

Recombinant adenoviruses

The HNF-4α expression vector (Ad/HNF4α-IRES-EGFP) contained the rat HNF-4α cDNA fragment (kindly provided by Dr. Ou JH)[25] in the parental adenovirus vector (Ad-IRES-EGFP) which was constructed based on the Adeno-X expression system (Clontech) containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus and EGFP gene. The adenoviruses were packaged and amplified in 293 cells and then purified by banding on CsCl gradients. The titer of recombinant adenoviruses was estimated according to the amount of viral genome. The 8w-induced MSCs were infected with purified adenoviruses at a 1500 multiplicity of infection (m.o.i.) for 1 h in serum-free medium. The overexpression of rHNF-4α was confirmed by real-time RT-PCR (forward primer: CAAGAGGTCCATGGTGTTCA; reverse primer: CCGAGGGACGATGTAGTCA; Taqman probe: #68). For the induction of P450 genes, 50 μmol/L of B-naphthoflavone or rifampicin (Sigma) was added to the culture medium for 2 d.

Statistical analysis

Statistical significance for the induction effect was determined by *t*-test. Differences were considered significant if the *P* value was less than 0.05.

RESULTS

The differentiation status of hepatocyte-like cells induced from human bone marrow MSCs

Human MSCs were isolated from bone marrow, expanded in growth medium, and differentiated into hepatic lineage according to the protocols previously described by Lee *et al*[3]. Under hepatic induction conditions, the fibroblastic morphology of MSCs (Figure 1A, 0 wk and 1 wk) gradually proceeded toward the polygonal shape of hepatocytes with the appearance of abundant granules in the cytoplasm (Figure 1A, 4 wk, 7 wk, and 10 wk). Interestingly, compared with human hepatoma cell lines of different differentiation status-HepG2, HA22T/VGH, and SK-Hep-1 cells; the morphology of induced MSCs at an early stage was similar to that of spindle-shaped SK-Hep-1 and HA22T/VGH cells. After 7 wk of induction, the morphology of MSCs gradually approached that of HepG2 cells (Figure 1B). These observations suggest that the induction of the differentiation process of human MSCs may have some similarities to human hepatoma cell lines with different stages of differentiation.

Beside the morphological differences, we also evaluated biological properties to characterize the hepatic maturation of induced MSCs including uptake of low-density lipoprotein (LDL) and albumin expression. After 10 wk of induction, most of the induced MSCs exhibited positive signals for albumin expression (Figure 1C) and LDL uptake (Figure 1D). These results also demonstrated that human MSCs have the potential to differentiate into hepatocyte-like cells as previously described[3].

Furthermore, to compare the differentiation status of human MSCs at a different period of induction with human hepatoma cell lines, the expression pattern of several hepatic genes was investigated using RT-PCR in human MSCs induced at 0, 1, 4, 7, and 10 wk together with HepG2, HA22T/VGH, and SK-Hep-1 cells (Figure 2). These hepatic genes included (1) an indicator for the early hepatic gene, albumin (Alb); (2) indicators for the middle hepatic gene, tyrosine-aminotransferase (TAT), and glucose 6-phosphatase (G6P); (3) indicators for the late hepatic gene, tryptophan 2,3-dioxygenase (TO)[26]; and (4) liver-enriched transcription factors including HNF-1α, HNF-3β, HNF-4, and C/EBPα. Our data indicated that most of the detected genes, including Alb, TAT, TO, HNF-3β, HNF-4, and C/EBPα, were expressed in MSCs 10 wk after induction, whereas the immature marker alpha-fetoprotein was not expressed during the entire period of induction (Figure 2A). Among them, the Alb gene was expressed as early as serum starvation treatment and maintained its expression during the whole induction period. The TAT gene was induced to be expressed 1 wk post-induction and the TO gene was activated 4 wk post-induction. This unique pattern is similar to the sequential expression of hepatic genes during hepatocyte maturation[26]. However, the expression of G6P was not detectable, even 10 wk after induction. The expression of liver-enriched transcription factors HNF-4 and C/EBPα was also activated 4 wk after induction. In contrast, HNF-3β was only weakly expressed until 7 wk post-induction and HNF-1α was not expressed 10 wk after induction. In the human hepatoma cells (Figure 2B), all genes except for TO were well expressed in HepG2 cells; Alb, TO, and HNF-4 genes were expressed in HA22T/VGH cells; and TAT, TO, HNF-3β, HNF-4, and C/EBPα genes were expressed in SK-Hep-1 cells. In general, the expression level of investigated hepatic genes was much lower in the hepatocyte-like cells derived from human MSCs than those in HepG2 cells, the well-differentiated hepatoma cell line, and relatively better than those in poorly differentiated hepatoma cell lines HA22T/VGH and SK-Hep-1. Taken together, human bone marrow MSCs can be specifically induced toward hepatic lineage and expressed certain hepatic-specific genes and liver-enriched transcription factors. However, the expression potency of hepatic genes was weak compared with well-differentiated hepatoma cells. Our results suggested that the differentiation status of induced MSCs seems to lie between the well-differentiated and poorly differentiated cell types of human hepatoma cell lines.

To confirm the specificity of these RT-PCR analyses, we performed a sequence analysis of the PCR fragments followed by nucleotide BLAST analysis. Interestingly, we found that the sequence of the PCR fragments from induced MSCs, HA22T/VGH, and SK-Hep-1 cells corresponded to the HNF-4γ gene, which is not expressed in adult liver[27], and that from HepG2 cells corresponded to the HNF-4α gene (data not shown). These results revealed that HNF-4α was only expressed in HepG2 cells, whereas HNF-4γ was expressed in the induced MSCs, HA22T/VGH and SK-Hep-1 cells. To further study the

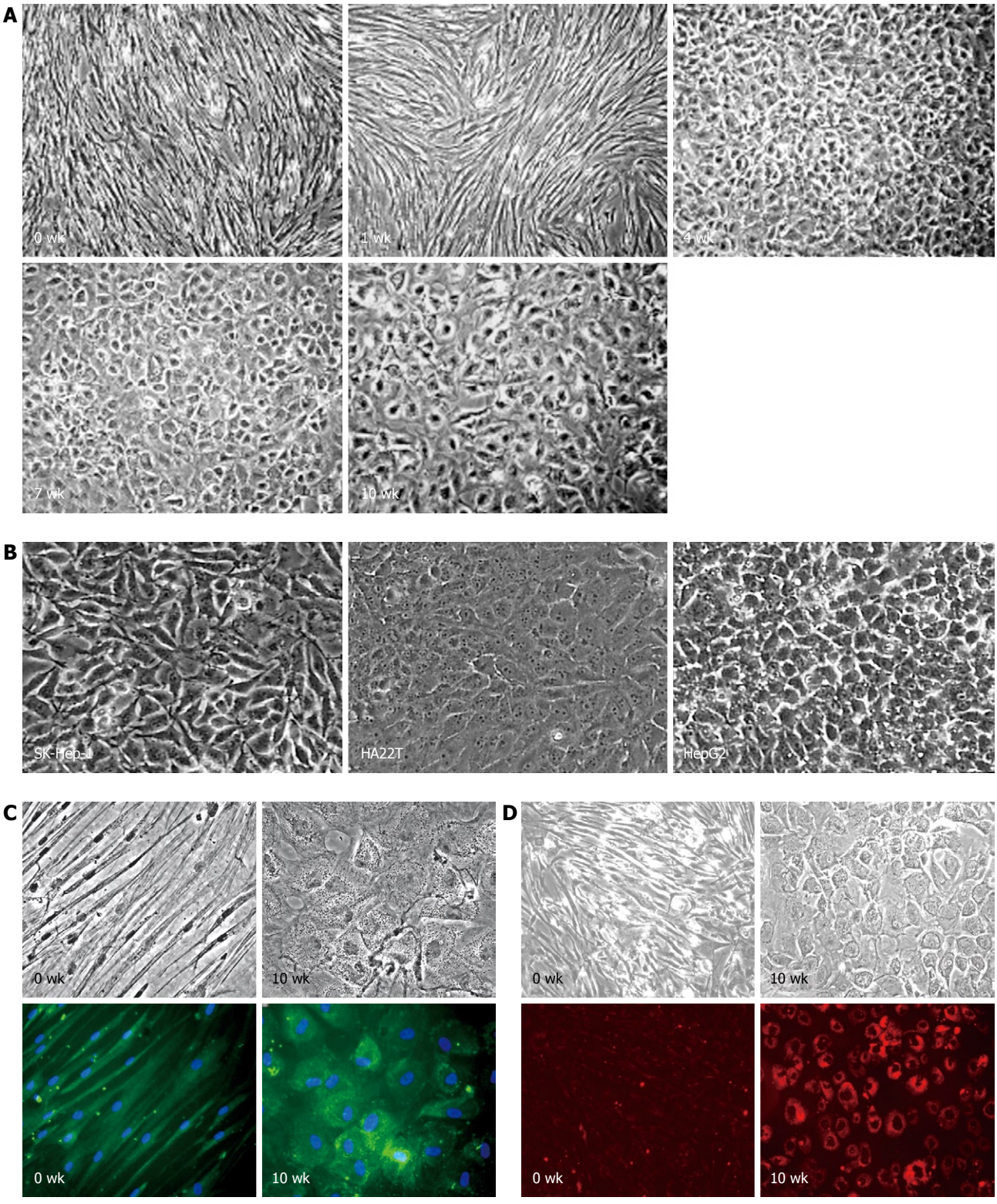


Figure 1 Hepatic differentiation of human bone marrow-derived mesenchymal stem cells. A: Morphological characterization of differentiated mesenchymal stem cells (MSCs) under hepatic induction. 0 wk: Undifferentiated MSCs; 1 wk: 1 wk post-induction; 4 wk: 4 wk post-induction; 7 wk: 7 wk post-induction; and 10 wk: 10 wk post-induction (original magnification, × 50); B: Morphology of human hepatoma cell lines: SK-Hep-1, HA22T/VGH, and HepG2 (original magnification, × 100); C: Production of albumin (green color) in differentiated MSCs after 10 wk induction (10 wk) and counterstained with Hoechst (blue color). The undifferentiated MSCs (0 wk) were used as negative control cells (original magnification, × 200); D: Uptake of low-density lipoprotein (red color) in differentiated MSCs after 10 wk induction (10 wk). The undifferentiated MSCs (0 wk) were used as negative control cells (original magnification, × 100).

kinetic expression of HNF-4, we use real-time RT-PCR analysis with specific isoform primers (Table 2) to estimate the expression level of HNF-4α or HNF-4γ in these samples. Similarly, expression of the HNF-4γ gene was

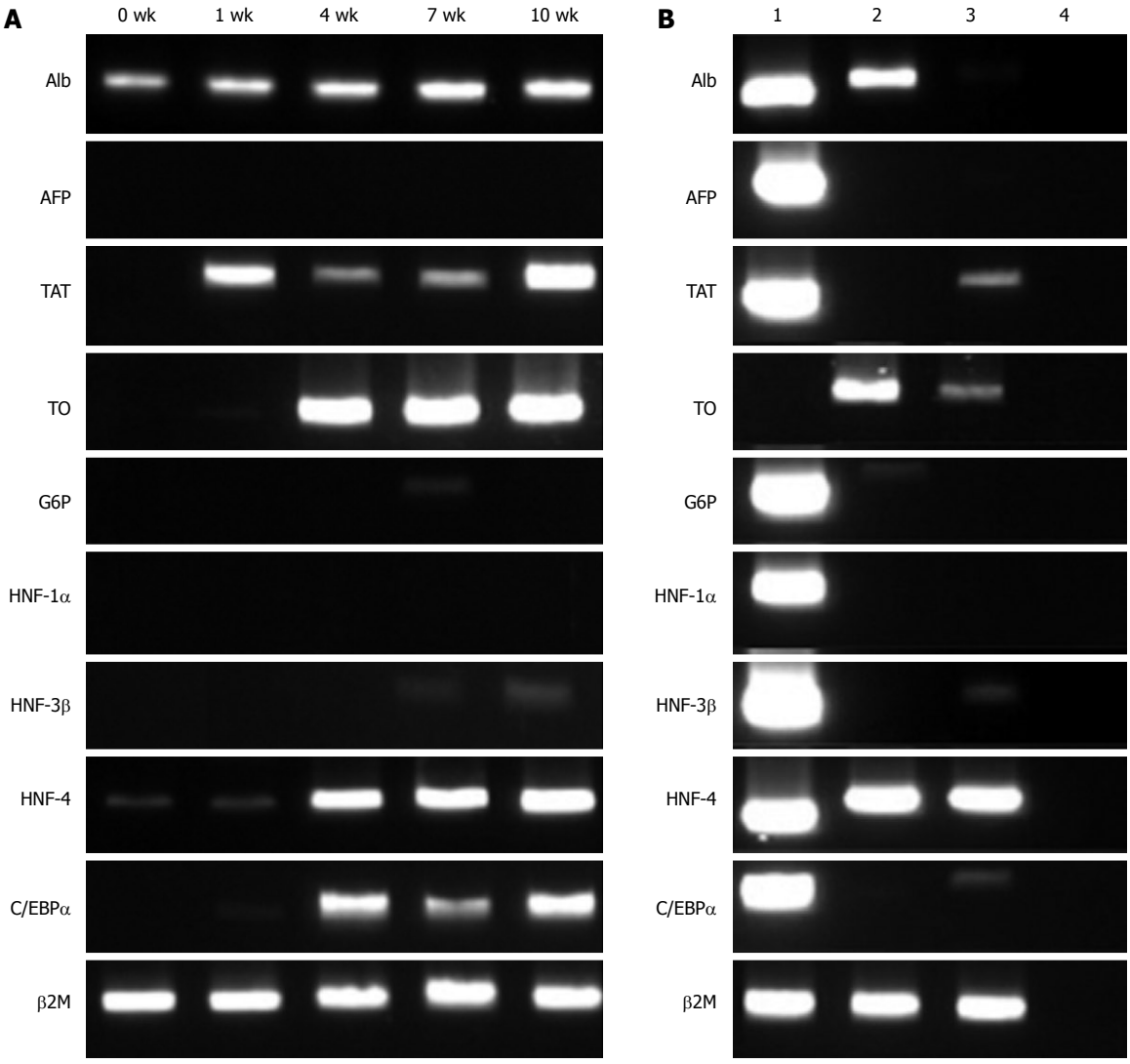


Figure 2 Detection of liver-specific genes and liver-enriched transcription factors in induced mesenchymal stem cells by reverse transcription-polymerase chain reaction. A: Induced mesenchymal stem cells at the indicated time point: 0, 1, 4, 7 and 10 wk post-induction; B: Human hepatoma cell lines: 1, HepG2; 2, HA22T/VGH; 3, SK-Hep-1; and 4, negative control. Expression of β2M was used as the control for adjustment of sample amount. Alb: Albumin; AFP: α-fetoprotein; TAT: Tyrosine-aminotransferase; TO: Tryptophan 2,3-dioxygenase; G6P: Glucose 6-phosphatase; β2M: β-2-microglobulin.

gradually increased during the hepatic induction of MSCs, and HA22T/VGH as well as SK-Hep-1 cells (Figure 3A). In contrast, the HNF-4α gene was not expressed during the hepatic induction of MSCs, whereas it was significantly expressed in HepG2 cells (Figure 3B). These results suggested that the hepatic induction of human MSCs in this study was not efficient because of the failed expression of HNF-4α during the induction process.

Enhancement of hepatic differentiation by overexpression of HNF-4α

Several studies have demonstrated that hepatic gene expression is regulated by the combinational action of liver-enriched transcription factors. In this study, we found that the expression of HNF-3β and C/EBPα was significantly weaker than that of well-differentiated hepatocytes in induced MSCs for 10 wk. Furthermore, the expression of HNF-1α and HNF-4α was undetectable in these cells. Our results clearly indicated that the induced MSCs were not well differentiated because of the low expression of these liver-enriched transcription factors. Among them, HNF-4α is crucial for hepatic differentiation because it exhibits a central determinant of hepatic gene expression including liver-enriched transcription factors[18]. Therefore, we investigated whether the transcription efficiency of hepatic genes could be simply activated by HNF-4α overexpression in induced MSCs and further improve their differentiation status. Overexpression of the rat HNF-4α gene was introduced into 8w-induced MSCs by adenovirus-mediated gene transfer (Ad/HNF4α-IRES-EGFP). Most of the cells (more than 80%) were successfully infected at a 1500 m.o.i. as monitored by the coexpressed EGFP gene, and the overexpression of HNF-4α was confirmed by real-time PCR analysis (Figure 4A). The expression level of other liver-enriched transcription factors in the HNF-4α-infected cells was examined by real-time RT-PCR analysis and compared with that of mock control cells infected with control adenoviruses (Ad-IRES-EGFP) (Figure 4B).

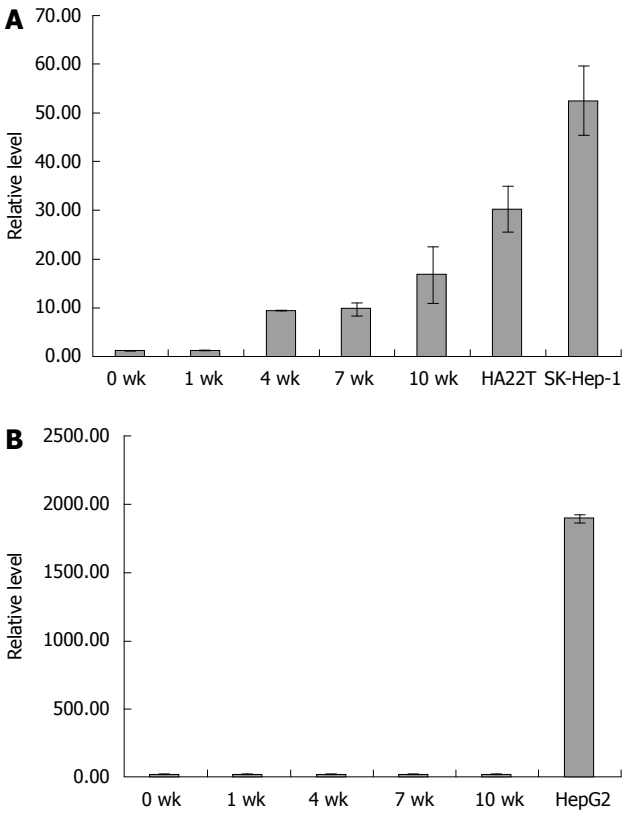


Figure 3 Detection of hepatocyte nuclear factor-4 isoforms in induced mesenchymal stem cells and human hepatoma cell lines by real-time reverse transcription-polymerase chain reaction. A: The relative level of hepatocyte nuclear factor (HNF)-4γ in induced mesenchymal stem cells (MSCs) at the indicated time point (0, 1, 4, 7 and 10 wk post-induction), HA22T/VGH and SK-Hep-1 cells; B: The relative level of HNF-4α in induced MSCs at the indicated time point (0, 1, 4, 7 and 10 wk post-induction) and HepG2 cells. The relative level at 0 wk was set to 1. The amount of input RNA was normalized using the housekeeping gene β2M. Each column represents the mean ± SD of three independent experiments.

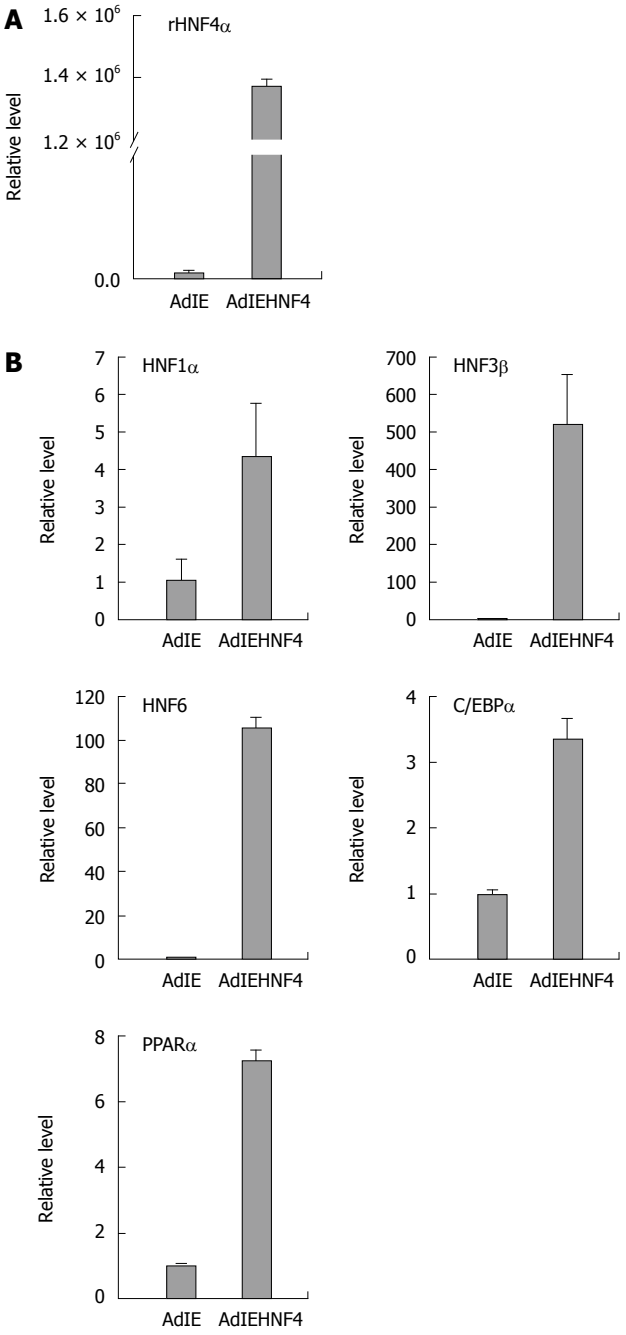


Figure 4 Detection of liver-enriched transcription factors in hepatocyte nuclear factor-4α overexpressing induced mesenchymal stem cells by real-time reverse transcription-polymerase chain reaction. The induced mesenchymal stem cells (8 wk post-induction) were infected with adenovirus containing rHNF-4α gene (Ad/HNF4α-IRES-EGFP) (indicated as AdIEHNF4) or parental control adenovirus (Ad-IRES-EGFP) (indicated as AdIE). The relative level in the control group was set to 1. A: The overexpression of rHNF4α was demonstrated in the Ad/HNF4α-IRES-EGFP infected cells; B: The induction effect of HNF-4α on the expression of liver-enriched transcription factors was represented as HNF1α, HNF3β, HNF6, C/EBPα and peroxisome proliferator-activated receptor α (PPARα), respectively. The amount of input RNA was normalized using the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase. Each column represents the mean ± SD of three independent experiments and the induction fold for each gene was significant ($P < 0.05$). HNF: Hepatocyte nuclear factor.

The expression of HNF-1α, C/EBPα, and peroxisome proliferator-activated receptor α (PPARα) was induced 3- to 7-fold in the HNF-4α-infected hepatocyte-like cells. Furthermore, the expression of HNF-3β and HNF-6 was dramatically induced by more than 100-fold (500-fold and 100-fold, respectively) in the HNF-4α-infected hepatocyte-like cells. The effect of HNF-4α overexpression on the induction of hepatic-specific genes was also evaluated by the same method. Among them, expression of Alb, TAT, and G6P genes was markedly induced in HNF-4α-infected cells, compared with mock control cells, by 12-, 40-, and 2000-fold, respectively (Figure 5).

Induction of the P450 gene family by HNF-4α overexpression

The drug-metabolizing enzymes, cytochrome P450 enzymes, are a superfamily of monooxygenases that play an important role in the detoxification of xenobiotics and the metabolic activation of chemical carcinogens in mature hepatocytes. Therefore, the expression of P450 genes is also designated as an indicator for the late hepatic genes. In this study, the expression patterns of P450 genes, including CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, were examined in induced MSCs, which were induced for 8 wk and then infected with Ad/HNF4α-IRES-EGFP for 1 wk, and compared with that in the mock control cells

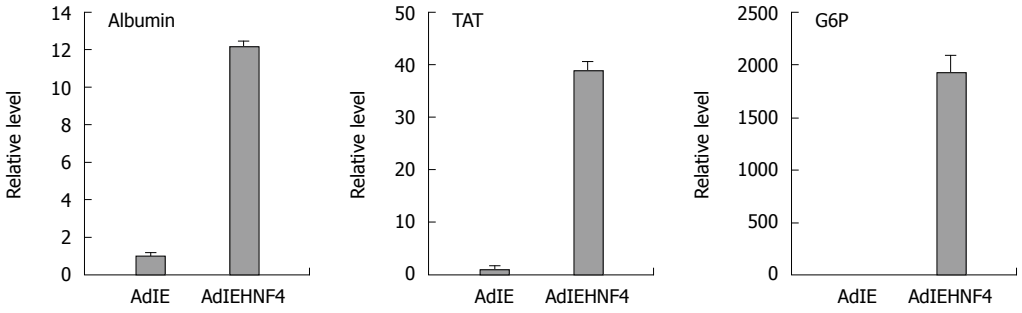
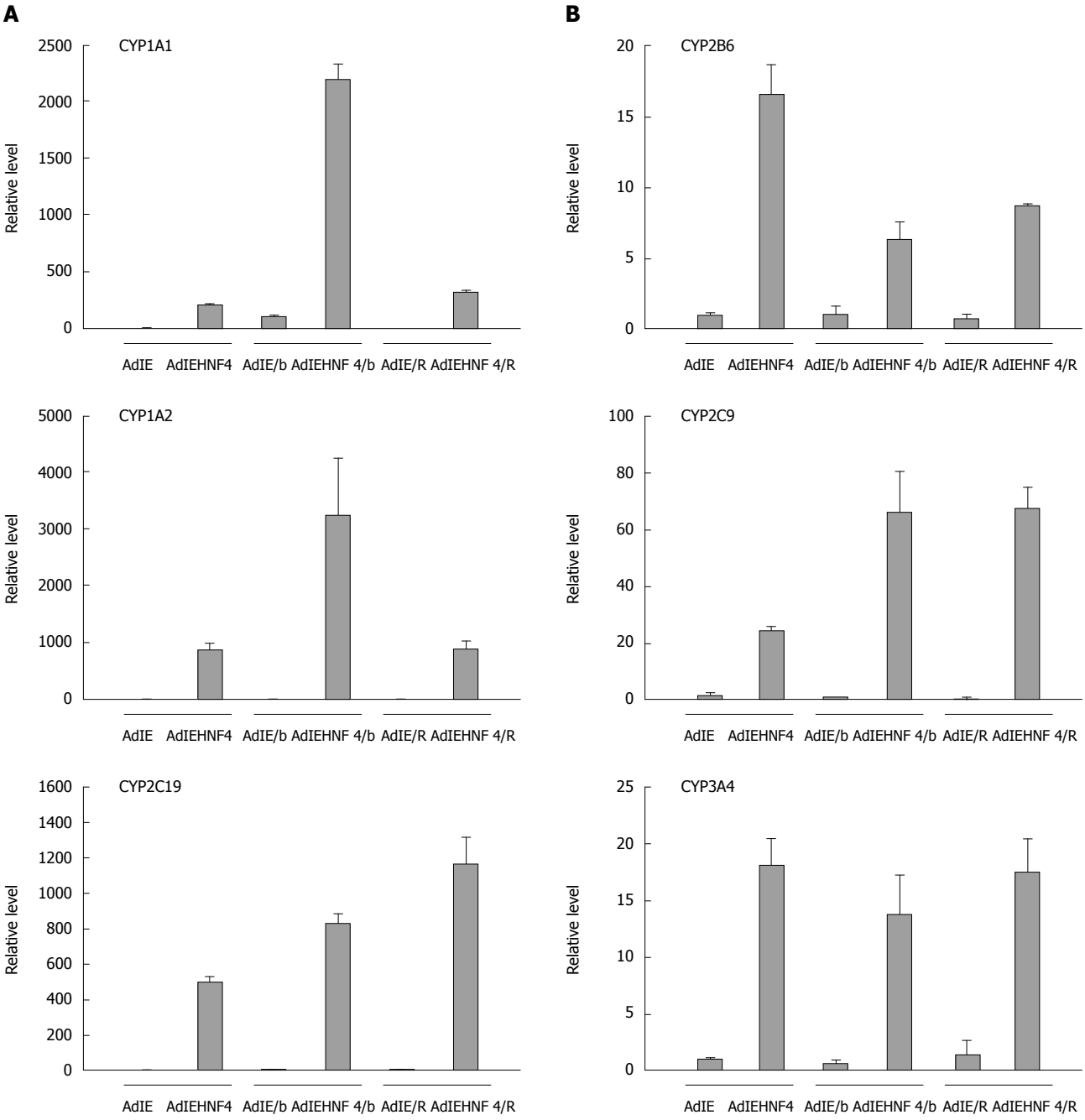


Figure 5 Detection of liver-specific genes in hepatocyte nuclear factor-4α overexpressing induced mesenchymal stem cells by real-time reverse transcription-polymerase chain reaction. The induced mesenchymal stem cells (8 wk post-induction) were infected with adenovirus containing rHNF-4α gene (indicated as AdIEHNF4) or parental control adenovirus (indicated as AdIE). The relative level of each gene in the control group was set to 1. The induction effect of HNF-4α on the expression of liver-specific genes was represented as Albumin, tyrosine-aminotransferase (TAT), and glucose 6-phosphatase (G6P), respectively. The amount of input RNA was normalized using the hypoxanthine-guanine phosphoribosyltransferase gene. Each column represents the mean ± SD of three independent experiments and the induction fold for each gene was significant ($P < 0.05$). HNF: Hepatocyte nuclear factor.



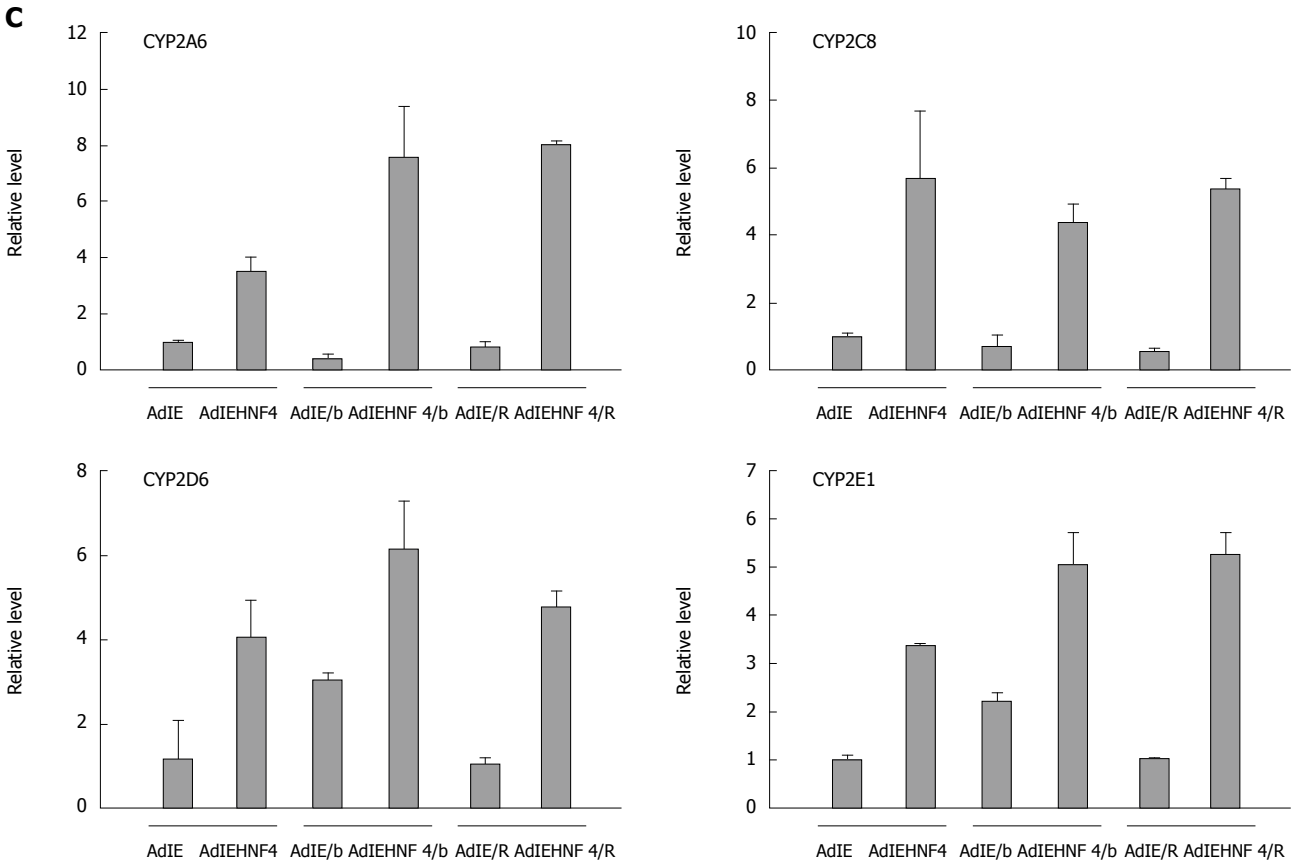


Figure 6 Detection of P450 genes in hepatocyte nuclear factor-4α overexpressing induced mesenchymal stem cells by real-time reverse transcription-polymerase chain reaction. The induced mesenchymal stem cells (8 wk post-induction) were infected with adenovirus containing rHNF-4α gene (indicated as AdIEHNF4) or parental control adenovirus (indicated as AdIE). The infected cells were further treated with inducer B-naphthoflavone (indicated as AdIE/b or AdIEHNF4/b) or rifampicin (indicated as AdIE/R or AdIEHNF4/R) for 2 d. The relative level of each gene in the control group was set to 1. The induction effect of HNF-4α and/or inducer on the expression of P450 genes is represented as (A) CYP1A1, CYP1A2, and CYP2C19; (B) CYP2B6, CYP2C9, and CYP3A4; (C) CYP2A6, CYP2C8, CYP2D6, and CYP2E1, respectively. The amount of input RNA was normalized using the HPRT gene. Each column represents the mean ± SD of three independent experiments and the induction fold by HNF-4α overexpression for each gene was significant ($P < 0.05$). HNF: Hepatocyte nuclear factor.

infected with Ad-IRES-EGFP. As shown in Figure 6, most of the detected genes were not expressed in the induced MSCs (indicated as AdIE). However, all of the detected genes were significantly activated by the overexpression of HNF-4α (indicated as AdIE/HNF4). Among them, CYP1A1, CYP1A2, and CYP2C19 genes were markedly activated by more than 100-fold (Figure 6A); CYP2B6, CYP2C9, and CYP3A4 genes were markedly activated by more than 10-fold (Figure 6B); and CYP2A6, CYP2C8, CYP2D6, and CYP2E1 genes were moderately activated by 3- to 6-fold (Figure 6C).

It is well known that expression of P450 genes can be activated by a range of chemicals[28]. Therefore, the induction potential of P450 gene expression was also investigated after cells were incubated for 48 h with either B-naphthoflavone (BNF) or rifampicin (RIF), which are reported to be inducers of selective P450 genes[29]. As shown in Figure 6, the inducibility of P450 genes was observed in the presence of BNF or RIF both in induced MSCs (indicated as AdIE/b or AdIE/R) and in HNF-4α-overexpressing cells (indicated as AdIEHNF4/b or AdIEHNF4/R). Among them, CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2D6, and CYP2E1 genes were significantly activated by BNF, whereas CYP2A6, CYP2C9, CYP2C19, and CYP2E1 genes were significantly induced by RIF ($P < 0.05$). However, the expression of CYP2C8 and CYP3A4 was not induced and that of CYP2B6 was reduced by either BNF or RIF. Taken together, in the presence of a high amount of HNF-4α, the MSCs-derived hepatocyte-like cells can be further induced toward a more differentiated hepatocytic status. The expression of liver-specific genes, liver-enriched transcription factors, and P450 genes could be significantly activated by HNF-4α overexpression and move toward that of well-differentiated cell types.

DISCUSSION

Recently, several sources of stem cells have been successfully driven to hepatic differentiation and display several hepatic functions such as LDL uptake, glycogen storage and albumin expression[3,5]. In this study, we induced the hepatic differentiation of human bone marrow MSCs according to the protocol previously described by Lee *et al*[3]. The expression pattern of hepatic genes in induced human MSCs seems to be correlated with the developmental pro-

cess of the liver *in vivo*. The differentiated cells expressed early hepatic genes as early as serum starvation treatment, middle hepatic genes at 1 wk post-induction, and late hepatic genes at 4 wk post-induction. However, their differentiation status was much less than that of HepG2 cells as evaluated by their potency of expression of hepatic-specific genes, liver-enriched transcription factors, and the P450 gene family. It is likely that the differentiation status of induced human MSCs lies between HepG2 cells and poorly differentiated hepatoma cells (HA22T/VGH and SK-Hep-1 cells).

It is well known that several liver-enriched transcription factors can coordinately regulate the expression of hepatic genes that are involved in liver-specific functions[15-17]. Among them, HNF-4α may act as a master gene in the transcriptional cascade that regulates constitutive expression of target genes[18-20]. Interestingly, we found that one of the HNF-4 isoforms, HNF-4γ was expressed in the middle stage of induced MSCs and poorly differentiated hepatoma cell lines. The human HNF-4γ gene encodes for a 408 amino acid protein with 70% overall identity to human HNF-4α. A previous study revealed that HNF-4γ is expressed in the kidney, pancreas, small intestine, and colon but not in the liver, while HNF-4α is significantly expressed in the liver[27]. Studies also demonstrated that HNF-4γ is able to activate transcription through the same binding sites as HNF-4α, however, the transactivation potential of HNF-4γ is significantly less active than HNF-4α[30]. These results suggest that the poorly differentiated status of hepatocyte-like cells induced from human MSCs is likely due to the expression of HNF-4γ instead of HNF-4α. Moreover, we demonstrated that the differentiation status of hepatocyte-like cells derived from human MSCs could be further progressed toward that of well-differentiated HepG2 cells by adenovirus-mediated HNF-4α overexpression. Most of the detected hepatic genes, liver-enriched transcription factors, and P450 genes were significantly activated by HNF-4α overexpression. These findings indicate that HNF-4α plays a key role in facilitating hepatic differentiation of human MSCs derived from bone marrow and are consistent with previous studies in rodents[31,32].

Metabolism by cytochrome P450 is a major route of detoxification for a large number of xenobiotics. The induction of P450 gene expression is a common cellular defensive mechanism of hepatocytes against the toxicity of foreign compounds. Hepatic expression of P450 genes can be activated following exposure to various classes of inducers including B-naphthoflavone (BNF), rifampicin (RIF), and phenobarbital (PB)[29]. Among them, expression of CYP1A1 and 1A2 can be significantly induced by exposure to BNF, but weakly induced by RIF and PB[33-35]. Expression of CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 can be increased by RIF and PB, whereas CYP2D6 was not obviously induced by RIF or PB[34-36]. In this study, the inducibility of P450 genes by either BNF or RIF was also demonstrated in the hepatocyte-like cells and HNF-4α-overexpressing cells (Figure 6). In addition, we found that CYP2A6, CYP2C9, CYP2D6, and CYP2E1 genes can be activated by BNF, which had not been examined in previous studies. Relatively little or no induction of CYP2C8 and CYP3A4 or a reduction in CYP2B6 was shown in the presence of RIF. The reason for this observation is unknown at the present time and remains to be further investigated. Taken together, hepatic differentiation of stem cells *in vitro* can be improved by the ectopic expression of a single liver-enriched transcription factor such as HNF-4α.

In conclusion, HNF-4α is a key factor in determining the differentiation status of hepatocyte-like cells derived from human MSCs. Overexpression of HNF-4α can activate various hepatic-specific genes and enhance the differentiation status in differentiated MSCs. This may provide a simple and convenient way to obtain better cell sources for clinical applications and drug screening *in vitro*. Adenoviral vectors are efficient systems for gene delivery both *in vitro* and *in vivo*. However, several limitations including innate immune responses by host cells, genomic integration into target cells, and cytotoxicity for target cells have impeded their clinical utility and studies regarding the determination of certain P450 detoxification. Therefore, delivery of HNF-4α genes by other systems should be considered for future studies. Recently, adeno-associated virus, which has the capacity to deliver genes to both dividing and non-dividing cells in numerous tissues, has shown significant promise in clinical trials because of its safety and delivery efficiency[37,38]. It will be a suitable strategy for the future application of HNF-4α overexpression.

COMMENTS

Background

Mesenchymal stem cells (MSCs) derived from bone marrow have the potential to differentiate into hepatocyte-like cells both *in vitro* and *in vivo*. These observations bring new hope for the possible application of cell-based therapy in severe liver diseases. However, the differentiation status of induced MSCs is poor and not sufficient for future clinical applications.

Research frontiers

The combinational action of several liver-enriched transcription factors regulates hepatic gene expression. Among them, hepatocyte nuclear factor (HNF)-4α plays a crucial role. However, the expression of HNF-4 isoforms in human hepatoma cell lines and induced MSCs has not been studied. In this study, the authors demonstrated that the overexpression of HNF-4α could enhance the hepatic differentiation of human MSCs.

Innovations and breakthroughs

This is the first report to show that the differentiation status of induced human MSCs is similar to poorly differentiated human hepatoma cell lines, all of which expressed HNF-4γ instead of HNF-4α. Overexpression of HNF-4α can significantly activate the expression of hepatic-specific genes, liver-enriched transcription factors and P450 genes and, therefore, enhance hepatic differentiation.

Applications

Overexpression of HNF-4α is a simple and convenient way to facilitate hepatic differentiation of human MSCs, and provide better cell sources for clinical application such as *in vitro* hepatotoxicity models for drug screening.

Peer review

This is a well-written paper that characterized human mesenchymal stem cells differentiating into hepatocyte-like cells. They found these cells express low levels of HNF-4α and high levels of HNF-4γ. Overexpression of HNF-4α induces more hepatocyte-like gene expression including p450-related genes.

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BRIEF ARTICLE

Intraoperative radiofrequency ablation combined with ^{125}iodine seed implantation for unresectable pancreatic cancer

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Abstract

AIM: To evaluate the feasibility, efficacy and safety of intraoperative radiofrequency ablation (RFA) combined with ^{125}iodine seed implantation for unresectable pancreatic cancer.

METHODS: Thirty-two patients (21 males and 11 females) at the age of 68 years (range 48-90 years) with unresectable locally advanced pancreatic cancer admitted to our hospital from January 2006 to May 2008 were enrolled in this study. The tumor, 4-12 cm in diameter, located in pancreatic head of 23 patients and in pancreatic body and tail of 9 patients, was found to be unresectable during operation. Diagnosis of pancreatic cancer was made through intraoperative biopsy. Patients were treated with FRA combined with ^{125}iodine seed implantation. In brief, a RFA needle was placed, which was confirmed by intraoperative ultrasound to decrease the potential injury of surrounding vital structures, a ^{125}iodine seed was implanted near the blood vessels and around the tumor border followed by bypass palliative procedure (cholangio-jejunostomy and/or gastrojejunostomy) in 29 patients.

RESULTS: The serum CA 19-9 level was decreased from 512 ± 86 U/mL before operation to 176 ± 64 U/mL, 108 ± 42 U/mL and 114 ± 48 U/mL, respectively, 1, 3 and 6 mo after operation ($P < 0.05$). The pain score on day 7 after operation, 1 and 3 mo after combined therapy was decreased from 5.86 ± 1.92 before operation to 2.65 ± 1.04, 1.65 ± 0.88 and 2.03 ± 1.16, respectively, after operation ($P < 0.05$). The rate of complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD) in 32 patients was 21.8% (7/32), 56.3% (18/32), 15.6% (5/32) and 6.3% (2/32), respectively, 6 mo after operation, with a median overall survival time of 17. 5 mo. The median survival time of patients at stage Ⅲ was longer than that of those at stage Ⅳ (19 mo *vs* 10 mo, $P = 0.0026$). The median survival time of patients who received and did not receive chemotherapy after operation was 20 mo and 16 mo, respectively ($P = 0.0176$). Of the 32 patients, 3 (10.6%) experienced postoperative complications including transient biliary leaks in 2 patients and acute pancreatitis in 1 patient. All the patients recovered well after conservative support treatment.

CONCLUSION: Intraoperative RFA combined with ^{125}iodine seed implantation is a feasible and safe procedure for unresectable pancreatic cancer with acceptable minor complications, and can prolong the survival time of patients, especially those at stage Ⅲ.

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Key words: Unresectable pancreatic cancer; Radiofrequency ablation; ^{125}iodine seed implantation

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INTRODUCTION

The prognosis of patients with pancreatic adenocarcinoma is dismal due to its delayed diagnosis and aggressiveness. Pancreatic adenocarcinoma, characterized by a late presentation, is one of the most lethal human cancers and currently the fifth and sixth most common causes of cancer-related death in men and women, respectively[1]. Only 10% of the tumors are confined to the pancreas at the time of presentation, 30%-40% are locally advanced and 50% have distant metastases. Surgical resection is the only potentially curative procedure for pancreatic cancer. However, only 5%-22% are resectable at the time of presentation[2]. The median survival time of such patients is 3-4 mo if they are untreated, and less than 5% of such patients can survive 5 years after treatment[3]. The 5-year survival rate for most patients who undergo resection and adjuvant therapy does not exceed 29%[4]. Some palliative therapeutic modalities have been applied in treatment of unresectable locally advanced pancreatic cancer, such as chemotherapy, chemoradiation therapy, external-beam radiation therapy, intraoperative radiation therapy (IORT), iodine-125 implantation and RFA[2,3,5-7]. However, these modalities have almost no effect on the overall survival rate of such patients, indicating that more effective treatment modalities should be developed for improving their prognosis.

The aim of this study was to evaluate the feasibility, efficacy and safety of intraoperative RFA combined with iodine-125 implantation for unresectable pancreatic cancer.

MATERIALS AND METHODS

Patients

Thirty-two patients (21 males and 11 females) at the age of 68 years (range 48-90 years) with unresectable locally advanced pancreatic cancer admitted to our hospital from January 2006 to May 2008 were enrolled in this study. The patients presented with variable symptoms including anorexia, nausea/vomiting, fatigue, weight loss, pruritus. Of these patients, 19 had painless obstructive jaundice, and 12 had mild epigastric or back pain with 2 almost addicted to pethidine or morphine because of intolerable pain. Abdominal ultrasound, contrast-enhanced computed tomography, abdominal and pelvis MRI imaging showed pancreatic cancer in all patients. Their mean serum CA 19-9 level was 512 ± 86 U/mL (range 344-1028 U/mL). The tumor, 4-12 cm in diameter, located in pancreatic head of 23 patients and in pancreatic body and tail of 9 patients, was found at operation to be unresectable due to infiltration of adjacent vessels and metastases in peritoneum or distant organs. Diagnosis of pancreatic cancer was thus made through intraoperative biopsy with hematoxylin and eosin staining. The tumor was classified as stage Ⅲ in 24 patients and stage Ⅳ in 8 patients according to the TNM staging system (UICC, 2002)[8]. The patients were treated with intraoperative FRA combined with ^{125}iodine seed implantation followed by a bypass palliative procedure (cholangio-jejunostomy and/or gastrojejunostomy) for 25 patients. All patients and their relatives were informed about the treatment options. All possible and potential complications were explained in detail to the patients and their relatives before they gave their written consent. The study was approved by the Hospital Ethical Committee.

RFA procedure

A RITA 1500X RF generator (RITA Medical Systems Inc., Mountain View, CA, USA), which generates 100-150 W of power, and a UniBlate™ electrode (AngioDynamics Inc., Queensbury, NY, USA) were used. The UniBlate™ electrode, consisting of a 17-gauge insulated cannula, 15 cm in length with a 1-3 cm exposure length for tumor destruction, is a unique new concept in RFA electrode design that provides linearly scalable ablations from 1 to 3 cm in length and 1 to 2.5 cm in diameter. A built-in thermocouple provides full temperature feedback and RF power control as well as a cool down cycle and track ablation capabilities. Two-four ablations were performed for each pancreatic mass depending on its size. A RFA needle was placed, which was confirmed by direct vision and intraoperative ultrasound to decrease the potential injury of surrounding vital structures (duodenum and vessels). The target temperature controlled with a thermosensor at tip of the needle was 90-100℃. Each application of RFA energy lasted for 12 min to gain an about 3 cm × 2 cm ablation zone. Coagulation necrosis was confirmed by intraoperative ultrasound after the procedure. Each needle tract was packed with thrombin-coated Gelfoam to prevent possible pancreatic leakages. A drainage tube was left in the ablated tumor.

Iodine-125 seed implantation

Type 6711 iodine-125 sealed seed sources were provided by HTA Co., Ltd (Beijing, China). The core source used is silver containing Na^{125}I and the package used is a titanium alloy tube sealed with laser. Each seed source is 4.5 ± 0.3 mm in length and 0.8 ± 0.03 mm in diameter. The half-life of radioactivity of each seed is 59.43 d. The mean photon energy of each seed is 27.4-35.5 KeV, with a human tissue penetration distance of 1.6 cm, an initial dose rate of 7 cGy/h and a mean radioactivity of 0.694 ± 0.021 mCi (25.6 MBq). After RFA, iodine-125 was implantated. The seeds were implanted at the site of tumor near blood vessels and around the tumor border, with each seed at a distance of 1.0 cm. The number of implanted seeds depended on the tumor size. Thirty-two patients were treated with a median number of 18 seeds (range 16-26).

Palliative surgery

Of the 23 patients, 15 with lesions in pancreatic head underwent common bile duct-jejunostomy and 8 re-

ceived both cholangio-jejunostomy and gastrojejunostomy. Of the 9 patients with lesions in pancreatic body and tail, 6 underwent gastrojejunostomy. The main criterion for cholangio-jejunostomy and/or gastrojejunostomy was anticipated tumor in pancreatic head with or without obstruction in bile duct and/or in duodenum or tumor invading in the retroperitoneal ligament of Treitz.

Postoperative management

After operation, all patients were observed in wards for 10-14 d. The patients were instructed to stop eating for 4-6 d. All patients received prophylactic intravenous antibiotics for 5-7 d. An analogue of sandostatin LAR (Novartis) was intravenously infused for 5-7 d. Serum amylase and lipase levels were measured on days 1, 3, and 7, respectively, after operation. Drain output was also closely monitored for any significant amount of fluid and sent for fluid amylase estimation to rule out pancreatic leak or ascites formation.

Postoperative systemic chemotherapy

Chemotherapy with gemcitabine (700 mg/m^2)[9] was conducted for patients 2 wk after surgery. Gemcitabine was given intravenously for over 30 min on days 1, 8 and 15, respectively, after operation. This therapy was repeated every 4 wk for 6 cycles if tolerated.

Follow-up

All patients were followed up for 6-33 mo (mean 18.2 mo). Follow-up CT scanning was performed 4 wk and then every 3-6 mo after operation to assess the effectiveness of treatment and monitor disease progression in all patients. Laboratory tests, such as a complete blood cell count, liver function and serum CA 19-9 level, were also repeated. Tumor diameter and general condition of the patients were recorded during follow-up. The pain was scored using a 10-point visual analog scale (VAS)[10,11] as 0 (none), 1-3 (mild), 4-7 (moderate), and 8-10 (severe). The effect of treatment on pain control was assessed by collecting pain score 7 d, 1 and 3 mo, respectively, after operation. The response evaluation criteria for solid tumor (RECIST)[12] were adopted in assessment of change in tumor burden. Complete response (CR) was defined as complete disappearance of all lesions lasting for at least 4 wk. Partial response (PR) referred to the situation where the sum of maximum diameter of all lesions was decreased by more than 30%. Stable disease (SD) was defined as the sum of maximum diameter of lesions was decreased by less than 30%. Progressive disease (PD) defined as at least 20% increase in the sum of maximum diameter of lesions, or the appearance of one or more new lesions.

Statistical analysis

Statistical analysis was performed using SPSS version17 software (SPSS, Chicago, USA). Pain score of patients and serum CA19-9 level before and after treatment were compared using paired *t*-test or nonparametric methods.

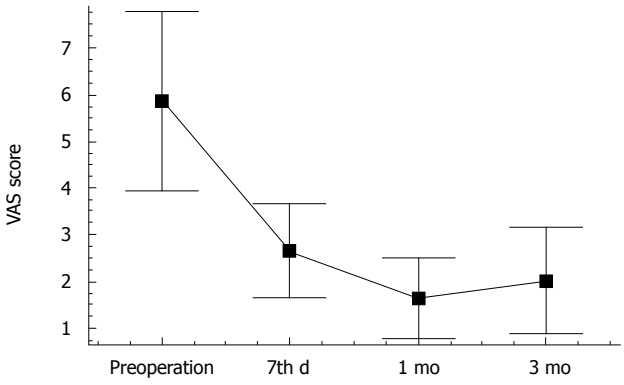

Figure 1 Pain score of patients before and after combined therapy.

Survival time was calculated using the Kaplan-Meier method[13] and difference was assessed with the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

No major procedure-related death occurred. Of the 32 patients, 3 (10.6%) experienced postoperative complications, 2 had surgical anastomosis-associated transient biliary leak which was treated nonoperatively, 1 had RFA-related acute pancreatitis which was treated with somatostatin infusion for 2 wk. The patients recovered well. None required any form of surgical or radiological intervention for minor complications. All patients were advised to receive postoperative adjuvant chemotherapy. However, 6 patients refused any further treatment. Of the 32 patients, 15 completed the full course of chemotherapy and 11 did not because of severe toxicity after one cycle.

The serum CA 19-9 level was decreased from 512 ± 86 U/mL before operation to 176 ± 64 U/mL, 108 ±42 U/mL and 114 ± 48 U/mL, respectively, 1, 3 and 6 mo after operation ($P < 0.05$). The mean pain score for the 26 patients with mild epigastric or back pain before and after operation is shown in Figure 1. The pain score was decreased from 5.86 ± 1.92 before operation to 2.65 ± 1.04, 1.65 ± 0.88 and 2.03 ± 1.16, respectively, 7 d, 1 and 3 mo after combined therapy ($P < 0.05$). CT showed partial necrosis after therapy in all patients. Most patients showed varying degrees of tumor necrosis 3 mo after operation (Figure 2). The rate of CR, PR, SD and PD for 32 patients was 21.8% (7/32), 56.3% (18/32), 15.6% (5/32) and 6.3% (2/32), respectively, 6 mo after operation. No significant difference was observed in tumor diameter of patients with SD before and 3 mo after operation. Two or more new lesions were found in 2 patients with PD after operation.

The survival time of all patients was calculated using the Kaplan-Meier method (Figure 3A). The mean and median survival time was 17.6 mo (95% CI: 15-20) and 17.5 mo (95% CI: 12-20), respectively. The overall 12 and 24 mo-survival rate was 65.6% and 21.9%, respectively. The maximun survival time was 33 mo in a patient who was still alive without evidence of disease progression at

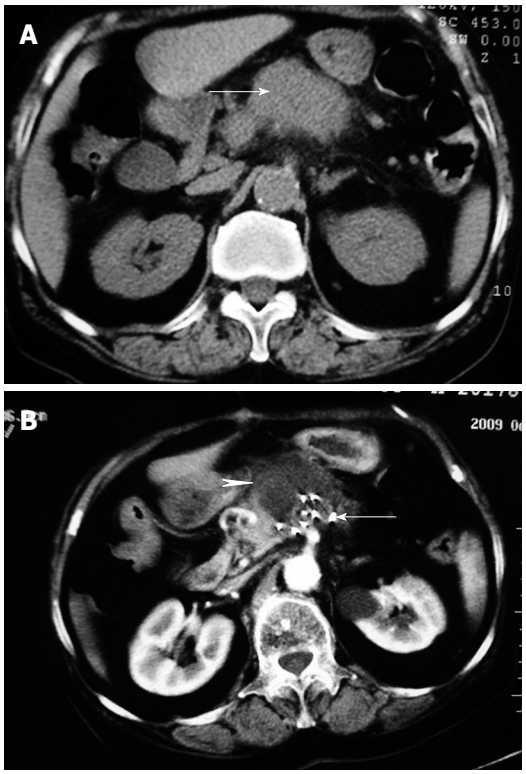

Figure 2 Computed tomography scan showing a tumor in pancreatic head (white arrow) before combined therapy (A) and a 4 cm × 4 cm necrosis with non-enhancement in tumor region of pancreas (arrowhead) 3 mo after combined therapy with ^{125}iodine seeds distributed around the tumor (arrow) (B).

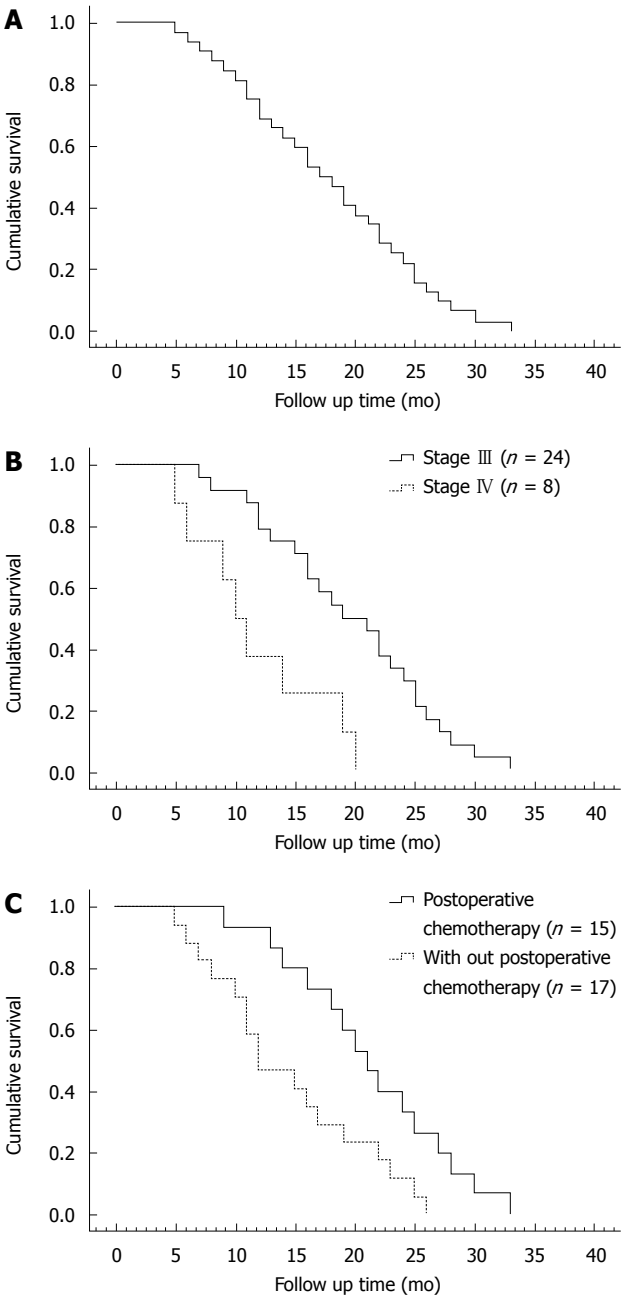

Figure 3 Total cumulative survival time of 32 patients with pancreatic cancer (A), Kaplan-Meier survival curves for patients with pancreatic cancer at stages Ⅲ and Ⅳ (B), and for those who received or did not receive chemotherapy after operation (C).

the time when we wrote this paper. The mean and median survival time of patients at stages Ⅲ and Ⅳ was 19 mo (95% CI: 16-22) and 19 mo (95% CI: 14-23), and 11 mo (95% CI: 7-15) and 10 mo (95% CI: 7-12), respectively. The 12 mo and 24 mo-survival rate was 75% and 33.3% for patients at stage Ⅲ, and 37.5% and 0% for patients at stage Ⅳ. Log-rank test showed that the survival rate for patients at stages Ⅲ and Ⅳ was significantly different (P = 0.0026, Figure 3B). The mean and median survival time of patients who received and did not receive chemotherapy after operation (n = 15) was 19 mo (95% CI: 15-23) and 20 mo (95% CI: 13-26), and 16 mo (95% CI: 13-19) and 16 mo (95% CI: 9-22), respectively. The 12 and 24 mo-survival rate was 80% and 40% for patients who received chemotherapy after operation, and 47.1% and 11.8% for those who did not receive chemotherapy after operation. Log-rank test showed a significant difference in survival rates for patients who received or did not receive chemotherapy after operation (P = 0.0176, Figure 3C).

DISCUSSION

Pancreatic cancer is difficult to diagnose at its early stage and would be unresectable in most patients when they present with symptoms. Traditional therapeutic options for patients with advanced unresectable pancreatic cancer include chemotherapy, combined chemoradiation, external beam irradiation, IORT and radioactive seed implantation. Palliative chemotherapy with gemcitabine can improve the outcome of patients with advanced pancreatic cancer. It was reported that the median survival time of patients is only about 6 mo with a 12-mo survival rate of less than 20% after chemotherapy with gemcitabine although it is superior to bolus 5-Fu[14,15]. The effect of combined gemcitabine and bolus Ⅳ 5-FU on advanced pancreatic cancer is similar to that of gemcitabine monotherapy. However, it has been shown that the 1-year survival rate of patients with advanced pancreatic cancer is 23% after combined erlotinib and gemcitabine therapy and is 17% after gem-

citabine monotherapy[15]. Chemotherapy with or without radiotherapy can improve the symptoms and quality of life of patients, but cannot prolong their survival time. Selection of patients, side effects and complications of chemotherapy and radiotherapy should also be considered[16]. IORT and conformal external-beam radiation therapy plus protracted 5-FU infusion for advanced pancreatic cancer have been evaluated in a phase Ⅱ study[17], showing that combined therapy could not prolong the survival time of such patients, the median survival time of all enrolled patients, those without and with metastasis was 7.8, 12.9, and 5.8, respectively. It was reported that I-125 seed implantation controls growth of the tumor but increases perioperative morbidity[18,19].

RFA is a relatively new procedure for liver and lung tumors[20,21] and other solid tumors such as small breast cancer, renal tumor, cancers of adrenal gland, spleen, prostate, bone and brain[22-28]. However, RFA has not been widely performed as a treatment procedure for pancreatic tumors because of the fragile pancreatic parenchyma and the proximity to some important structures such as duodenum, common bile duct and vessels. The technical feasibility and effect of RFA have been studied in normal porcine pancreatic tissues with encouraging results because discrete zones of coagulation necrosis can be noticed with no major complications[29]. In 2000, Matsui *et al*[30] first reported 20 patients with stage Ⅳ pancreatic adenocarcinoma treated with RFA. Complications were observed only in 2 patients (a cyst and an abscess formation). No significant difference was found in the prognosis of these patients compared with those at the same stage who did not receive RFA, indicating that RFA is relatively safe and can be used in treatment of unresectable tumors without metastasis or benign pancreatic tumors. Since then, some reports on RFA for pancreatic cancer have been published[7,31-33]. However, the number of patients who were treated with RFA was small in most reports. It is difficult to comment on the improvement in survival. RFA for pancreatic tumors has also various complications such as acute pancreatitis, pancreatic fistula, and pancreatic ascites. It was more recently reported that the effect of RFA and palliative therapy on advanced pancreatic cancer is similar and RFA can prolong the survival time of patients with stage Ⅲ unresectable pancreatic cancer[34].

In the present study, intraoperative FRA in combination with ^{125}iodine seed implantation was performed in order to evaluate its feasibility, efficacy and safety in patients with unresectable pancreatic cancer.

In our series, no major complications occurred except for RFA-associated acute pancreatitis. In our experience, it is important to adequately expose the tumor before RFA by liberal kocherization and/or opening the mesocolon depending upon its location. Under intraoperative ultrasound guidance, the electrode can be accurately placed into the tumor. The ablation should be restricted within the tumor to avoid damage to normal pancreatic tissue, surrounding organs and nearby large blood vessels. To minimize the residual tumor near the blood vessels, ^{125}iodine seed was implanted into duodenum and normal pancreatic tissue. Distal common bile duct injury caused by ablation was taken into consideration in patients with tumors in pancreatic head. A cholangio-jejunostomy was performed to circumvent this effect after RFA. Somatostatin analogues were used after operation to reduce possible complications such as acute pancreatitis, pancreatic fistula. Our results demonstrate that RFA combined with ^{125}iodine seed implantation for unresectable pancreatic tumors is feasible and safe with acceptable minor complications.

Better tumor responses, significantly decreased tumor marker levels and pain score, were observed during follow-up. The pain was relieved in all patients, and 3 patients who were almost addicted to pethidine or morphine for intolerable pain stopped any related medication after combined treatment. Objective local cytoreduction was confirmed in tumor of all patients with contrast-enhanced CT during follow-up. The rate of CR, PR, SD and PD was 21.8%, 56.3%, 15.6% and 6.3%, respectively, 6 mo after operation. The median and maximun survival time of 32 patients was 17.5 and 33 mo in a patient who was still alive at the time when we wrote this paper. The 12 and 24 mo-survival rate was 75% and 33.3% for patients at stage Ⅲ, and 37.5% and 0% for patients at stage Ⅳ, indicating that chemotherapy after operation can improve the survival rate of patients with pancreatic cancer[9]. In this study, the median survival time of patients who received and did not receive chemotherapy after operation was 20 and 16 mo, respectively, suggesting that systemic chemotherapy after operation can prolong their survival time. In this study, some patients did not receive chemotherapy due to their older age and organ dysfunction as well as toxicity of the therapy. Thus, further study is needed to verify the effect of postoperative chemotherapy on unresectable pancreatic cancer after intraoperative RFA combined with ^{125}iodine seed implantation in.

In conclusion, RFA combined with ^{125}iodine seed implantation is a feasible and safe procedure for unresectable pancreatic cancer. However, a greater number of patients and long-term follow-up are needed to confirm its effect on the survival time and quality of life of such patients.

COMMENTS

Background

The prognosis of pancreatic cancer is dismal due to its late diagnosis and aggressiveness. Surgical resection is the only potentially curative procedure for pancreatic cancer. However, only 5%-22% are resectable and less than 5% of patients can survive for 5 years after operation. The 5-year survival rate for most patients does not exceed 29% after resection and adjuvant therapy. Some palliative therapeutic modalities can be used in treatment of unresectable locally advanced pancreatic cancer, such as chemotherapy, chemoradiation, external-beam radiation therapy, intraoperative radiation therapy, iodine-125 implantation and radiofrequency ablation (RFA). However, they cannot improve the overall survival rate of such patients, thus more effective treatment modalities should be developed.

Research frontiers

RFA is commonly used in treatment of liver and lung tumors with encouraging results but not in treatment of pancreatic tumor because of the fragile pancreatic parenchyma and the proximity to some important structures. A few reports are available on RFA in treatment of pancreatic cancer but the number of patients is small in most reports. It is difficult to comment on its feasibility

and safety. Moreover, no report is available on ^{125}iodine seed implantation as a complementary therapy for pancreatic cancer after RFA.

Innovations and breakthroughs

This is the first study on RFA combined with ^{125}iodine seed implantation for pancreatic cancer, which has a complementary effect on pancreatic cancer with different mechanisms of action.

Applications

Surgical resection is the only potentially curative procedure for pancreatic cancer, but only 5%-22% are resectable at the time of presentation, 30%-40% are locally advanced and 50% have distant metastases. RFA combined with ^{125}iodine seed implantation is a feasible and safe procedure for unresectable pancreatic cancer.

Terminology

Pancreatic adenocarcinoma accounts for 90% of all pancreatic cancers with 80% occurring in pancreatic head. Carcinoma in pancreatic body and tail is much less common. Microscopically, these tumors may vary from well-differentiated to undifferentiated tumors. Approximately two thirds of all pancreatic cancer patients will have metastasis at the time of diagnosis, and the tumor will become unresectable in the majority of the remaining patients. Ultrasound, computed tomography and magnetic resonance imaging are the currently used methods in diagnosis and staging of pancreatic cancer.

Peer review

The authors described a series of pancreatic cancer patients with unresectable cancer who received intraoperative RFA along with i-125 seed implantation. The data are fairly compelling and the article is well written.

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BRIEF ARTICLE

Temporary self-expanding metallic stents for achalasia: A prospective study with a long-term follow-up

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Abstract

AIM: To compare the efficacy of self-expanding metallic stents (SEMSs) for the long-term clinical treatment of achalasia.

METHODS: Ninety achalasic patients were treated with a temporary SEMS with a diameter of 20 mm (n = 30, group A), 25 mm (n = 30, group B) or 30 mm (n = 30, group C). Data on clinical symptoms, complications and treatment outcomes were collected, and follow-up was made at 6 mo and at 1, 3-5, 5-8, 8-10 and > 10 years, postoperatively.

RESULTS: Stent placement was successful in all patients. Although chest pain occurrence was high, stent migration was less in group C than in groups A and B. The clinical remission rate at 5-8, 8-10 and > 10 years in group C was higher than that in the other two groups. The treatment failure rate was lower in group C (13%) than in groups A (53%) and B (27%). SEMSs in group C resulted in reduced dysphagia scores and lowered esophageal sphincter pressures, as well as normal levels of barium height and width during all the follow-up time periods. Conversely, these parameters increased over time in groups A and B. The primary patency in group C was longer than in groups A and B.

CONCLUSION: A temporary SEMS with a diameter of 30 mm is associated with a superior long-term clinical efficacy in the treatment of achalasia compared with a SEMS with a diameter of 20 mm or 25 mm.

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Key words: Achalasia; Dysphagia; Self-expanding metallic stents; Comparison

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INTRODUCTION

Self-expanding metallic stents (SEMSs), bare or covered,

have been used safely and effectively in the treatment of malignant esophageal dysphagia and fistula for the last two decades[1-6]. However, the management of benign esophageal strictures with stent placement has not been well established primarily due to complications, such as stent migration, reflux, perforation, bleeding, and most importantly, the development of new strictures as a result of stent-induced tissue hyperplasia[7-12].

Recently, a novel strategy using retrievable stents has been successfully applied in the treatment of benign esophageal strictures[7-10]. Temporary stent placement also seems to be an alternative approach for the treatment of patients with esophageal achalasia. However, there are only a few reports of this disease being treated with SEMSs[11-19]. Commencing in July 1994, we designed and manufactured (Youyan Yijin Advanced Materials Co. Ltd, Beijing, China) a temporary SEMS using three diameters specialized for the treatment of esophageal achalasia[13-16,20]. Because relatively little is known about the long-term efficacy of patients treated with SEMS, we designed a prospective study to compare the long-term clinical outcome of the stents with different diameters in the treatment of achalasic patients.

MATERIALS AND METHODS

Study design

This pilot study was approved by the Institutional Review Board of the Sixth Affiliated People's Hospital of Shanghai Jiao Tong University, and informed consent was obtained from each patient. From July 1994 to December 2007, 90 consecutive achalasic patients were treated with temporary SEMSs with a diameter of 20 mm (n = 30, group A), 25 mm (n = 30, group B) or 30 mm (n = 30, group C). Patients with achalasia agreed to undergo a prospective study to evaluate the use of fluoroscopically-placed SEMS over a 13-year follow-up period. The preoperative diagnosis was based on clinical presentations, barium swallows, gastroscopies or esophageal manometric.

The inclusion criteria for stent placement were as follows: (1) documented primary esophageal achalasia; (2) recurrent dysphagia following pneumatic balloon dilation; and (3) patient life expectancy of more than 6 mo. The exclusion criteria were (1) a lesion longer than 6 cm; (2) dysfunction of blood coagulation, active infection, significant cardiac or pulmonary disease, malignancy, and significant psychological or psychosocial dysfunction; and (3) World Health Organization performance score ≥ 3. The preoperative dysphagia scores were evaluated by three radiologists, including grade 0, no dysphagia; grade 1, some solid food; grade 2, liquids only; grade 3, difficulty with liquids and saliva; grade 4, complete dysphagia. These procedures were performed by an interventional radiologist (Cheng YS) who has 15 years of experience in gastrointestinal interventional radiology.

Stent construction and insertion procedure

Each SEMS was woven from a single thread of 0.16 mm highly elastic nitinol wire. As shown in Figure 1, the stent had a tubular configuration with an elliptical structure, proximally and distally. The body of the stent was covered with polyethylene measuring 20, 25 or 30 mm in diameter and 80 mm in length when fully expanded. The elliptic structure at both ends was 1 cm in length and 2 mm larger in diameter than the body of the stent. For implantation under fluoroscopic guidance, each stent was compressively mounted on a guiding tube by a 24-French (Fr) introducer sheath (8 mm in diameter).

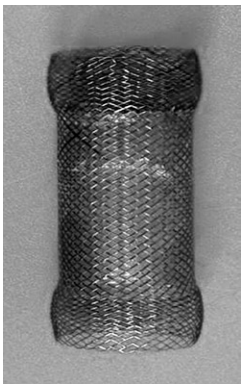

Figure 1 Photograph of a partially covered self-expanding metallic stent.

The SEMSs were specifically designed for placement in the esophageal cardia. The details of the stent placement techniques are described elsewhere[20]. Briefly, after topical anesthesia, a 0.035-inch guide wire (Radiofocus M; Terumo, Tokyo, Japan) with a straight 5-Fr catheter (Torcon NB; Cook, Bloomington, USA) was advanced perorally until the tip reached the gastric body, after which it was exchanged for a stiffer guide wire (0.035-inch Amplatz super-stiff). Under fluoroscopic control, a 24-Fr delivery system (Youyan Yijin, Beijing, China) was inserted over the guide wire until the proximal and distal edges of the stent bridged the esophageal achalasia. The stent was then deployed by withdrawing the introducer sheath. Patients ate semisolid food on the day following stent placement and were given a prophylactic H_2 receptor blockade to prevent reflux esophagitis. Chest radiography was performed 1, 3 and 7 d after the stent placement to verify the state of the stent expansion and migration.

Stent retrieval was performed by gastroscopy 4-5 d after placement. Ice-cold water (500-1000 mL) was injected *via* the bioptic hole to retract the stent, and the stent was gently removed by grasping the proximal wire or by using a retrieval lasso. Usually less than 10 min were required for this procedure.

Postoperative outcome evaluations

Postoperative outcomes were assessed by responses to a standardized questionnaire for symptoms at the initial presentation and during the follow-up periods. In the questionnaire, the clinical symptoms were recorded, including those of dysphagia score, chest pain, barium swallow, esophageal emptying, esophageal manometry and, if necessary, endoscopy. Outcome assessments were performed postoperatively at 6 mo and at 1, 3-5, 5-8, 8-10 and > 10 years. A slightly modified grading system of Vantrappen and Hellemans[21] was used to estimate the effectiveness of

treatment, including: (1) excellent, completely free of symptoms; (2) good, dysphagia or chest pain ⩽ once per week without regurgitation; (3) moderate, dysphagia 2-4 times per week; and (4) poor, dysphagia daily and/or regurgitation. Ratings of excellent and good were considered as an indication of treatment success, and ratings of moderate and poor were an indication of treatment failure.

Timed barium esophagram

Details of the timed barium esophagram[22,23] as an objective assessment of esophageal emptying were as previously described. Briefly, after fasting overnight, patients ingested a low density barium sulfate suspension (45% w/v) for 30-45 s while maintaining an upright position. Patients were instructed to drink the amount of barium they could tolerate without regurgitation or aspiration (usually between 100 and 250 mL). With the patient upright in a slightly left posterior oblique position, radiographs of the esophagus were taken at 1, 2 and 5 min after the last swallow of barium. The maximal esophageal width (barium width) and the distance from the distal esophagus (identified by a bird's beak appearance of the esophagogastric junction) to the top of a distinct barium column (barium height) were measured. The same volume of barium was given to each patient for both the preoperative and postoperative studies. The 5 min barium heights and widths (normal ⩽ 3 cm) were used for analysis of the degree of esophageal emptying and of reduction in esophageal diameter. In most normal subjects, barium could be completely emptied out of the esophagus by 1 min, and emptied from all individuals by 5 min.

Esophageal manometry

Esophageal manometry was performed in all patients with an overnight fast using a low compliance, pneumohydraulic water infusion system (Arndofer, Medical Specialties, Milwaukee, WI, USA) and an 8-lumen, manometric catheter. The catheter had four ports radially oriented (90°) near the tip and four centrally positioned 5 cm apart (5, 10, 15 and 20 cm from the tip). The recording sites were connected to an 8-channel polygraph (Synetics Medical AB, Stockholm, Sweden). The manometric catheter assembly was passed transnasally without any sedation into the stomach. The lower esophageal sphincter (LES) pressure was determined using the station pull through technique and recorded as the mean of four measurements at mid-respiration. Completeness of LES relaxation (normal > 85%) was assessed as the percent decrease from the resting LES pressure to the gastric baseline following wet swallows. Esophageal body motility was recorded at 3, 8, 13 and 18 cm above the LES in response to 5 mL swallows of water at 30 s intervals[24]. LES pressures and peristalsis were determined at the time of diagnosis, at 6 mo, and 1, 3-5, 5-8, 8-10 and > 10 years after the procedures.

Statistical analysis

All the data were expressed as the mean ± SD. Comparisons of the variables between the two groups were performed by the Mann-Whitney test, χ^2 test or the Fisher's exact test as appropriate. The cumulative remission rate was determined by the Kaplan-Meier estimator and the difference between their curves was tested by the log rank test. Statistical analyses were performed using SPSS statistical software (version 13.0 for Windows, SPSS Inc., Chicago, IL, USA). The *P* value was considered statistically significant if ⩽ 0.05.

Table 1 Clinical characteristics of 90 patients treated with a temporary self-expanding metallic stent with a diameter of 20, 25, or 30 mm (mean ± SD) *n* (%)

	Group A (*n* = 30)	Group B (*n* = 30)	Group C (*n* = 30)	*P* value
Age (yr)	43.37 ± 15.84	36.23 ± 10.58	37.30 ± 13.13	> 0.05
Gender (M/F)	16/14	17/13	18/12	> 0.05
Duration of symptoms	6.01 ± 3.69	4.74 ± 2.76	5.33 ± 3.65	> 0.05
Symptoms				
Chest pain	23 (77)	21 (70)	18 (60)	> 0.05
Regurgitation	15 (50)	12 (40)	15 (50)	> 0.05
Heartburn	7 (23)	9 (30)	10 (33)	> 0.05
Weight loss	3.80 ± 2.90	2.96 ± 2.76	3.57 ± 3.36	> 0.05
Dysphagia score	2.93 ± 0.45	2.87 ± 0.43	2.83 ± 0.53	> 0.05
Lesion diameter (mm)	5.33 ± 2.14	5.97 ± 1.90	6.0 ± 2.38	> 0.05
Lesion length (mm)	16.93 ± 6.50	19.73 ± 5.98	18.57 ± 6.52	> 0.05

RESULTS

Clinical characteristics

The clinical characteristics of the patient population are summarized in Table 1. A total of 90 patients who underwent stent placement for achalasia were enrolled in this prospective analysis. These included 51 men and 39 women with a mean age of 37.96 ± 13.32 years (range: 11-85 years). The mean duration of the symptoms between a significant dysphagia confirmation and stent placement was 5.36 ± 3.39 years (range: 1.1-15.7 years). There were no significant differences among the three groups in clinical symptoms, duration of the symptoms, lesion diameter, lesion length or the length of the stent (Table 1).

Technical and initial clinical outcomes

Technical and initial clinical outcomes among the three groups are shown in Table 2. Fluoroscopic stent placement in the esophageal cardiac gland was technically successful in all patients without procedure-related complications. Complete expansion of the stent occurred within 24 h after placement. The mean time of the procedure was 19 ± 6 min (range: 10-30 min).

Stent migration was significantly less in group C than in group A ($P = 0.039$), whereas chest pain occurrence was significantly higher in group C than in group A ($P = 0.0047$). There were no significant differences in reflux, bleeding or food impaction among the three groups ($P > 0.05$). No perforation occurred among the three groups after stent placement, and the 30-d mortality was nil.

Table 2 Technical and clinical outcome among the three groups (mean ± SD) *n* (%)

	Group A (*n* = 30)	Group B (*n* = 30)	Group C (*n* = 30)	*P* value
Early outcome				
Technique success	100%	100%	100%	1.0
Complications				
Stent migration	8 (27)	4 (13)	2 (7)[a] (*P* = 0.039)	0.096
Chest pain	5 (17)	10 (33)	12 (40)[a] (*P* = 0.047)	0.130
Reflux	7 (23)	5 (17)	6 (20)	0.814
Bleeding	3 (10)	5 (23)	6 (20)	0.557
Perforation	0	0	0	1.0
Food impaction	2 (7)	0	0	0.132
30-d mortality	0	0	0	1.0
Late outcome				
Primary patency (yr)	5.45 ± 0.41	6.67 ± 0.56[c] (*P* = 0.023)	7.15 ± 0.50[a] (*P* = 0.003)	0.006
Cumulative treatment failures	16 (53)	8 (27)[c] (*P* = 0.037)	4 (13)[a] (*P* = 0.001)	0.003
Follow-up (yr)	7.13 ± 2.61	7.24 ± 2.95	7.30 ± 2.46	0.997

[a]*P* < 0.05, Group C *vs* Group A; [c]*P* < 0.05, Group B *vs* Group A.

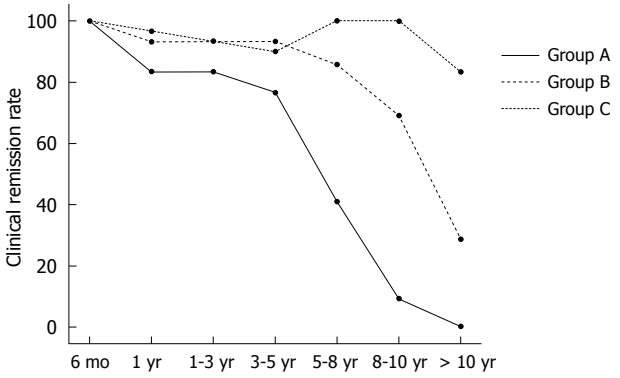

Figure 2 Clinical remission rates in comparison with self-expanding metallic stents with a diameter of 30 mm (Group C), 25 mm (Group B) or 20 mm (Group A).

Treatment success was achieved in all patients with a patency of the esophageal cardiac gland at 1 mo after stent removal, and the dysphagia scores significantly improved for all patients. There were no significant differences in clinical success among the three groups ($P > 0.05$).

Long-term follow-up and final outcomes

Figure 2 shows the long-term follow-up and clinical outcomes at various follow-up periods and the curves of the clinical remission rates among the groups. The mean time from stent removal to the last follow-up assessment was 7.23 ± 2.65 years (range: 3-12.7 years). All patients were assessed at 6 mo and at 1, 1-3, 3-5, 5-8 (67 patients), 8-10 (38 patients) and > 10 years (19 patients), prospectively. There were no significant differences in these rates at 6 mo or at 1, 1-3 or 3-5 years among the three groups ($P > 0.05$). However, the clinical remission rates were significantly higher in group C than in groups A and B at 5-8, 8-10 and > 10 years (Figure 2).

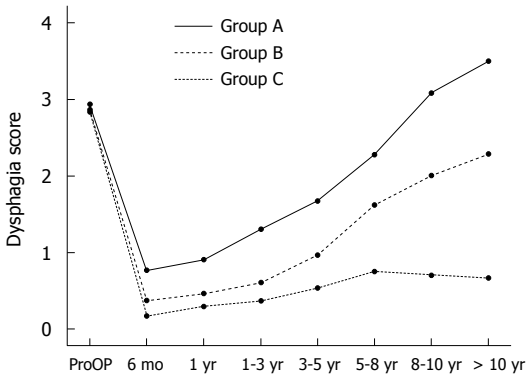

Figure 3 Dysphagia scores among the three groups before self-expanding metallic stents placement at different follow-up time intervals.

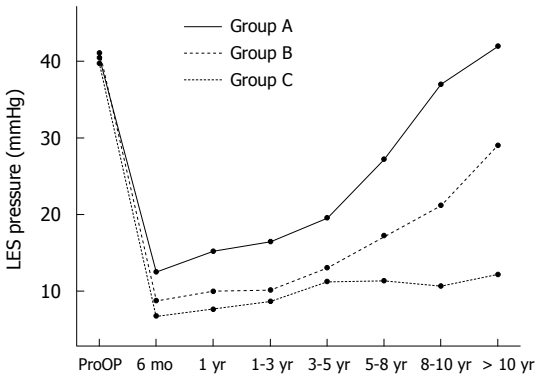

Figure 4 Lower esophageal sphincter pressures assessed by manometry among the three groups before self-expanding metallic stent placement at different follow-up time intervals. LES: Lower esophageal sphincter.

Figures 3-5 exhibit the curves of the dysphagia scores, the LES pressures and the barium height and width measurement among the three groups. The LES pressure was less than 12 mmHg in group C at all times of measurement. In group C, the esophageal barium height and diameter also remained consistently lower than the preoperative values and below normal levels. Similarly, the dysphagia score for group C remained at a lower level for all later measurements. Conversely, the dysphagia score, LES pressure and esophageal emptying (as assessed by the barium column length and width) in group A increased and gradually returned to the preoperative values. The same parameters in group B increased more slowly than those in group A. However, they were significantly increased at 8-10-year and > 10-year follow-up evaluations, although they remained below the preoperative values.

Within the 13-year follow-up period, the stent treatment was considered to have failed in 16 (53%) patients in group A, 8 (27%) in group B and 4 (13%) in group C after 5.53 ± 3.74 years (range: 7 mo-12.7 years). The overall cumulative treatment failure rate was significantly higher in group A than in groups B and C ($P = 0.0037$ and $P = 0.0001$). Nine patients with poor clinical results (seven in group A and two in group B) after 8.28 ± 3.51 years (range: 3.1-11.4 years) received an additional stent treatment (diameter of 30 mm). Although they were considered as treat-

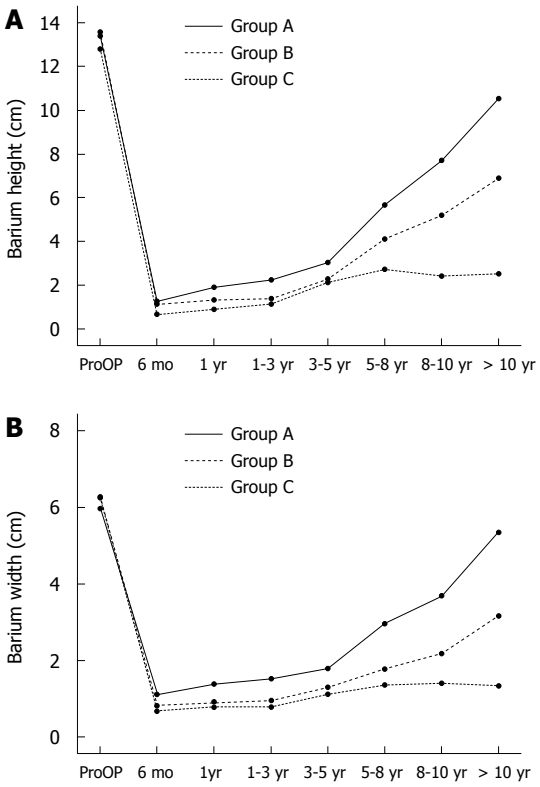

Figure 5 The barium height (A) and width (B) assessed by a timed barium esophagram among the three groups before self-expanding metallic stent placement at different follow-up time intervals.

ment failures, the remaining 19 patients were included in the follow-up assessments because they were experiencing mild recurrent dysphagia. One patient died at the end of this study due to old age.

The mean survival in groups A, B and C was 7.13 ± 0.48 years (95% CI: 6.19-8.06), 7.24 ± 0.54 years (95% CI: 6.19-8.30) and 7.31 ± 0.45 years (95% CI: 6.43-8.12), respectively; and the median survival was 6.60 ± 0.61 years (95% CI: 5.39-7.81), 6.90 ± 1.30 years (95% CI: 4.35-9.45), and 7.10 ± 0.61 years (95% CI: 5.89-8.31), respectively. There were no significant differences in patient survivals among the three groups ($P = 0.828 > 0.05$, log rank test). The primary patency in groups C and B was significantly longer than that in group A (Table 2, $P = 0.001$ and $P = 0.02$, log rank test).

DISCUSSION

In this prospective study, the overall cumulative clinical remission rate was 47%, 73% and 87% in groups A, B and C, respectively. This rate was significantly lower in group A than that in groups B and C, whereas the clinical remission rate at the > 10-year follow-up period in group C (83.3%) was substantially higher than that in groups B (28.6%) and A (0%). The curve of the clinical remission rate in group A dropped quickly from 100% at 6 mo to 0% at the > 10 years assessment, whereas the curve of the clinical remission rate in group C declined slowly from 100% at 6 mo to 83.3% at the > 10 years assessment. The curve of the clinical remission rate in group B fell between the curves of groups A and C (Figure 2). Notably, the mean primary patency in group C was longer than that in groups A and B. These results demonstrate that the clinical remission rate in group C was higher than in groups A and B over the long-term follow-up periods.

Moreover, SEMS treatment in group C resulted in a reduced dysphagia score and LES pressure, and normal levels of barium height and width during all follow-up time points, whereas these parameters increased and gradually returned to the preoperative values in group A. Although, these parameters increased more slowly in group B than in group A, they increased significantly at the 8-10-year and > 10-year follow-up evaluations. These results indicate a superior long-term effectiveness for the clinical symptomatic remission of esophageal achalasia in group C compared with groups A and B.

The data in group C demonstrated a successful long-term clinical remission rate comparable with the results of other published studies which required repeated pneumatic dilations[25-34], and our results were even better than previous reports of achalasic patients treated with SEMS placement[11,12,18,19]. Furthermore, the long-term efficacy of SEMSs with a diameter of 30 mm is comparable with those of laparoscopic esophageal myotomy, which results in a success rate of about 90% after a mean follow-up period of 5-14 years[35-39]. The higher long-term clinical remission rate in group C may be attributed to the use of a large-diameter SEMS. The stent expanded to its full size within 24 h after placement, and we believe that the radial expansile force was generated spontaneously, slowly and evenly during stent expansion. Unlike pneumatic dilation, which can tear the cardiac muscular acutely and suddenly, we speculate that the SEMS opened the cardiac musculature slowly and gently. Thus, it is likely that the cardia muscularis was separated evenly, resulting in less restenosis and a satisfactory long-term therapeutic efficacy.

In this study, we compared the long-term clinical outcome of the stent with different diameters placed once for the treatment of achalasic patients. The question remains if smaller stents for a different time frame can give the same results as short duration wider stents? According to our experience, the same result may not be obtained from the wider stent due to insufficient radial expansile force and enough time to tear cardiac musculature. Moreover, it is unlikely to be adopted by the patients due to repeated implants and retrieval procedures as well as a high cost.

Stent migration has been the most frequent complication in stent placement for benign strictures, ranging from 18.7%-81.8%[7,11,40,41]. As expected, the migration rate was lower in group C (6.6%) than in groups A (26.7%) and B (13.3%). These results indicate that as the diameter of the SEMS increases, the potential of stent migration may be reduced. We speculate that the large-sized SEMSs provided a substantial radial expansile force and friction (due to the uncovered nitinol-wire) against the esophageal wall, and the temporary stent placement prevented the risk of late migration. Notably, previous reports have confirmed

that SEMSs with larger diameters (generally ⩾ 25 mm) employed in the esophagus may minimize the risk of migration[7,11,40-42].

The present study had several limitations. First, this was a single center study with no control studies. Although a prospective study was applied to compare the efficacy of three different sized SEMSs, future randomized trials in the use of our stent and pneumatic dilation are needed to compare the long-term clinical efficacy, the risk of complications and recurrent dysphagia that are involved in the treatment of achalasia. Second, larger SEMS may result in a high rate of chest pain, bleeding and perforation, while small SEMS may lead to a high rate of stent migration and food impaction. In addition, regurgitation may occur after SEMS placement.

In conclusion, we found that a temporary SEMS, 30 mm in diameter, was associated with a superior long-term clinical efficacy for the treatment of achalasia compared with SEMSs with diameters of 20 or 25 mm. Randomized trials comparing temporary stent placement with pneumatic dilation are needed.

COMMENTS

Background

Retrievable self-expanding metallic stents (SEMSs) have been successfully applied in the treatment of benign esophageal strictures, but they are rarely used for the treatment of patients with esophageal achalasia.

Research frontiers

The authors designed temporary SEMSs for the treatment of patients with esophageal achalasia; however, little is known about the long-term efficacy in patients treated with SEMS. This prospective study compared the long-term clinical outcome of the stent with different diameters in the treatment of achalasic patients.

Innovations and breakthroughs

The authors designed and manufactured the temporary SEMSs of three diameters in size specialized for the treatment of patients with esophageal achalasia. The SEMSs were inserted under fluoroscopic control and retrieved by gastroscopy 4-5 d after placement. A temporary SEMS, 30 mm in diameter, was associated with a superior long-term clinical efficacy for the treatment of achalasia compared with SEMSs with diameters of 20 or 25 mm.

Applications

A temporary SEMS, 30 mm in diameter, was associated with a superior long-term clinical efficacy for the treatment of achalasia compared with SEMSs with diameters of 20 or 25 mm.

Terminology

Achalasia is a disorder of esophageal motility characterized by aperistalsis, elevated lower esophageal sphincter (LES) pressure, and failure of LES relaxation upon swallowing.

Peer review

This manuscript describes the effectiveness of LES dilation in patients with achalasia on placing a self-expanding metal stent. It is a well-written manuscript with a clear objective, although some gray-points were detected and could be discussed in order to improve the present version.

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BRIEF ARTICLE

Resistance of *Helicobacter pylori* to antibiotics from 2000 to 2009 in Shanghai

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Abstract

AIM: To investigate the resistance of *Helicobacter pylori* (*H. pylori*) to 6 commonly used antibiotics from 2000 to 2009 in Shanghai.

METHODS: A total of 293 *H. pylori* strains were collected from 2000 to 2009 in Shanghai and tested for their susceptibility to metronidazole, clarithromycin, amoxicillin, furazolidone, levofloxacin and tetracycline using agar dilution.

RESULTS: The resistant rates of *H. pylori* to clarithromycin (8.6%, 9.0% and 20.7%) and levofloxacin (10.3%, 24.0% and 32.5%) increased from 2000 to 2009 in Shanghai. The resistant rate of *H. pylori* to metronidazole remained stable (40%-50%). Only one strain of *H. pylori* isolated in 2005 was resistant to tetracycline. All strains were sensitive to amoxicillin and furazolidone. The resistant rate of *H. pylori* to antibiotics was not related with the sex, age and clinical outcome of patients.

CONCLUSION: Resistance of *H. pylori* to antibiotics plays an important role in making treatment strategies against *H. pylori*-associated diseases.

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Key words: *Helicobacter pylori*; Antibiotic resistance; Agar dilution; Metronidazole; Clarithromycin; Levofloxacin; Tetracycline; Amoxicillin; Furazolidone

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INTRODUCTION

Chronic gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma can be relieved or even cured after eradication of *Helicobacter pylori* (*H. pylori*)[1,2]. Although triple therapy with proton pump inhibitor (PPI), amoxicillin and clarithromycin or metronidazole is still recommended, it should only be used when the local prevalence of resistance of *H. pylori* to antibiotics is below a certain level[3]. Recently, levofloxacin, tetracycline and furazolidone have been recommended in primary and/or rescue therapies in some areas. Resistance of *H. pylori* to antibiotics is the main reason for the failure of therapies

for *H. pylori*-associated diseases. Since the initial eradication of *H. pylori* can no longer be achieved due to its increasing resistance to antibiotics, it is necessary to investigate the local resistance of *H. pylori* to antibiotics for choosing the effective therapy for *H. pylori*-associated diseases.

MATERIALS AND METHODS

Patients and H. pylori strains

Clinical *H. pylori* strains were isolated from 293 patients who visited Renji Hospital (Shanghai, China) for dyspeptic symptoms in 2000, 2005 and 2009. Their dyspeptic symptoms included abdominal pain, abdominal distention, eructation, nausea, sour regurgitation and disgorging. Patients who used antibiotics, bismuth, H2-receptor antagonist (H2RA) or PPI for *H. pylori* in the past month, or administered non-steroidal anti-inflammatory drugs and alcohol, were excluded from the study. A total of 293 patients (male: 182, female: 111), enrolled in this study, were diagnosed as gastritis, peptic ulcer and gastric cancer, respectively, by endoscopy. All patients gave their written informed consent for participation in the study.

One biopsy was taken from the antrum of each patient for *H. pylori* culture. The biopsy specimens were cultured with a brain heart infusion (BHI) agar medium (OXOID, Basingstoke, UK) containing 5% defibrinated sheep blood under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) at 37℃. All stocks were kept in BHI broth (Difco Laboratory, Detroit, MI, USA) supplemented with 30% glycerol at -80℃. Clinical *H. pylori* strains were identified when the tests and Gram staining were positive for urease, oxidase, and catalase.

Agar dilution and minimal inhibitory concentrations

Minimal inhibitory concentration (MIC) of metronidazole (Met), clarithromycin (Cla), amoxicillin (Amo), levofloxacin (Lev), tetracycline (Tet) and furazolidone (Fur), was measured with the two-fold agar dilution method. *H. pylori* strains were suspended in saline and detected with a spectrophotometer. The bacterial suspensions (10^8 colonies per milliliter) were then plated onto agar plates containing various concentrations of the above antibiotics with an inoculator (Sakuma Seisaku, Tokyo, Japan). Three days after microaerophilic incubation, MIC was defined as the lowest drug concentration that prevented visible growth of the bacteria. ATCC43504 was used as a quality control. Met > 8 μg/mL, Cla > 2 μg/mL, Lev > 2 μg/mL, Amo > 8 μg/mL, Tet > 2 μg/mL and Fur > 2 μg/mL were determined as resistance breakpoints as previously described[4,5]. Antibiotics and their solvents used in this study are shown in Table 1.

Statistical analysis

Data were analyzed by chi square test using SPSS13.0. $P < 0.05$ was considered statistically significant.

RESULTS

The demographic data about the patients are shown in Table 2. No significant difference was observed in background of the patients.

Table 1 Antibiotics used in this study

Antibiotic	Manufacturer	Solvent
Metronidazole, Met	Wuhan Pharmaceutical Ltd.	Distilled water
Clarithromycin, Cla	Livzon Syntpharm Co., Ltd.	Acetone
Levofloxacin, Lev	Livzon Syntpharm Co., Ltd.	Distilled water
Tetracycline, Tet	SIGMA-ALDRICH Co., Ltd.	0.1 mol/L phosphate buffer saline (pH 6.0)
Amoxicillin, Amo	Kunming Baker Norton Pharmaceutical Ltd.	0.1 mol/L phosphate buffer saline (pH 6.0)
Furazolidone, Fur	Shenzhen Jiangmen Pharmaceutical Ltd.	N,N-dimethyl-formamid (DMF)

Table 2 Demographics of strains

	2000	2005	2009
No. of strain	58	100	135
Male/Female	46/12	61/39	75/60
Age (average, range)	44.3 (20-78)	45.2 (18-81)	42.5 (20-74)
Gastritis/Ulcer/Gastric cancer	36/20/2	46/44/10	81/53/1

Resistance rate of H. pylori to antibiotics

No significant difference was observed in the resistance rate of *H. pylori* to metronidazole during the past 10 years (28/58 in 2000, 49/100 in 2005 and 57/135 in 2009). The resistance rate of *H. pylori* strains isolated in 2009 to clarithromycin was 20.7% (28/135), which was significantly higher than that of *H. pylori* strains isolated in 2000 (8.6%, 5/58) and 2005 (9.0%, 9/100) ($P < 0.05$). The resistance rate of *H. pylori* strains to levofloxacin was higher in 2009 (32.6%, 44/135) than in 2005 (24.0%, 24/100) ($P < 0.05$), and the resistant rate of *H. pylori* strains to levofloxacin was significantly higher in 2005 (24.0%, 24/100) than in 2000 (10.3%, 6/58) ($P < 0.05$). No *H. pylori* strain was resistant to amoxicillin and furazolidone and only one *H. pylori* strain isolated in 2005 was resistant to tetracycline. No significant difference was observed in gender and age of the patients and in antibiotic resistance-associated diseases (Figure 1).

Multidrug resistance

Multidrug resistance means that one strain is resistant to two or more antibiotics. In this study, the multidrug resistance of *H. pylori* was 27.9% (36/129) in 2009, which was significantly higher than that (10.3%, 6/58) of *H. pylori* in 2000 ($P < 0.05$). The multidrug resistance of *H. pylori* to metronidazole and levofloxacin was the highest (43.1%, 25/58), followed by the combination of metronidazole/levofloxacin and metronidazole/clarithromycin with a resistance rate of 24.1% (14/58).

DISCUSSION

The resistance of *H. pylori* to metronidazole has been

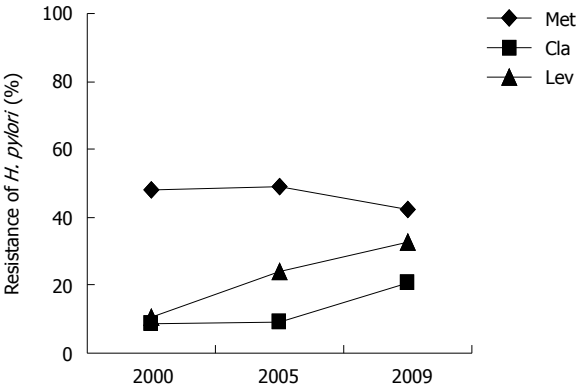

Figure 1 Resistance of *Helicobacter pylori* to Met, Cla and Lev in different years. *H. pylori: Helicobacter pylori.*

increasing in Shanghai since 1990s. The resistant rate of *H. pylori* has increased from 42% in 1995 to 70% in 1999[6,7]. In this study, agar dilution showed that the resistance of *H. pylori* to metronidazole was slightly decreased in recent years[8], but was still higher than 40%. According to the Maastricht Ⅲ Consensus Conference report, in an area with a resistance rate of *H. pylori* to metronidazole of over 40%, the efficacy of triple therapy with metronidazole may decrease. However, the eradication rate of *H. pylori* for metronidazole-containing therapy can be improved by increasing its dosage, prolonging its duration or adding bismuth salts[9-11].

It has been shown that triple therapy with clarithromycin is the first choice of treatment for eradication of *H. pylori* with an ITT rate of over 90%[12]. However, the resistance of *H. pylori* to clarithromycin has been increasing all over our country[13]. For example, the resistance of *H. pylori* to clarithromycin was 10% in 1999 and 36% in 2005 in Beijing[14], and 0% in 1995 and 10% in 1999 in Shanghai[6]. In this study, the resistance of *H. pylori* to clarithromycin was 8.6% in 2000, 9.0% in 2005 and 20.7% in 2009, respectively. The guidelines recommend that if the resistance of *H. pylori* to clarithromycin is over 15%-20%, therapies with clarithromycin should not be used as an empirical treatment. However, therapies with combined clarithromycin and bismuth can improve the bactericidal activity of clarithromycin[10].

Levofloxacin, a new broad-spectrum antibiotic with a strong antimicrobial activity, has been used in eradicating *H. pylori* in recent years. The guidelines suggest that levofloxacin-containing therapies as the first-line therapy and rescue therapy have good efficacy. However, it was reported that the resistance of *H. pylori* to levofloxacin is high in many areas (29.1% in Beijing and 21.7% in Xi'an)[15]. In the present study, the resistance rate of *H. pylori* to levofloxacin in Shanghai was 10.3% in 2000, 24.0% in 2005 and 32.6% in 2009, respectively, indicating that eradication of *H. pylori* may fail when its resistance to levofloxacin is over 20% and that levofloxacin should not be used in treatment of *H. pylori* when susceptibility test is not performed[16,17].

Antimicrobial susceptibility testing is an effective method that tests if *H. pylori* strains are resistant to some antibiotics. Romano *et al*[18] showed that antimicrobial susceptibility testing before treatment improves the rate of response to therapy and is cost-saving. However, this testing cannot be done in large areas because the culture of *H. pylori* is very costly.

In this study, the resistance of *H. pylori* to metronidazole in Shanghai was over 40%, which showed a decreasing trend. The resistance rate of *H. pylori* to clarithromycin was 20.7% in 2009 which was on the warning level. The resistance of *H. pylori* to levofloxacin has increased rapidly in the past 10 years with a resistant rate of 30.7% in 2009. Almost all *H. pylori* strains were not resistant to amoxicillin, furazolidone and tetracycline, indicating that these antibiotics may become good candidates against *H. pylori*. Treatment strategies should be made and changed according to the resistance of *H. pylori* to antibiotics. Further study is needed in more centers.

COMMENTS

Background

Many alimentary diseases are associated with *Helicobacter pylori* (*H. pylori*) infection. The resistance of *H. pylori* to antibiotics is increasing as the spreading of eradication treatment and is the main reason for the failure of *H. pylori* eradication. The authors need to know the local area resistance rate of *H. pylori* to antibiotics in different places and treatment strategies should be made according to antimicrobial susceptibility testing.

Research frontiers

The resistance of *H. pylori* to 6 antibiotics from 2000 to 2009 in Shanghai was studied, which may help us chose antibiotics for eradication of *H. pylori*.

Innovations and breakthroughs

This study showed the resistance rates of *H. pylori* to 6 antibiotics from 2000 to 2009 in Shanghai and highlighted the importance of the resistance of *H. pylori* to antibiotics in making treatment strategies against *H. pylori*.

Applications

The resistance rate of *H. pylori* to metronidazole, clarithromycin, amoxicillin, furazolidone, levofloxacin and tetracycline from 2000 to 2009, shown in the present study, may help the clinicians carry out experiential therapies.

Peer review

In this manuscript, the authors reported the resistance pattern of *H. Pylori* to six antibiotics from 2000 to 2009 in Shanghai and highlighted the importance of the resistance of *H. pylori* to antibiotics in making treatment strategies against *H. pylori*, which may help the clinicians carry out experiential therapies for *H. pylori*-associated diseases.

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BRIEF ARTICLE

Inhibition of KIT RNAi mediated with adenovirus in gastrointestinal stromal tumor xenograft

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Abstract

AIM: To investigate a therapeutic method for gastrointestinal stromal tumor (GIST) based on KIT RNA interference (RNAi) with AdMax adenovirus.

METHODS: KIT short hairpin RNA (shRNA), whose lateral sides were decorated with restriction endonuclease sequences, was designed. T_4 DNA ligase catalyzed the joint of the KIT shRNA and the green fluorescent protein-containing PDC316-EGFP-U6 to form PDC316-EGFP-U6-KIT. Homologous recombination of AdEGFP-U6-KIT was performed with the AdMax system. Heterotopically transplanted GISTs were established in nude mice. AdEGFP-U6-KIT was intratumorally injected. The volume, inhibition ratio of tumor and CD117 expression of GIST graft tumor in nude mice were compared between test and control groups.

RESULTS: The length of KIT shRNA was determined to be about 50bp by agarose electrophoresis. Gene sequencing detected the designed KIT RNAi sequence in PDC316-EGFP-U6-KIT. After transfection with AdEGFP-U6-KIT, 293 cells displayed green fluorescence. The physical and infective titers of AdEGFP-U6-KIT were 5×10^{11} viral particles/mL and 5.67×10^7 plaque forming units/mL, respectively. The mean volume of the grafted tumor was significantly smaller in test mice than in control mice (75.3 ± 22.9 mm^3 *vs* 988.6 ± 30.5 mm^3, $t = -18.132$, $P < 0.05$). The inhibition ratio of the tumors was 59.6% in the test group. CD117 positive expression was evident in two cases (20%) in the test group and 10 cases (100%) in the control group ($\chi^2 = 10.2083$, $P < 0.005$).

CONCLUSION: AdEGFP-U6-KIT is successfully constructed, and KIT RNAi mediated with Admax vector system can effectively inhibit the expression of the KIT gene and the growth of GIST in nude mice.

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Key words: Gastrointestinal stromal tumor; RNA interference; KIT; Adenoviral vector; Nude mice

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is a mesenchymal

neoplasm and is the most prevalent gastrointestinal mesenchymal tumor. Its annual incidence is about 10-20 per million people[1-3]. However, it is difficult to give an exact incidence, since both the definition and classification of GIST are contentious[1]. GIST occurs in the stomach (50%-60%), small intestine (30%-40%), colon and rectum (5%-10%), and esophagus (5%)[1,2]. The gold standard therapy for GIST is complete resection, depending on the lesion size and location. It is unnecessary to dissect the lymph node since lymph node metastasis is rare. During surgery, tumor rupture must be avoided as it is the main factor resulting in post-operative recurrence. The 5-year survival rate for en bloc resection of GIST is 48%-65%[3]. In some cases, because of the anatomic site or the tumor size, only a partial resection can be performed. The two most important prognostic features of primary GIST are tumor size and mitotic index. GIST is an easily recurrent disease, which is found in the liver (65%), peritoneal surface (50%), or both (20%)[1,4]. Conventional chemotherapy is of little benefit for GIST because it is a non-epithelial neoplasm[1]. The use of Imatinib mesylate in advanced GIST produces a response in 50% of treated patients, and stabilizes the disease in 75%-85% of patients. The 2-year survival after Imatinib therapy is approximately 70%[5]. Imatinib therapy after 1 year is associated with a high risk of relapse[6]. Primary resistance to Imatinib affects about 15% of patients and 50% of patients become secondarily resistant by 2 years after Imatinib therapy[3,7]. Resistance to Imatinib is a major clinical problem, which has prompted the search for alternate drugs for Imatinib resistant cases.

The KIT proto-oncogene on chromosome 4 (4q11-q12) encodes the KIT protein, and appears to play an important role in the early stages of tumor formation as well as in late tumor progression. The sites of mutation are exon 11 (65%-70%), 9 (10%-20%), 13 (1%-2%) and 17 (< 1%)[8]. Inhibition of the KIT gene may block the formation and development of GIST. RNA interference (RNAi) is the most effective method to silence a target gene. RNAi can block KIT gene expression in GIST, while it is still uncertain if KIT RNAi can become an effective therapeutic method for GIST[9,10]. It was also reported that an adenovirus vector can effectively introduce RNAi to cancer gene therapy[11].

In this study, an adenovirus vector was successfully constructed to mediate KIT RNAi in GIST xenografts. The results demonstrated that the adenovirus system can silence efficiently KIT and inhibit the growth of grafted GIST. This approach may have therapeutic potential in GIST.

MATERIALS AND METHODS

Plasmids, medium and reagents

Plasmids pDC316-EGFP-U6 and pBHGlox_E1,3Cre, DH5α strain and HEK293 cells were purchased from Vector Gene Technology. Plasmid DNA extraction kit, RPMI1640, agarose, fetal bovine serum, and DMEM were purchased from Shanghai Sangon Biological Engineering Technology & Services. CD117 (mouse monoclonal IgG), T4 DNA ligase, *Hin*dⅢ, *Bam*HⅠ, and *Bg*Ⅲ were purchased from New England Biolabs.

Synthesis of KIT (B/H)

The sequence of KIT RNAi was previously reported as 5'-GGCCGACAAAAGGAGATCTTTCGAGA-TCTCCTTTTGTCGGCCTTTTT-3'[12]. These sense and antisense oligonucleotides were ligated to *Hin*dⅢ and *Bam*HⅠ and annealed to form double strains of DNA that designated KIT (B/H). Agarose gel electrophoresis was employed to identify the length of KIT (B/H).

*Hin*dⅢ and BamH1 digestion of PDC316-EGFP-U6 to form PDC316-EGFP-U6 (B/H)

Distilled deionized water (22 μL), 10 × K Buffer (5 μL), PDC316-EGFP-U6 (20 μL), *Bam*HⅠ (1.5 μL), and *Hin*dⅢ (1.5 μL) were added into 0.2 mL Eppendorf (EP) tube. The EP tube was incubated at 37℃ overnight. One hundred microliters of solution BD was added to the tube, and then the solution was transferred to a DNA purification pillar. After 2 min, the pillar was centrifuged at 12000 r/min at room temperature for 1 min. After removal of the filter liquor and the addition of 500 μL of solution PE, the pillar was centrifuged at 12000 r/min at room temperature for 1 min. The step was repeated and the pillar was centrifuged at 12000 r/min for 1 min. The pillar was put into a 1.5 mL EP tube, and 30 μL of 60℃ sterile water was added into pillar. After centrifugation at 13400 × *g* for 1 min, PDC316-EGFP-U6 (B/H) solution was acquired and identified with agarose electrophoresis.

Insertion of KIT (B/H) to PDC316-EGFP-U6 (B/H) to form PDC316-EGFP-U6-KIT

Distilled deionized water (2 μL), 10 × T4 DNA Ligation Buffer (1 μL), PDC316-EGFP-U6 (B/H) (3 μL), KIT (B/H) (3 μL), and T4 DNA Ligase (1 μL) were added to a 0.2 mL EP tube. The tube was incubated at 16℃ for 2 h. The product was named PDC316-EGFP-U6-KIT.

PDC316-EGFP-U6-KIT transformation

Six microliters of PDC316-EGFP-U6-KIT was put into a 30 μL suspension of DH5α competent cells and rotated slightly for 30 min in an ice bath, prior to transfer to a 42℃ water bath for 90 s and then to an ice bath for 2 min. Two hundred microliters of LB medium was added to the tube. The mixture was cultured with shaking (200 r/min) at 37℃ for 1 h. The bacterial liquid was spread on a LB agar plate containing ampicillin (100 μg/mL). After absorption of liquid at room temperature, the agar was incubated overnight at 37℃.

PDC316-EGFP-U6-KIT identification

Four colonies on the plate were collected and put separately into a tube containing 3 mL of LB, and cultivated in a rocking bed overnight at 37℃. The plasmid extraction kit was used to obtain plasmid DNA. Three microliters of bacterial liquid was put into a 1.5 mL EP tube, and

centrifuged at 12000 r/min for 1 min. After removal of the supernatant, 250 μL of solution I /Rnase A was added to suspend the bacteria. Then, 250 μL of solution II was inverted gently six times and placed at room temperature for 2 min. Two hundred and fifty microliters of solution III was then added to the tube and the contents were mixed by gentle inversion six times. After centrifugation at 12000 r/min for 10 min, the supernatant was removed, added to a DNA purifying pillar, held for 2 min, centrifuged at 12000 r/min for 1 min, and the filter liquor was removed. Five hundred microliters of PB solution was added to the pillar, the suspension was centrifuged at 12000 r/min for 1 min, and the filter liquor was removed. Then, 500 μL of solution W was put into the pillar, centrifuged at 12000 r/min for 1 min, and the filter liquor was removed. The step was repeated and the contents of the pillar were centrifuged at 12000 r/min for 3 min. The pillar was put into a 1.5 mL EP tube and 50 μL of 60℃ sterile water was added. After 2 min, the contents were centrifuged at 13400 × *g* for 1 min. The resulting solution contained PDC316-EGFP-U6-KIT. Distilled deionized water (4.6 μL), 10 × K Buffer (1 μL), PDC316-EGFP-U6-KIT (4 μL), and *Sal*I (0.4 μL) were added to a 0.2 mL EP tube and incubated at 37℃ for 2 h. Agarose gel electrophoresis was used to identify the recombinant plasmid.

PDC316-EGFP-U6-KIT sequencing test

PDC316-EGFP-U6-KIT sequencing was done by Life Technologies Corporation using ABI377DNA.

Preparation of recombinant adenovirus AdEGFP-U6-KIT

Transfection was performed according to the manufacturer's instructions. Approximately 5 × 10^5 HEK293 cells were seeded in 60-cm plates 24 h before transfection, with a 80% confluency. Four micrograms of shuttle plasmid PDC316-EGFP-U6-KIT and 6 μg rescue plasmid pBHGlox(delta) E1, 3Cre were mixed well, then DMEM was added to a total volume of 300 mL and left at room temperature for 5 min. Three hundred microliters of DMEM and 10 μL Lipofectamine 2000 was added slowly to the tube with constant mixing, and the mixture was left at room temperature for 5 min. The mixed plasmids and diluted Lipofectamine 2000 were blended and kept at room temperature for 30 min. Afterwards, the mixture was added to a plate containing cultured HEK293 cells. The second day after transfection, the HEK293 cells were transferred to 75 cm^2 cell culture bottles and cultured in DMEM containing 10% fetal calf serum. The bottles were monitored daily for the appearance of cytopathic effect (CPE), which was evident by a rounded and refractile appearance of the cells, and would begin to lift off the surface of the bottle. The CPE cells were observed under fluorescence microscope for green fluorescence. When > 90% of the cells showed CPE, the cells were harvested and subjected to three freeze (methanol/dry ice bath)/thaw(37℃ water bath) cycles. After the cell debris was sedimented, the supernatant containing the adenovirus particles comprised the AdEGFP-U6-KIT stock. The stock was stored in small aliquots at -70℃ after 10% glycerol was added.

Preparation of purified high-titer AdEGFP-U6-KIT stocks

When HEK293 cells had attained a 90% confluency in a 75 cm^2 cell culture bottle, 2 mL of the stock unfrozen AdEGFP-U6-KIT supernatant was added. About 44 h after infection, the HEK293 cells presented total CPE and were harvested for three freeze/thaw cycles as described above. Supernatant was collected and added to four 75 cm^2 cell culture bottles containing HEK293 cells and treated to recover supernatant as described above. Ten milliliters of AdEGFP-U6-KIT supernatant was added to 10 cell culture bottles, which were inoculated with 1.8 × 10^8 HEK293 cells that has attained a 90% confluency. About 70 h after infection, the cell suspension was centrifuged at 3000 r/min for 10 min. The precipitate of cells was suspended in Tris buffer and treated with three freeze/thaw cycles as described previously. After centrifugation at 6000 r/min for 10 min, the supernatant was collected, digested with 20 units Dnase, filtered through a 0.45 μm filter membrane, and purified with ion exchange chromatography. Further purification was achieved using molecular sieving. The purified AdEGFP-U6-KIT was stored in virus preservation fluid. After desalination and sterilization using a 0.22 μm sterile filter, the purified and sterile virus fluid was stored in small aliquots at -70℃ after 10% glycerol was added.

Titer determination of recombinant adenovirus AdEGFP-U6-KIT

According to the manufacturer's instructions, 10 × virolysis solution was used to purify virus samples. The physical titer was calculated based on the equation of OD260 × 1.1 × 10^{12} (vp/mL), and 50% tissue culture infective dose [TCID50, plaque forming units (PFU)/mL] was also determined.

Influence of AdEGFP-U6-KIT on growth of GIST in nude mice

The study was approved by the Ethics Board of the First Affiliated Hospital of Sun Yat-sen University. Prior written informed consent was obtained from patients with gastric stromal tumors for use of samples. Balb/c-nu/nu mice were purchased from the Chinese Academy of Medical Sciences. All mice were maintained according to the "NIH Guide for the Care and Use of Laboratory Animals". Patient-derived GIST xenografts were established in Balb/c-nu/nu mice as described[13]. Briefly, primary GISTs were obtained and immediately placed in chilled RPMI 1640. The tumors were kept in an ice bath and quickly transferred to the laboratory. Thin slices of tumor were diced into 2-3 mm pieces and washed three times with RPMI 1640. These tumor pieces were minced into fine fragments that would pass through an 18-gauge needle and were then mixed 1:1 (v/v) with Matrigel to give a total volume of 0.1 mL/injection. The tissue mixture was subcutaneously injected into the flank of 9-10-wk-old Balb/c-nu/nu mice. In 6 primary gastric GISTs obtained

at operation, only 1 was successfully grafted. Its size and frequency of mitosis was 20 cm × 12 cm × 14 cm and 14/50HPF, respectively. The tumor tissue showed CD117 positive staining. According to Miettinen *et al*'s report, it belonged to high risk GIST for recurrence and metastasis. For serial transplantation, tumor-bearing animals were anesthetized with diethyl ether and sacrificed by cervical dislocation. Tumors were minced under sterile conditions and injected into Balb/c-nu/nu mice as described above. Growth of established tumor xenografts was monitored at least twice a week by vernier caliper measurement of the length (a) and width (b) of tumor. Tumor volumes were calculated as $(a \times b^2) /2$[14]. Twenty-four days after graft, 20 mice harboring grafted tumors were randomly selected and divided into two groups. Fifty microliters of AdEGFP-U6-KIT (2.5×10^9 viral particles) was intratumorally injected into the test group of mice ($n = 10$), while blank AdEGFP-U6 was used in the control group of mice ($n = 10$). Forty-five days after injection, tumors were harvested, frozen in liquid nitrogen, fixed in buffer containing 10% formalin, and embedded in paraffin for histological study. The tumor inhibition rate referred to reduced degree of tumor [(tumor volume before injection - tumor volume at finish of test)/tumor volume before injection]. Positive value presented contraction of tumor, while a negative value indicated tumor growth.

Immunohistochemistry of CD117 in grafted tumors

Immunohistochemistry was performed as described[15]. Briefly, each grafted tumor specimen was fixed in 10% formaldehyde and embedded in paraffin. Sections 4 μm thick were cut and mounted on glass slides. Immunohistochemical staining was performed using a standard avidin-biotin method. The formalin-fixed, paraffin-embedded 4 μm thick tissue sections were deparaffinized with xylene, dehydrated in ethanol, and incubated with 3% hydrogen peroxidase for 5 min. After being washed with phosphate buffered saline (PBS), tissue sections were incubated in 10% normal bovine serum for 20 min, followed by an overnight incubation with a 1:100 dilution of CD117 antibody. Biotinylated goat antimouse immunoglobulin was used as the secondary antibody. Peroxidase-conjugated avidin was at a 1:500 dilution. Finally, 0.2 g/L DAB and 10 mL/L hydrogen peroxide in PBS were used as the substrate. Specimens positive for CD117 served as the positive control, and those with the first antibody substituted by PBS as negative control. Brown granules in the cytoplasm of a tumor cell were considered indicative of a positive cell, and brown staining of more than 20% of the tumor cells was regarded as positive.

Statistical analysis

The χ^2 or Fisher exact test was used to compare categorical variables, and Student's *t* test was used to analyze continuous variables. Statistical analyses were performed using SPSS software version 11.5. Results were considered statistically significant at $P < 0.05$.

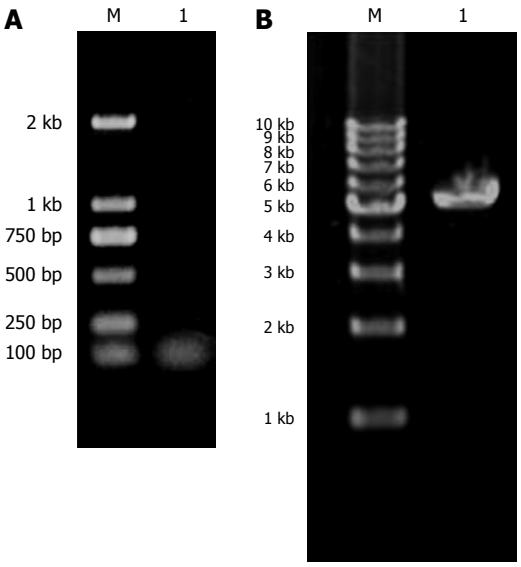

Figure 1 Agarose gel electrophoresis. A: KIT (B/H) was about 50 bp in length in lane 1. Lane M: DNA Marker DL2000; B: PDC316-EGFP-U6 was about 5300 bp in lane 1, consistent with the vector length. Lane M: 1 kb DNA Ladder Marker.

RESULTS

Synthesis of KIT (B/H)

The length of KIT (B/H) band shown in agarose gel electrophoresis was 50 bp or so, which gave evidence that KIT (B/H) was synthesized correctly (Figure 1A).

*Hind*Ⅲ and BamH1 digestion of PDC316-EGFP-U6 to form PDC316-EGFP-U6 (B/H)

In agarose gel electrophoresis, PDC316-EGFP-U6 (B/H) was about 5300 bp, consistent with the vector length (Figure 1B).

PDC316-EGFP-U6-KIT identification

There was incision enzyme site *Sal* I between *Hind*Ⅲ and *Bam*H I in blank PDC316-EGFP-U6 plasmid. After recombination, restrictive endonuclease site *Sal*I was eliminated. The blank plasmid could be linearized by *Sal* I digestion, while the recombinant one could not. Figure 2 shows that the plasmids on lanes 3 and 4 were the recombinant PDC316-EGFP-U6-KIT.

PDC316-EGFP-U6-KIT sequencing test

The sequencing graph showed that the KIT RNAi sequence in PDC316-EGFP-U6-KIT plasmid was correct (Figure 3).

Recombinant adenovirus AdEGFP-U6-KIT

The CPE and green fluorescence in HEK293 cells were observed 8 d after recombination of PDC316-EGFP-U6-KIT and pBHGlox(delta)E1,3Cre (Figure 4A and B). The physical titer and TCID50 was 5×10^{11} (viral particles/mL) and 1.26×10^{10}/mL, respectively.

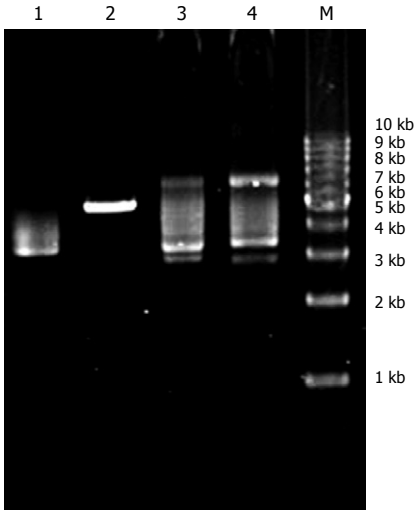

Figure 2 After recombination, restrictive endonuclease site *SalI* was eliminated. The blank plasmid could be linearized by SalI digestion, while the recombinant one could not. The plasmids on lanes 3 and 4 were the recombinant PDC316-EGFP-U6-KIT. Lane 1: Blank PDC316-EGFP-U6; Lane 2: Blank PDC316-EGFP-U6 digested with *SalI*; Lane 3: Recombinant PDC316-EGFP-U6-KIT; Lane 4: Recombinant PDC316-EGFP-U6-KIT digested with *SalI*; Lane M: DNA Marker DL2000.

Influence of AdEGFP-U6-KIT on growth and CD117 expression of GIST in nude mice

The GIST xenograft was incubated for 9-15 d. There was no histological difference between primary gastric GIST and xenografts, and both presented CD117 positive expressions (Figure 5A and B). An expansive, mobile and hard mass was observed in all animals. The mean tumor volume was similar between the test and control groups before injection of AdEGFP-U6-KIT (186.3 ± 33.6 mm^3 *vs* 176.8 ± 30.9 mm^3, $t = 0.3642$, $P > 0.10$). Twenty-one days after the intervention with recombinant virus, the mean volume of graft tumor was smaller in the test animals than in the control animals (75.3 ± 22.9 mm^3 *vs* 988.6 ± 30.5 mm^3, $t = -18.132$, $P < 0.05$). The tumor inhibition rate in the test and control mice was +59.6% and -459.2%, respectively. There were two cases in the test group and 10 cases in the control group who presented positive CD117 expression (20% *vs* 100%, $\chi^2 = 10.2083$, $P < 0.005$).

DISCUSSION

GIST is the most common mesenchymal tumor of the digestive tract. Metastasis of tumor cells to lymph nodes in GIST is rare, however, recurrence and liver metastasis of GIST often occured[15]. Heinrich *et al*[10] reported that GIST is generally distinguished from other abdominal sarcomas by the expression of KIT receptor tyrosine kinase. This kinase is important not only as a diagnostic marker for GIST, but as a primary oncogene in approximately 80% of these tumors, as evidenced by activating mutations of the *KIT* gene. About 90% of GIST had gain-of-function mutations of the *KIT*, and half of GIST without *KIT* mutation presented gain-of-function mutations in the *PDGFRA* (platelet-derived growth factor receptor-α) that encodes another receptor tyrosine kinase[16]. Other studies also showed that approximately 85% of GISTs gain activating mutations in *KIT* or the homologous *RTK* gene, with the PDGFRA. *KIT* activation was associated with proliferation, apoptosis, adhesion and chemotaxis[17,18]. With GIST's resistance to conventional chemotherapy, tyrosine kinase inhibitors are an emerging class of anticancer therapies that have shown a pivotal role in clinical practice. Imatinib, which inhibits the enzymatic activity of KIT, can present a satisfactory response, while about 15% of patients show initial resistance, and many patients who respond positively at first show secondary resistance later[5-7,19]. In clinical studies, 75%-90% of patients with advanced GISTs treated with Imatinib experienced a clinical benefit[10]. The Imatinib induced responses correlated with tumor kinase mutational status. Patients with *KIT* exon 11-mutant GIST have a higher response rate and a significantly longer median survival compared with patients with exon 9-mutant GISTs, and those whose GISTs lack *KIT* or *PDGFRA* mutations[20]. The duration and dose of Imatinib in the neoadjuvant setting are yet undecided, however, less than 5% patients have complete clinical response to Imatinib[1]. Due to the unfavorable status of GIST therapy, a new method is necessary, especially one based on management to inactivate KIT expression.

RNAi is a process in which double-stranded RNA is used to generate degradation of cognate mRNA[11]. Synthetic 21-23 nucleotide (siRNA) has been demonstrated to induce transient and efficient RNAi[21]. Plasmid vector designed to produce siRNA presents transient siRNA expression and low transfection efficiency. For the high titer and level of recombinant adenoviruses in transgene expression, they currently are widely used in gene interventions, including RNAi[11,22]. In the present study, plasmids pDC316-EGFP-U6 and pBHGlox-E1,3Cre were employed to generate recombinant adenovirus. Shuttle plasmid pDC316-EGFP-U6 was reconstructed based on pDC316, in which the U6-promotor was designed to drive the goal gene and enhanced green fluorescent protein was employed to observe transfection. The multiple cloning site (MCS) for shRNA in pDC316-EGFP-U6 was U6 promoter-*Bam*H Ⅰ-*Sal* Ⅰ-*Hin*dⅢ. KIT (B/H) was correctly inserted into the MCS between *Bam*H Ⅰ and *Hin*dⅢ, and DNA sequencing and agarose gel electrophoresis could provide a strong evidence. The physical titer and TCID50 of recombinant virus AdEGFP-U6-KIT was 5×10^{11} viral particles/mL and 1.26×10^{10}/mL, respectively. Wang *et al*[23] reported that a recombinant adenovirus vector designed for expression of a fusion gene was constructed successfully using the AdMax Adenovirus Vector Creation System, and its titer was 8×10^{10} PFU/mL. Other studies also demonstrated that AdMax system has a high recombinant efficiency and is comparatively simple[24-26]. It is reasonable to draw a conclusion that the Admax system could effectively recombine adenovirus in molecular organism research.

Based on the pivotal role of KIT in some diseases,

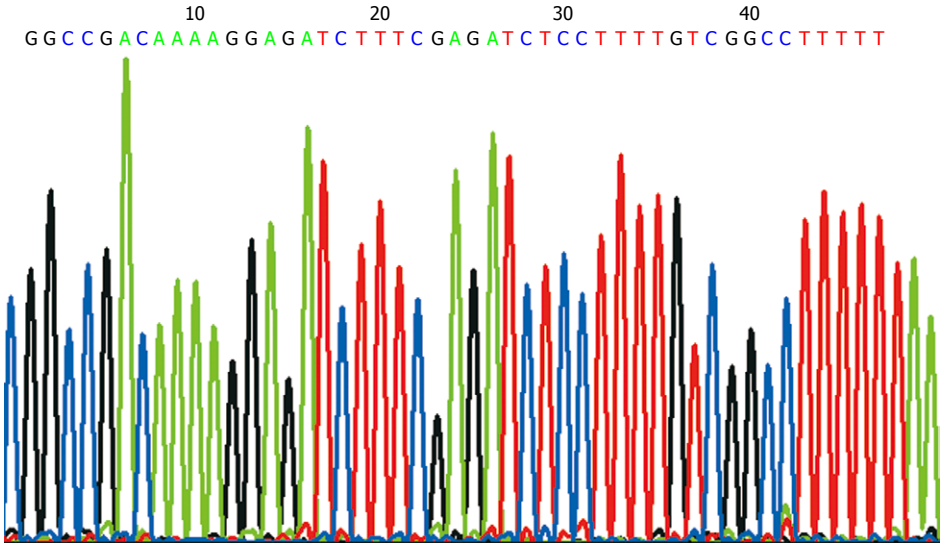

Figure 3 Sequencing graph shows that the KIT RNAi sequence in PDC316-EGFP-U6-KIT plasmid was correct.

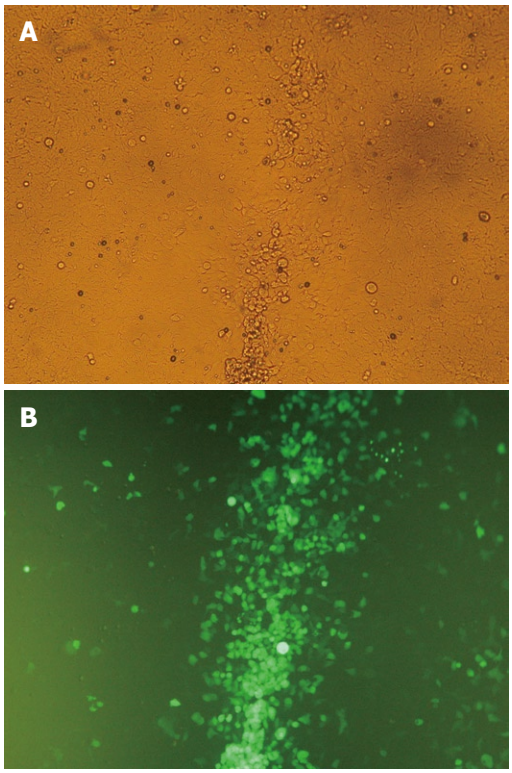

Figure 4 Cytopathic effect and green fluorescence in HEK293 cells observed after recombination of PDC316-EGFP-U6-KIT and pBHGlox(delta) E1,3Cre. A: Cytopathic effect of HEK293; B: Fluorescence in HEK293 cell, × 200.

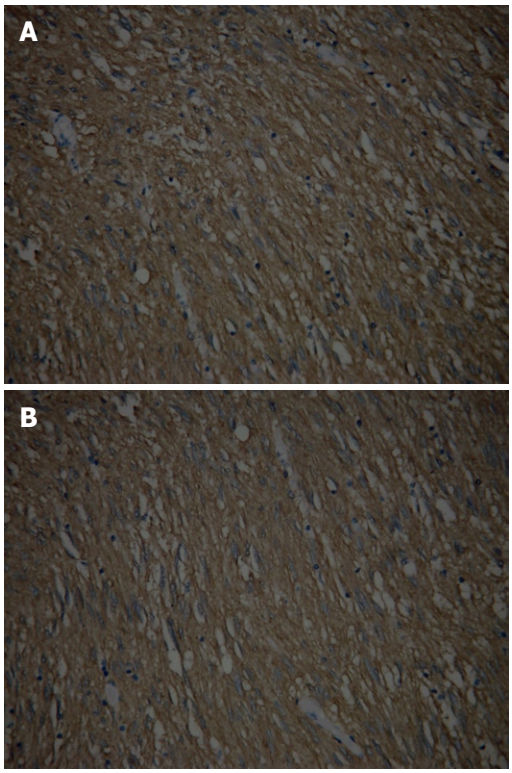

Figure 5 Positive staining of CD117 in gastrointestinal stromal tumor. A: Primary gastric gastrointestinal stromal tumor; B: Xenograft, × 200.

including mast cell leukemia and GIST[1,27], KIT RNAi might be an alternative to control disease development. Ruano *et al*[27] discovered that retroviral transduction of HMC1.1 and HMC1.2 cell lines with vectors carrying DNA to be transcribed for RNAi against the wild type or mutant KIT messengers lowered KIT protein levels considerably, decreased cell proliferation, and raised the apoptotic levels. Furthermore, the same study suggested that the highly specific effect of RNAi in reducing KIT mRNA could be used for the treatment of other cancers resistant to Imatinib mesylate, such as GIST[27]. A study reported that KIT protein was detected in spermatogonial cells and knocked down to undetectable levels at 24 h after transfection with KIT siRNA[28]. Catalano *et al*[12] showed that exposure of a malignant mesothelioma cell line to KIT siRNA was associated with down-regulation of KIT expression and an increase in apoptosis. In the present study, KIT RNAi whose addition was mediated

with Admax adenovirus suppressed dramatically KIT protein (CD117) expression, providing evidence that the KIT mRNA was knocked down by RNAi. Furthermore, the GIST xenograft was reduced markedly in the test group of animals and the tumor inhibition rate was 59.6%, while in the control mice, the xenograft grew rapidly and the mean volume increased by 5.6 times at the end of the experiment compared with that observed before intervention with KIT RNAi. The sequence of KIT RNAi in our study was not located in the mutant regions, therefore, the recombinant adenovirus could be used in most GISTs[1,8,10]. Zhu *et al*[9] evaluated interactions with the KIT oncoproteins and determined signaling pathways that are dependent on KIT oncogenic activation in GIST. Tyrosine-phosphorylated KIT oncoproteins interacted with PDGFRA, PDGFRB, phosphatidylinositol 3-kinase and PKCtheta in GIST cells, and these interactions were abolished by KIT inhibition with Imatinib or KIT RNAi. Another study used a KIT lentiviral shRNA to infect GIST882, shRNA knockdown of total KIT expression in Imatinib sensitive GIST882 cell line resulted in parallel decreases in phosphorylated-KIT. KIT knockdown in the cell lines also provided flow cytometric evidence for G1 block, decreased S phase, and markedly increased apoptosis[10]. Yang *et al*[29] reported that, excepting mutations of *KIT* or *PDGFRA* gene, there were cytogenetic aberrations and molecular genetic aberrations. A new paradigm of classification integrating the standard clinical and pathological criteria with molecular aberrations may permit personalized prognosis and treatment.

In summary, GIST is prevalent and serious, and efficacious therapy is still required. The recombinant adenovirus AdEGFP-U6-KIT was correctly constructed and potently inhibited KIT expression and growth of GIST xenografts. AdMax adenovirus vector can effectively introduced RNAi into cancer gene therapy. KIT RNAi mediated with adenovirus might become a method for GIST treatment.

COMMENTS

Background

About 90% of gastrointestinal stromal tumor (GIST) had gain-of-function mutations of KIT. The gold standard therapy for GIST is complete resection, depending on the lesion size and location. GIST is an easily recurrent disease, which is frequently found in the liver (65%), peritoneal surface (50%), or both (20%). Conventional chemotherapy is of little benefit for GIST because it is a non-epithelial neoplasm. The use of Imatinib mesylate in advanced GIST produces a response in 50% patients. Primary resistance to Imatinib affects about 15% of patients, and 50% of patients become secondarily resistant by 2 years after Imatinib therapy. Clearly, a new strategy is required.

Research frontiers

The KIT proto-oncogene on chromosome 4 (4q11-q12), which encodes for the KIT protein, appears to play an important role in early stages of tumor formation as well as in late tumor progression. Inhibition of *KIT* gene may block the formation and development of GIST. RNAi is the most effective method to silence a special gene. Two documents gave evidences that RNAi could block *KIT* gene expression in GIST, but all of them are not involved in the growth of GIST. Up to now, it is still uncertain if KIT RNAi could become an effective therapeutic method for GIST.

Innovations and breakthroughs

In this study, the authors successfully constructed an adenovirus vector to mediate KIT RNAi in GIST xenografts. The results demonstrate that an adenovirus system that induces KIT RNAi can silence efficiently KIT, inhibit the growth of GIST xenografts, therefore it may be a promising method for GIST treatment. This may be the first investigation about KIT RNAi mediated with adenovirus for the treatment of GIST xenograft.

Applications

The results of the present study show that it is possible that KIT RNAi mediated by adenovirus might become a treatment method for diseases related to KIT, including GIST, and it is also a useful method to study the KIT function in cytogenic and molecular research.

Peer review

The authors described that an adenovirus vector was successfully constructed to mediate KIT RNAi in GIST xenografts. The results demonstrated that the adenovirus system can silence efficiently KIT and inhibit the growth of grafted GIST. This approach may have therapeutic potential in GIST. Their results are very attractive. However, additional data should be required.

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CASE REPORT

Soft tissue sarcoma with metastasis to the stomach: A case report

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Abstract

Soft tissue sarcomas are unusual malignancies comprising 1% of cancer diagnoses in the United States. Undifferentiated pleomorphic sarcoma accounts for approximately 5% of sarcomas occurring in adults. The most common site of metastasis is the lung, with other sites being bone, the brain, and the liver. Metastasis to the gastrointestinal tract has rarely been documented. We present an unusual case of high-grade pleomorphic sarcoma with metastasis to the stomach, complicated by upper gastrointestinal bleeding.

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Key words: Gastrointestinal bleeding; Metastatic sarcoma; Undifferentiated pleomorphic sarcoma

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INTRODUCTION

Soft tissue sarcomas are unusual malignancies comprising 1% of cancer diagnoses in the United States, and metastasis to the gastrointestinal tract has rarely been documented. The gastroenterologist may encounter metastatic sarcoma while investigating the etiology of GI bleeding. Distal gastrectomy is sometimes performed for life threatening hemorrhage, however long term survival is poor due to widespread metastatic disease. We present an unusual case of high-grade pleomorphic sarcoma with metastasis to the stomach, complicated by upper gastrointestinal bleeding.

CASE REPORT

A 60-year-old male presented to the outpatient surgery clinic complaining of an enlarging mass on his left posterior shoulder, accompanied by increasing pain and decreased range of motion. He stated that the mass had grown from a small "pea sized" nodule to its current size within the last 6 mo. He denied any weight loss. His past medical and surgical histories were noncontributory. He reported a 40 pack-year history of tobacco use, but

Figure 1 Large left shoulder mass.

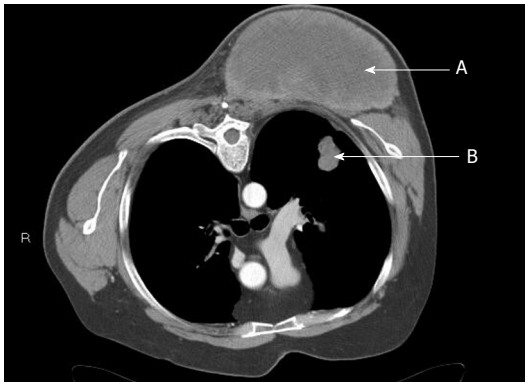

Figure 2 Chest computed tomography scan showing mass of left posterior chest (A) and suspected left pulmonary metastasis (B).

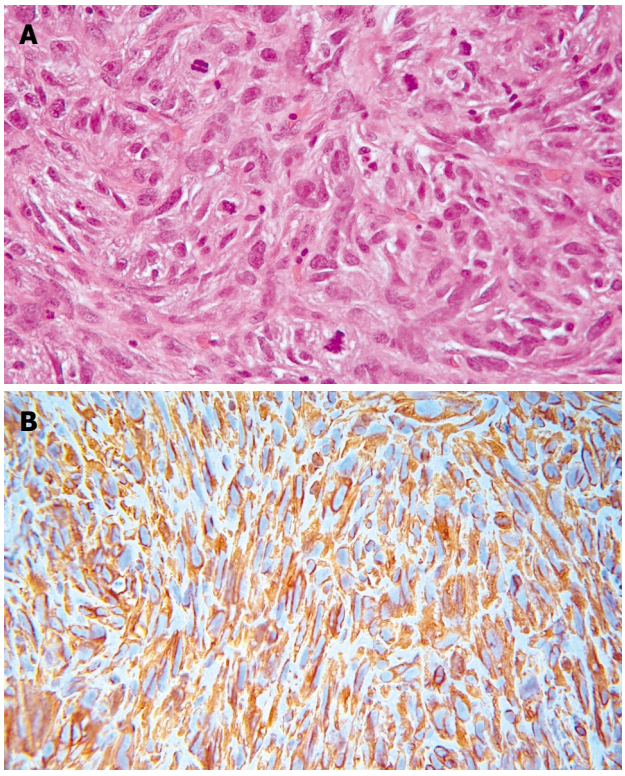

Figure 3 High-grade pleomorphic sarcoma show large pleomorphic tumor cells in a fibrous stroma with numerous mitotic figures (A), tumor cells show a strong diffuse cytoplasmic immunoreactivity to vimentin (B). Other immunoreactions failed to discern any line of differentiation. The "vimentin only" immunophenotype leads to a diagnosis of undifferentiated pleomorphic sarcoma.

denied alcohol use or occupational chemical exposure. He denied any family history of malignancy.

The physical examination was significant only for a firm, 10 cm × 20 cm mass on the left posterior shoulder, extending from the upper scapular border to the inferior tip, and from the left paraspinal muscles to the posterior axillary line (Figure 1). His range of motion was limited due to severe pain. Computed tomography (CT) performed several weeks prior to his surgical clinic evaluation demonstrated a 13.6 cm × 7.7 cm × 10 cm heterogeneous soft tissue mass located posterior to the left scapula, extending from the upper to the lower scapular borders, adjacent to or arising from the left paraspinous muscles (Figure 2). Rapid growth of the mass was apparent, but there was no evidence of bony involvement. Also noted was a 2.5 cm × 1.8 cm ill-defined nodule in the posterior left upper lobe of the lung (Figure 2), a 0.83 cm left perihilar nodule, and a sub centimeter peripheral density of the right posterior mid lung close to the pleura. Emphysematous changes and blebs were seen in the apices of the lungs bilaterally.

A diagnosis of sarcoma was suspected and subsequently confirmed by incisional biopsy, which revealed a grade 3 undifferentiated pleomorphic sarcoma. Microscopically the lesion was a highly cellular malignant mesenchymal neoplasm composed of large irregular, plump elongated to spindle shaped cells arranged in short fascicles with focal vague cartwheel and storiform patterns. The lesion was predominately diffuse and pleomorphic with no morphologic pattern or cytologic line of differentiation. The nuclei were large, crowded, and pleomorphic with shapes including vesicular, elongated, spindled and occasionally bizarre and multinucleate giant cells. More than 20 mitoses per 10 high power field were present (Figure 3A). The immunohistochemical reactivity showed 100% vimentin cytoplasmic reactivity (Figure 3B), 30% CD68 reactivity, 80% of nuclei showed MIB-1 reactivity and 50% of the sample exhibited necrosis. Immunohistochemical stains were nonreactive for epithelial, smooth muscle, striated muscle, neural, or lipoblast differentiation. The morphologic and immunohistochemical reactivity supported a diagnosis of undifferentiated pleomorphic sarcoma grade 3.

As metastasis to the lung was suspected, the patient was referred for cardiac and pulmonary evaluation in anticipation of a palliative resection with potential chest wall involvement. As part of the preoperative evaluation, the chest CT was repeated, revealing an increase in the primary tumor size compared to the prior CT, as well as multiple new lung nodules consistent with metastatic disease. After consulting with medical oncology, thoracic surgery and plastic surgery, the patient was offered a palliative resection of the mass due to severe pain and disability of his left shoulder. The resection involved removal of parts of the trapezius, latissimus dorsi, rhomboid, serratus

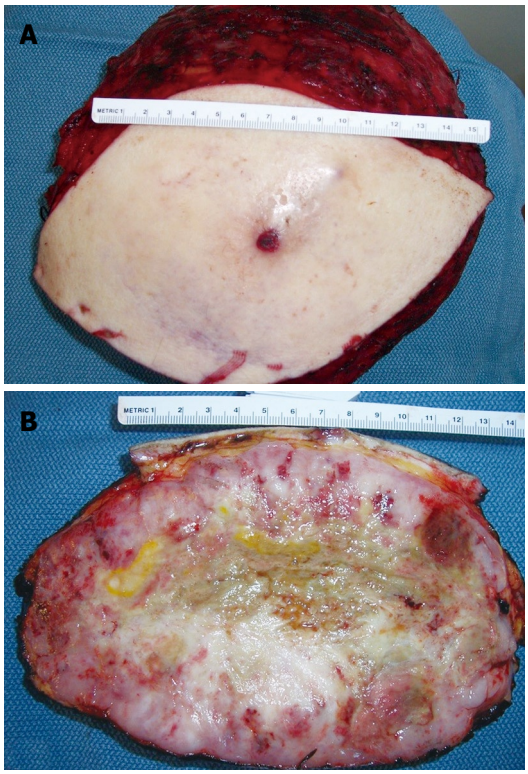

Figure 4 Resected left shoulder mass (A), cross section of resected shoulder mass showing central necrosis and smooth glistening dense fish flesh like tumor at the periphery (B).

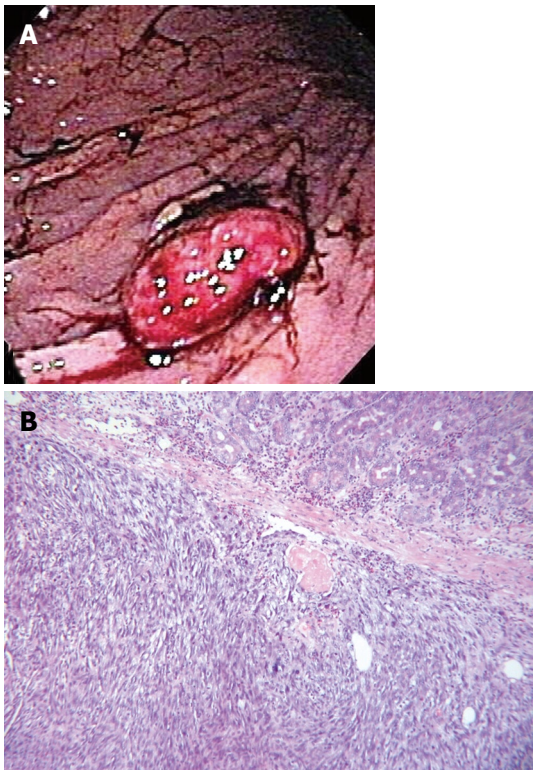

Figure 5 Endoscopic view of the stomach shows a mass with surface ulceration and blood clots (A), the photomicrograph (B) reveals a malignant neoplasm, composed of densely cellular stroma with spindle cells containing pleomorphic nuclei.

posterior, paraspinal, and superficial scapular muscles. Using extensive undermining and creation of skin flaps, the wound was closed primarily over drains. The resected mass measured 23.0 cm × 24.0 cm × 12.0 cm and weighed 2730 g (Figure 4A and B) and the tumor involved the deep resection margin. Brachytherapy catheters were inserted in the wound adjacent to the chest wall. His immediate postoperative course was uncomplicated and he was discharged home on postoperative day seven.

Two weeks after discharge, the patient presented for follow up complaining of burning upper abdominal pain and melanotic stools. Upper endoscopy was notable for mild diffuse gastritis and a 5 cm × 2 cm greater curvature mass (Figure 5A). The surface of the mass was ulcerated, hemorrhagic, with focal blood clots. The lesion was removed with snare and cautery with resolution of the bleeding. Biopsy revealed a grade 3 pleomorphic sarcoma consistent with metastatic disease (Figure 5B), as well as *Helicobacter pylori* (*H. pylori*) infection. He received blood transfusion due to severe anemia and treatment for the *H. pylori* infection. A few weeks later, while awaiting the initiation of chemotherapy the patient suffered a cerebrovascular accident and his condition deteriorated rapidly. He received only comfort care and was discharged to hospice.

DISCUSSION

Soft tissue sarcomas are unusual malignancies comprising 1% of cancers diagnosed in the United States[1]. Most primary sarcomas involve an extremity (59%); the next most common sites are trunk (19%), retroperitoneum (13%), and head and neck (9%)[2]. In a study of 1240 patients from the French Federation of Cancer Centers Sarcoma Group, a multivariate analysis showed in the order of importance the following independent predictors for metastasis and survival: histologic grade, tumor size, bone or neurovascular involvement, and tumor depth for the overall group. The predictors for metastasis and survival for malignant fibrous histiocytoma were histologic grade and neurovascular or bone invasion[3]. In this study, the metastasis-free 5-year survival rate was 91% for grade 1, 71% for grade 2, and 44% for grade 3; this study along with others show that histologic grade is the most reliable predictor of metastatic risk and overall tumor-free survival in adult soft tissue sarcomas[3-5]. Several reported studies show that the quality of surgical margins is the most important factor for predicting local recurrence[3-7].

The most commonly used and critically analyzed histologic grading systems are the French grading[8,9] and the National Cancer Institute grading[10]. Both are 3-grade systems and are quoted in the latest edition of the World Health Organization (WHO) classification of STS[11]. The National Cancer Institute system is based on tumor histologic type and subtype, location, and the amount of tumor necrosis. Cellularity, nuclear pleomorphism, and mitotic index are considered for some tumor types. The French grading system[8,9] is based on 3 components: tumor differentiation, mitotic index, and tumor necrosis. Tumor

differentiation and mitotic index are scored 1 to 3 and necrosis 0 to 2. The 3-grade system is obtained by summing the scores for each of the components. Grade 1 is defined as a score of 3 or less; grade 2 has a score of 4 or 5; and grade 3 has a score of 6 to 8. A MIB-1 score, as recently used by Hasegawa *et al*[12] using the French grading system, could replace the mitotic index, which may be difficult to evaluate in small biopsies. These authors found that grading using the MIB-1 score was more reproducible, and it had a better predictive value than the classical grading using mitotic index[13].

Malignant fibrous histiocytoma (MFH) accounts for approximately 20% of all adult soft tissue sarcomas[5], and occurs most commonly in the extremities, trunk or retroperitoneum[2]. The term MFH was introduced by Ozzello *et al*[14] and described by O'Brien *et al*[15], to describe a group of malignant mesenchymal lesions (sarcomas) which manifested features of both fibroblastic and histocytic differentiation. Kempson *et al*[16] further characterized the lesion as having a storiform growth pattern with varying proportions of histiocytes and giant cells. The origin and existence of the MFH as a distinct pathological entity is controversial. The current consensus is that the MFH, especially the pleomorphic storiform subtype, is a common "morphologic pattern" shared by a number of pleomorphic neoplasms, irrespective of their histologic origin and represents a "final common pathway" for tumor growth. Every effort must be employed to identify a line of differentiation in this group of lesions, and those neoplasms in which no line of differentiation can be identified are designated storiform-pleomorphic MFH. Therefore, storiform-pleomorphic MFH is a diagnosis of exclusion[17]. In such cases, the WHO classification of soft tissue tumors[11] advocates an alternate name undifferentiated high-grade pleomorphic sarcoma. After this conceptual shift, this subtype, which was considered to be the most common soft tissue tumor in adults now accounts for no more than 5% of adult soft tissue sarcomas[18].

The use of immunohistochemistry is essential in the diagnostic workup of any MFH-like tumor because a diagnosis based on morphology alone is unacceptable. Since the diagnosis of MFH is one of exclusion, an extensive immunohistochemistry panel is required to identify a definitive line of differentiation. The immunohistochemical marker panel must include broad markers such as vimentin and cytokeratins for mesenchymal and epithelial differentiation, respectively. The panel may then be narrowed to add specific markers related to the clinical setting and the histologic suspicion. Undifferentiated pleomorphic sarcoma typically demonstrates immunoreactivity to vimentin but fails to show immunoreactivity of other lines of differentiation. Traditional histocytic markers such as CD68, α_1-antitrypsin, α_1-antichymotrypsin, and factor XIII no longer play a useful role in the diagnosis of MFH. Immunoreactivity of these markers is found to be nonspecific and therefore, will not support a definitive diagnosis of MFH[19,20]. This lesion showed 100% cytoplasmic vimentin reactivity and the other markers were nonreactive or displayed insignificant reactivity.

There have been occasional reports of MFH occurring as primary malignancies in the stomach[4], however, metastatic MFH to the stomach is rare. The case presented here is the third case of a metastatic gastric MFH that presented with gastroduodenal bleeding. The other two cases were published in 1983 by Adams *et al*[21] and in 2009 by Karanlik *et al*[22]. A review of the literature yielded 10 cases of MFH with metastasis to the stomach[23]. Of the 10 reported cases of gastric metastasis, six were classified as storiform-pleomorphic. The case reported here is likewise of the storiform-pleomorphic subtype. Undifferentiated storiform-pleomorphic sarcoma as seen in our case presentation, accounts for approximately 5% of sarcomas occurring in adults. The most common sites of metastasis of pleomorphic sarcoma are the lung (80%) and lymph nodes (32%).

The pathologic characteristic of MFH is that of a spindle-cell sarcoma with no distinct line of differentiation and typically presents in adults over 40 years old. The proliferative activity in soft tissue sarcoma has been assessed with the monoclonal antibody MIB-1[10]. The MIB-1 index ranges from 1% to 85% and correlates with mitotic score and tumor grade. The MIB-1 index of this patient's tumor was > 80%, which explains its rapid growth, large size, and widespread metastasis. Like most patients with large (> 5 cm), high-grade sarcomas, our patient had metastatic disease on presentation.

Soft tissue sarcomas metastasize *via* hematogenous spread. Of the 10 previously reported cases of gastric metastasis, eight were found to be metastatic to the antrum and greater curvature suggesting a hematogenous pathway through the gastroduodenal or gastroepiploic vessels[23]. Likewise, the patient reported here had metastasis to the greater curvature. Sixty percent (6/10) of the reported cases of gastric metastasis were treated with distal gastrectomy and only two of these patients survived more than two years. The average length of survival for the other 4 patients who underwent resection was 7 mo[23]. Gastric metastasis portends a grave prognosis with only 20% (2/10) of patients surviving more than 16 mo. Our patient was too debilitated to undergo gastric resection; however, it is unlikely that resection would have resulted in any clinical benefit. This patient was 60 years old and male, which is consistent with other cases of gastric metastasis for MFH in which the average age was 66 and 8/10 cases were men.

The management of high-grade sarcoma usually involves a multidisciplinary approach, including surgical resection, radiation therapy, and adjuvant chemotherapy. Therapy is hampered by the fact that 20% of patients with high-grade soft tissue sarcoma have pulmonary metastatic disease at the time of initial diagnosis[24]. The endoscopist should consider the possibility of gastric metastasis in patients with a history of sarcoma who present with upper gastrointestinal bleeding. Nearly all patients who present with bleeding from gastric metastasis due to

high-grade sarcomas have widespread disease at presentation, thus the role of surgery is limited. Surgical resection of gastric metastasis may be performed to control life-threatening bleeding, but most patients do not receive long-term benefit.

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CASE REPORT

Local recurrence of hepatocellular carcinoma after radiofrequency ablation

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Abstract

A 51-year-old Chinese male with a 20-year history of hepatitis B was diagnosed with hepatocellular carcinoma in the right anterior portion of the liver, sized 3.5 cm × 3.2 cm, and was treated with radiofrequency ablation (RFA) on December 18, 2001. The patient did not receive antiviral therapy for hepatitis B virus after RFA. The treated lesion reduced gradually and reached its minimum size of 1.7 cm × 1.5 cm seven years later on November 18, 2008. However computed tomography findings revealed that a recurrence lesion of 6.0 cm × 4.8 cm which was histologically confirmed overlapped the previous treated lesion at the 8th year on December 3, 2009. Although recurrence at 8 years after curative RFA is a rare event, such a possibility must be kept in mind. To find and treat the recurrence lesion promptly, long-term and close monitoring is warranted after RFA. Meanwhile, the recurrence-prevention therapy is as important as close monitoring for those patients with a history of hepatitis B.

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Key words: Hepatocellular carcinoma; Radiofrequency ablation; Recurrence

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a worldwide health problem and a poor prognostic disease. Local treatments are the main approach for early HCC. Percutaneous radiofrequency ablation (RFA) is a potentially curative therapy for early HCC. Emerging clinical evidence has demonstrated that RFA can obtain the same survival rate as surgical resection[1-3]. However, follow-up after RFA is not yet adequately addressed by international guidelines. How long and how often should the follow-up be taken remains unclear. Here, we report a rare case of HCC locally recurring 8 years after RFA and review the literature.

CASE REPORT

A 51-year-old Chinese male with a 20-year history of hepatitis B was diagnosed with HCC in his annual checkup. Spiral computed tomography (CT) revealed a 3.5 cm × 3.2 cm mass in the right anterior portion of the liver (Figure 1A). Liver biopsy was performed and the diagnosis of well-differentiated hepatocellular carcinoma was histologically confirmed (Figure 2A). Laboratory findings revealed the following: platelets: 207 000/mm^3 [normal value: (100-300) × 10^3/mm^3]; Prothrombin time: 13.9 s (normal values: 10.0-13.0 s); Activated partial thromboplastin time -26.8 s (normal values: 23.0-36.0 s); Total bilirubin: 11.3 μmol/L (normal values: 5.1-17.1 μmol/L); Direct bilirubin: 6.5 μmol/L (normal values: 0-6.0 μmol/L); Serum alpha-fetoprotein (AFP): 5.6 μg/L (normal values: 0-8.1 μg/L);

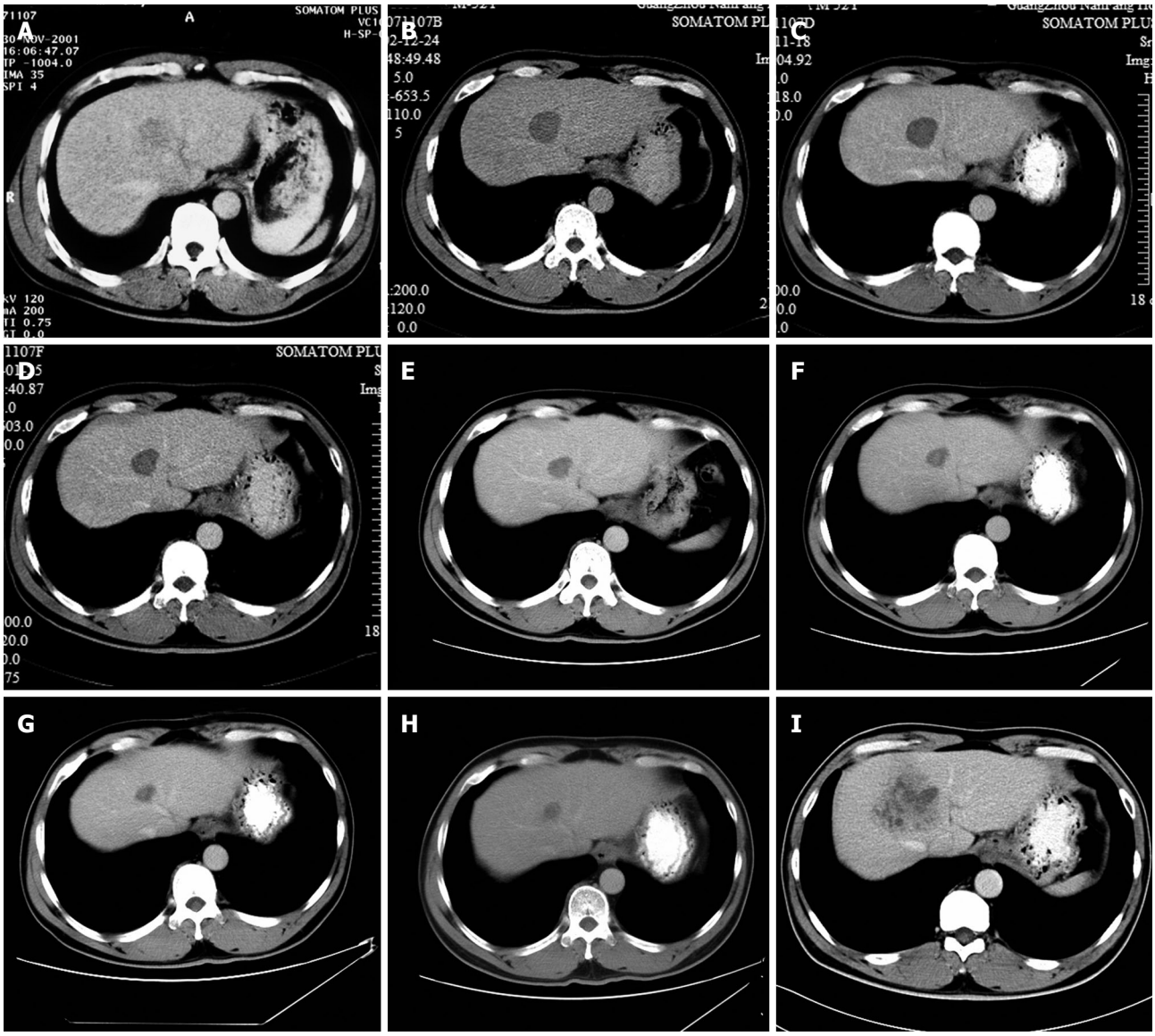

Figure 1 Computed tomography images of the hepatocellular carcinoma. A: Tumor size was 3.5 cm × 3.2 cm before radiofrequency ablation (RFA) (2001-11); B: Tumor size was 3.2 cm × 3.1 cm at 12 mo after RFA (2002-12); C: Tumor size was 3.1 cm × 3.0 cm at 2 years after RFA (2003-11); D: Tumor size was 2.8 cm × 2.5 cm at 3 years after RFA (2005-1); E: Tumor size was 2.4 cm × 2.2 cm at 4 years after RFA (2005-11); F: Tumor size was 2.1 cm × 2.0 cm at 5 years after RFA (2006-09); G: Tumor size was 1.9 cm × 1.6 cm at 6 years after RFA (2007-11); H: Tumor size was 1.7 cm × 1.5 cm at 7 years after RFA (2008-11); I: Tumor size was 6.0 cm × 4.8 cm at 8 years after RFA (2009-12).

Albumin: 33.5 g/L (normal values: 35.0-55.0 g/L). HBsAg (+), HBsAb (-), HBeAg (+), HBeAb (-), HBcAb (+); Hepatitis B Virus (HBV)-DNA: 3.28 × 10^5 copies/mL; Hepatic function: Child-Pugh A.

Following written informed consent, RFA was performed on December 18, 2001. Local anesthesia and conscious sedation were achieved with subcutaneous 1% lidocaine and intravenous midazolam and fentanyl. The RF delivery system was an RF 2000 generator system (Radio Therapeutics, USA), used in conjunction with a LeVeen needle electrode and two large grounding pads placed on the patient's thighs. The RF generator supplies voltage at a frequency of 460 KHz and a maximum power output of 90 W. The active expandable needle electrodes have a 15 cm long stainless steel insulated cannula with 10 retractable lateral hooks. The maximum deployment diameter of the hooks was 3.5 cm. Using a multi-frequency convex probe, RFA was performed with real-time ultrasound guidance. The needle was inserted *via* the intercostal approach according to the ultrasonographic picture. When the end of the needle electrode was located in the center of the lesion, the hooks were deployed and the RF generator was activated. After maintaining a baseline power output at 50 W for 1 min, the output was increased by 10 W/min until it reached 90 W. Then the power output was kept at 90 W until radiofrequency generator system roll-off automatically[4].

No severe complication was observed after RFA procedure. One year after RFA, CT findings demonstrated the treated lesion as a low-attenuation area measuring 3.8 cm × 3.5 cm on December 24, 2002, with no contrast enhancement, overlapping the previous enhancing

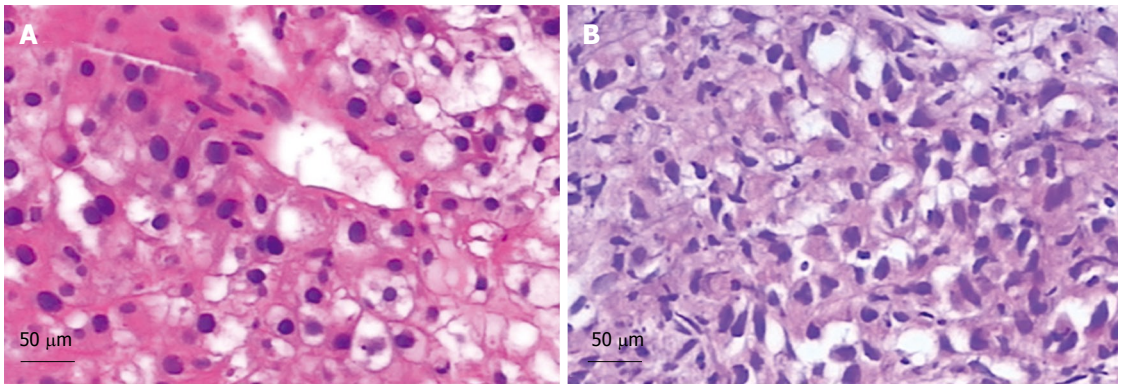

Figure 2 Pathological examinations (HE stain). A: A well-differentiated hepatocellular carcinoma (HCC). Scale bar = 50 μm; B: A well-moderately differentiated HCC. Scale bar = 50 μm.

lesion (Figure 1B). No antiviral therapy for hepatitis B virus (HBV) was given after RFA.

The patient was regularly followed up at oncology outpatient department by performing examinations of abdominal ultrasound (US), contrast-enhanced CT and AFP. US and AFP tests were taken every 3-6 mo for 2 years, then annually for the next 3 years. Contrast-enhanced CT of the liver was performed annually.

The annual CT scan revealed that the treated lesion reduced gradually from 3.8 cm × 3.5 cm, December 24, 2002 to 1.7 cm × 1.5 cm, November 18, 2008 (Figure 1B-H). Serum AFP levels were normal throughout the follow-up period. However, on December 3, 2009, CT findings demonstrated that a recurrence lesion of 6.0 cm × 4.8 cm overlapped the previous treated lesion (Figure 1I). Liver biopsy proved the diagnosis of well-moderately differentiated HCC (Figure 2B). Laboratory findings: HBsAg (+), HBsAb (-), HBeAg (+), HBeAb (-), HBcAb (+), HBV-DNA: 7.36 × 10^5 copies/mL.

DISCUSSION

HCC is the fifth most common cancer worldwide and is particularly prevalent in China where most HCC are associated with chronic HBV infection. Local treatments are the main therapeutic approach for early HCC. Surgical resection is considered as the only potentially curative therapies[5] and hepatic resection (HR) is generally considered as the first option for early HCC in most guidelines. However many patients can not undergo a surgical operation because of the limited inclusion criteria. Consequently, as a potentially curative nonsurgical therapy, RFA has been developed[6,7]. Nowadays, RFA is recommended by the guidelines established by the American Association for the Study of Liver Diseases (AASLD)[6], European Association for the Study of the Liver (EASL)[8] and the Japanese "Evidence-based guidelines"[9] for HCC measuring 3 cm or smaller.

Accumulated clinical evidence indicates that the efficacy of RFA is equal to that of a surgical resection for early HCC[1,10]. Meanwhile, the efficacy of RFA is tumor-size dependent. The risk factors for tumor recurrence after RFA include tumor size, insufficient safety margin, multinodular tumor and tumor location[11]. Among these risk factors, tumor size > 2.3-3.0 cm is the main risk factor for local recurrence[12-15]. To reduce the risk of recurrence after RFA, it is important to choose the right case according to the status of the lesions. Moreover, tumor recurrence was associated with hepatitis virus infection. Generally, it is recognized that tumor factors were associated with early HCC recurrence (within two years of local treatment) while high viral loads and hepatic inflammatory activity were associated with late recurrence (> 2 years after local treatment)[16]. Therefore, in view of the relation of HCC recurrence and hepatitis virus infection, secondary prevention of HCC after curative RFA is another essential clinical issue. Antiviral therapy may block the procedure of multicentric carcinogenesis in which new lesions are formed as a result of underlying hepatitis[17]. Lamivudine is a popular choice for the secondary prevention of recurrence after local treatment for HCC in chronic hepatitis B patients. A multicenter randomized controlled trial showed that lamivudine can reduce HCC incidence by approximately 50% in patients with chronic hepatitis B cirrhosis[18]. Lamivudine is also beneficial for preventing HCC in patients without cirrhosis[19]. Though its effect on survival of patients with HCC is to be proven, lamivudine after RFA for hepatitis B-related HCC was safe[20].

In this case, though no sufficient safety margin was obtained, CT findings showed that the lesion was completely necrotic one year after RFA and the treated lesion reduced gradually over the next seven years. It reduced to its minimum size of 1.7 cm × 1.5 cm on November 18, 2008. On December 3, 2009, CT image showed a 6.0 cm × 4.8 cm recurrence lesion, which was histologically confirmed. The lesion overlapped the previously treated one. The growth process of the recurrence lesion between November 18, 2008 and December 3, 2009 remains unknown because of the lack of imaging examination. Based on previous studies, multicentric carcinogenesis relating to hepatic inflammatory activity resulting from HBV infection is the most likely reason for recurrence in this case. We suppose that the recurrence lesion was very close to the previous treated one at the beginning and, as the accretion of the recurrence tumor, overlapped the previous treated lesion. Long-term survival without safety margins in this case may

contribute to clinicopathological factors such as normal AFP and well-differentiated hepatocellular carcinoma. This case reminds us that after RFA, antiviral therapy should be given to patients with hepatitis B in order to reduce the recurrence rate of HCC. Both high-level and low-level viremia are associated with significant risk for HCC[21]. At the moment, evidence shows that antiviral therapy can lower the incidence of HCC in patients with viral hepatitis and that the therapy is rather safe[11,19,20]. Post-operative antiviral therapy may be crucial in reducing late recurrence[16]. The follow-up period after RFA should be extended. So far, it is reported that the longest time to recurrence occurred within 3 years of RFA[11]. Although rare, recurrence can occur 8 years later after RFA as with this case. Thus, a long-term follow-up is necessary. Intervals between follow-ups should be shortened. At present, local treatments work well in treating liver tumors smaller than 5 cm in diameter. In this case, the follow-up interval was set to be one year. When the recurrence was detected, the patient had missed the best chance of treatment due to the recurrence lesion being larger than 5 cm in diameter.

In conclusion, close monitoring and recurrence-prevention therapy after RFA is warranted.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

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Prague, Czech Republic
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October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

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Barcelona, Spain
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October 29-November 02
Boston, Massachusetts, United States
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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