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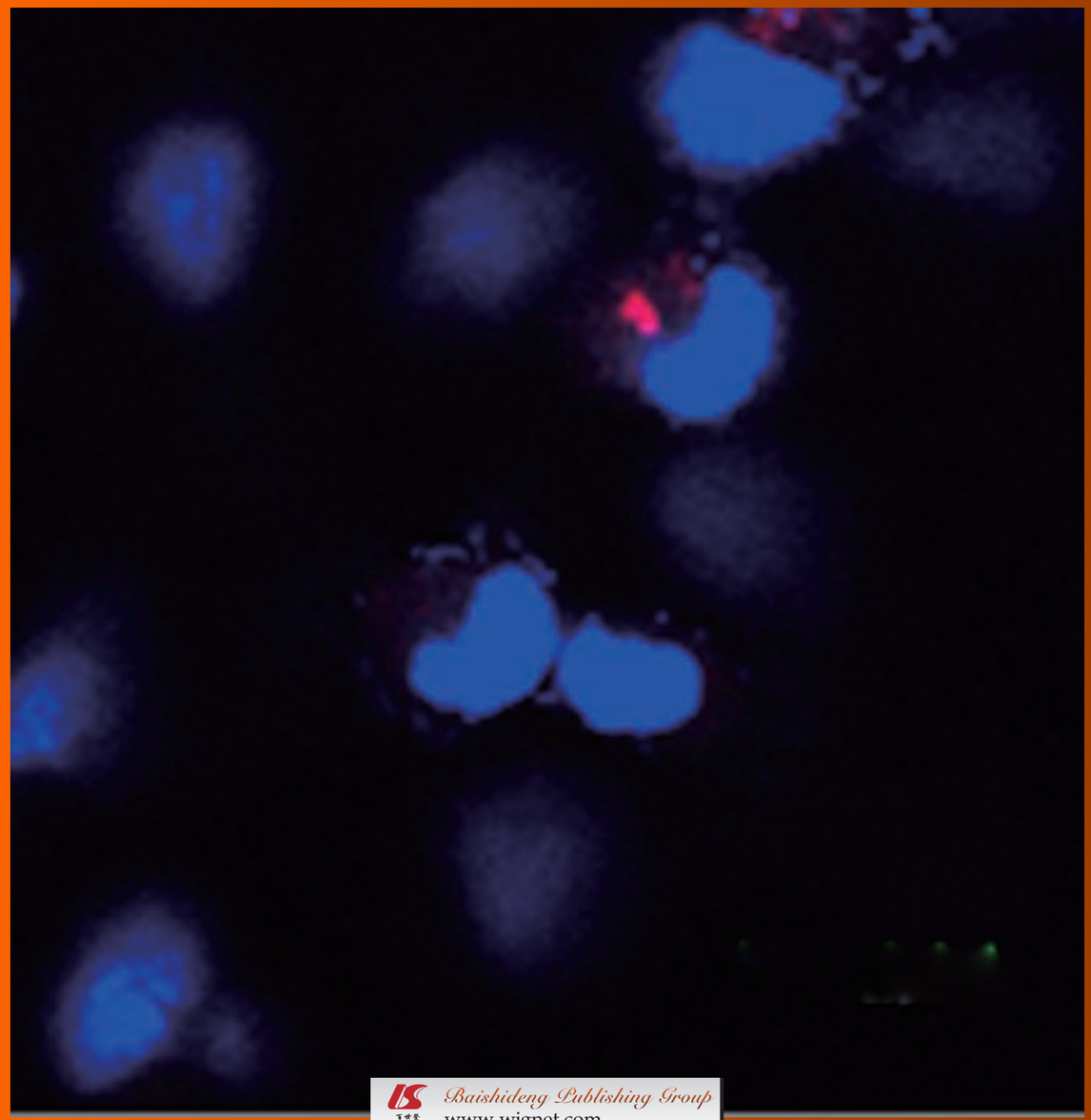

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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Beijing Baishideng BioMed Scientific Co., Ltd.
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
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Beijing 100025, China
Telephone: +86-10-5908-0039
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EDITORIAL

Pathophysiology and prevention of postoperative peritoneal adhesions

Willy Arung, Michel Meurisse, Olivier Detry

Willy Arung, Department of General Surgery, Cliniques Universitaires de Lubumbashi, University of Lubumbashi, Lubumbashi 1825, Katanga Province, Congo
Michel Meurisse, Olivier Detry, Department of Abdominal Surgery and Transplantation, CHU de Liège, University of Liege, Liège B4000, Belgium
Author contributions: Arung W performed the review and wrote the manuscript; Meurisse M and Detry O supervised the review and corrected the manuscript.
Correspondence to: Olivier Detry, Professor, Department of Abdominal Surgery and Transplantation, CHU de Liège, University of Liege, Liège B4000, Belgium. oli.detry@chu.ulg.ac.be
Telephone: +32-4-3667645 Fax: +32-4-3664069
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Abstract

Peritoneal adhesions represent an important clinical challenge in gastrointestinal surgery. Peritoneal adhesions are a consequence of peritoneal irritation by infection or surgical trauma, and may be considered as the pathological part of healing following any peritoneal injury, particularly due to abdominal surgery. The balance between fibrin deposition and degradation is critical in determining normal peritoneal healing or adhesion formation. Postoperative peritoneal adhesions are a major cause of morbidity resulting in multiple complications, many of which may manifest several years after the initial surgical procedure. In addition to acute small bowel obstruction, peritoneal adhesions may cause pelvic or abdominal pain, and infertility. In this paper, the authors reviewed the epidemiology, pathogenesis and various prevention strategies of adhesion formation, using Medline and PubMed search. Several preventive agents against postoperative peritoneal adhesions have been investigated. Their role aims in activating fibrinolysis, hampering coagulation, diminishing the inflammatory response, inhibiting collagen synthesis or creating a barrier between adjacent wound surfaces. Their results are encouraging but most of them are contradictory and achieved mostly in animal model. Until additional findings from future clinical researches, only a meticulous surgery can be recommended to reduce unnecessary morbidity and mortality rates from these untoward effects of surgery. In the current state of knowledge, pre-clinical or clinical studies are still necessary to evaluate the effectiveness of the several proposed prevention strategies of postoperative peritoneal adhesions.

Key words: Abdominal surgery; Laparoscopy; Complication; Occlusion; Abdominal pain

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INTRODUCTION

Peritoneal adhesions represent an important clinical challenge in gastrointestinal surgery. Peritoneal adhesions are a consequence of peritoneal irritation by infection or surgical trauma. They are a major cause of morbidity, resulting in multiple complications, many of which may manifest several years after the initial surgical procedure[1,2].

Development of peritoneal adhesions has been studied extensively, but to date, there has been no definitive strategy to prevent their formation, as controversies concerning the effectiveness of available preventive agents still exist. In addition, most of the available clinical literature concern gynecological patients; for patients undergoing general and/or abdominal surgery, no recommendations or guidelines exist[3]. The aim of this review is to present the epidemiology, pathogenesis and various prevention strategies of adhesion formation. We performed a literature search for this review in Medline and PubMed, using the key words: "adhesions", "intraperitoneal adhesions", "intra-abdominal adhesions", "adhesion reduction", "adhesion prevention", "adhesion formation", "adhesion pathophysiology". We also reviewed the reference lists in all articles retrieved in the search, as well as those of major texts regarding peritoneal adhesion formation. Both clinical and experimental studies upon adhesion formation were retained. There was no restriction regarding publication language.

Definition, epidemiology and consequences of peritoneal adhesions

Peritoneal adhesions are pathological bonds usually between omentum, loops of bowel and the abdominal wall. These bonds may be a thin film of connective tissue, a thick fibrous bridge containing blood vessels and nerve tissue, or a direct contact between two organ surfaces[4]. According to their etiology, peritoneal adhesions may be classified as congenital or acquired, which can be postinflammatory or postoperative (the most frequent)[5]. Among postoperative adhesion formation, three processes may be distinguished: *adhesion formation* (adhesions formed at operative sites); *de novo adhesion formation* (adhesions formed at non-operative sites); and *adhesion reformation* (adhesions formed after the lysis of previous adhesions)[6] . Diamond *et al*[7] have distinguished type 1 and type 2 formation of postoperative peritoneal adhesions. Type 1 or *de novo* adhesion formation concerns adhesions formed at sites that did not have previous adhesions, including type 1A (no previous operative procedure at the site of adhesions) and type 1B (previous operative procedures at the site of adhesions). Type 2 involves adhesion reformation, with two separate subtypes: type 2A (no operative procedure at the site of adhesions besides adhesiolysis) and type 2B (other operative procedures at the site of adhesions besides adhesiolysis)[7].

Peritoneal adhesions are mostly induced by surgical procedures in the peritoneal cavity, and their prevalence after major abdominal procedures has been evaluated at 63%-97%[8,9]. Overall, approximately one-third of patients who underwent open abdominal or pelvic surgery were readmitted an average of two times over the subsequent 10 years for conditions directly or possibly related to adhesions, or for further surgery that could potentially be complicated by adhesions; > 20% of all such readmissions occurred during the first year after initial surgery, and 4.5% of readmissions were for adhesive small bowel obstruction (ASBO)[1,10-13]. Colorectal surgery has proved to be the most important type of surgery that may cause intra-abdominal adhesions[14]. This surgery has the highest total number of inpatient episodes, inpatient days, operating time, theater time, and costs due to peritoneal adhesion-related intestinal obstruction[14]. Among open gynecological procedures, ovarian surgery had the highest rate of readmissions directly related to adhesions (7.5/100 initial operations)[13].

Small bowel obstructions (SBO) is the most common complication of peritoneal adhesions[1,2,8,9]. At Westminster Hospital (London, United Kingdom), intestinal obstruction accounted for 0.9% of all admissions, 3.3% of major laparotomies, and 28.8% of cases of large or SBO over 24 years[5]. A 1992 British survey has reported an annual total of 12 000-14 400 cases of adhesive intestinal obstruction. Barmparas *et al*[15] have studied the incidence and risk factors for ASBO following laparotomy. The overall incidence of ASBO was 4.6% and the risk of ASBO was highly influenced by the type of procedure, with ileal pouch-anal anastomosis being associated with the highest incidence of SBO[15]. In 1988 in the United States, admissions for adhesiolysis accounted for nearly 950 000 d of inpatient care[5]. All these studies have demonstrated that ASBO is a significant health issue both in the developed and developing world. However, ASBO risk factors, such as the type of past surgical procedure, the site of adhesions, as well as the timing and recurrence rate of adhesive obstruction, remain unpredictable or poorly understood[5].

In addition to ASBO, peritoneal adhesions may cause pelvic or abdominal pain, and infertility[1,2,16]. Peritoneal adhesions may also prolong the time needed to gain access to the abdominal cavity at subsequent surgery[17,18], and may increase the risk of bowel injury during subsequent surgery[19]. Controversy remains on the role of peritoneal adhesions on abdominal pain. Adhesions have been implicated as a significant cause of chronic pelvic pain, and their surgical lysis has been proposed as the therapeutic modality of choice[20,21]. However, chronic pelvic pain is one of most common gynecological complaints and yet remains an enigma. A comparison of chronic pelvic pain patients and asymptomatic infertility patients has not revealed a significant difference in the density or the location of adhesions[22]. Thus, it is possible that a common mechanism for pelvic pain exists and that adhesions are only associated features. Bradykinin, histamine and other autocoids are able to stimulate pain receptors. For Rapkin *et al*[22], these findings question the role of pelvic adhesions as a cause of chronic pelvic pain. According to other authors, although adhesions are thought to cause pain indirectly by restricting organ motion, thus stretching and pulling smooth muscle of adjacent viscera or the abdominal wall, adhesions themselves are capable of generating pain stimuli. Sulaiman *et al*[23] have studied the distribution, location, size and type of nerve fibers present in human peritoneal adhesions, associated or not with chronic pelvic pain. They have found that nerve fibers,

identified histologically, ultrastructurally, and immunohistochemically, were present in all examined peritoneal adhesions. Furthermore, fibers expressing the sensory neuronal markers calcitonin gene-related protein and substance P were present in all adhesions irrespective of reports of chronic abdominopelvic pain. That study has suggested that these structures may be capable of conducting pain after appropriate stimulation, and peritoneal adhesions are implicated as a cause of chronic abdominopelvic pain. In addition, many patients are relieved of their symptoms after adhesiolysis[23].

As consequence, peritoneal adhesions have a significant economic impact. Their direct costs in Sweden can be estimated to be $13 million annually[24]. It has been estimated that in the United States, there are 117 hospitalizations for adhesion-related problems per 100 000 people, and the total cost for hospital and surgical expenditure is about $1.3 billion[25]. In some European countries, the direct medical costs for adhesion-related problems are more than the surgical expenditure for gastric cancer and almost as much as for rectal cancer[3,26,27]. Indeed, postoperative adhesions have a profound economic impact, including the surgical procedure itself, hospitalization, recuperation and lost productivity[25]. During 1988, excluding patient and indirect costs, hospitalization in the United States, accounting for 948 727 d of inpatient care, was responsible for an estimated $1179.9 million in expenditure, of which $925 million was associated with hospital costs and $254.9 million with surgeons' fees[25]. The study of Ray *et al*[28] has demonstrated substantial costs associated with surgical procedures and hospitalization for adhesiolysis. During 1996, the total annual cost of adhesions management exceeded $2 billion, excluding recuperation and lost productivity[28]. Hospitalization for adhesiolysis alone cost > $700 million. Furthermore, > 300 000 patients are estimated to undergo surgery to treat adhesion-induced SBO in the United States annually[25]. Thus, developing effective strategies for adhesion prevention may help to reduce adhesions management costs and unnecessary morbidity and mortality rates.

Postoperative peritoneal adhesion pathophysiology

The first peritoneal adhesions were described at postmortem examination of a patient with peritoneal tuberculosis in 1836. To explain this finding, it was suggested in 1849 that coagulated lymphatic vessels may turn into fibrinous adhesions[29,30]. Until now, the exact pathophysiology of peritoneal adhesions has remained elusive. Despite many clinical and experimental studies, peritoneal adhesions pathophysiology remains controversial.

Aside from the normal peritoneal regeneration, the process of postoperative peritoneal adhesion formation may be considered as the pathological part of healing following any peritoneal injury, particularly due to abdominal surgery[5,31]. The balance between fibrin deposition and degradation is crucial in determining normal peritoneal healing or adhesion formation. If fibrin is completely degraded, normal peritoneal healing may occur. In contrast, incompletely degraded fibrin may serve as a scaffold for fibroblasts and capillary in growth to form peritoneal adhesions.

Peritoneal injury, due to surgery, infection or irritation, initiates inflammation with fibrinous exudate and fibrin formation[32]. Fibrin results from coagulation cascade activation that is activated in the peritoneal cavity, resulting in the formation of thrombin that triggers conversion of fibrinogen into fibrin. However, owing to activation of the fibrinolytic system, any intra-abdominal fibrin deposits must be lysed. After abdominal surgery, however, the equilibrium between coagulation and fibrinolysis is disturbed, in favor of the coagulation system. Thus, fibrin forms deposits are a matrix for ingrowth of fibrocollagenous tissue. Indeed, fibroblasts invade the fibrin matrix and the extracellular matrix (ECM) is produced and deposited. This ECM can still be completely degraded by the proenzymes of matrix metalloprotease (MMP), leading to normal healing. However, if this process is inhibited by tissue inhibitors of MMPs, peritoneal adhesions may be formed[33]. Generally, if fibrinolysis does not occur within 5-7 d of the peritoneal injury, the temporary fibrin matrix persists and gradually becomes organized with collagen-secreting fibroblasts. This process leads to peritoneal adhesion formation[34,35] and growth of new blood vessels mediated by angiogenic factors[13].

Activation of the fibrinolytic system results in the conversion of plasminogen into plasmin that is highly effective in the degradation of fibrin into fibrin degradation products. Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen (uPA) are both plasminogen activators. They are expressed in endothelial cells, mesothelial cells and macrophages. tPA, a serine protease, is the main plasminogen activator and has a high affinity for fibrin. It binds to a specific receptor, which exposes a strong plasminogen-binding site on the surface of the fibrin molecule. Therefore, in the presence of fibrin, the activation rate of plasminogen is strikingly enhanced, whereas in the absence of fibrin, tPA is a poor activator of plasminogen[36,37]. This results in higher plasminogen activation at the sites where it is required, whereas systemic activation is prevented. In the peritoneal cavity, tPA is responsible for 95% of plasminogen-activating activity[38]. uPA is equally effective in the degradation of fibrin[39], but its much lower affinity for fibrin results in a significantly lower plasminogen-activating activity. Besides activation of plasminogen, uPA may play an important role in tissue remodeling[40].

Plasminogen activation is hampered by plasminogen-activating inhibitor (PAI)-1 and 2 throughformation of inactive complexes. The most potent inhibitor of tPA and uPA is the glycoprotein PAI-1. PAI-2 is less effective in counteracting plasminogen activators. It probably plays a role in peritoneal tissue repair[41]. Both PA-1 and PAI-2 are produced by endothelial cells, mesothelial cells, monocytes, macrophages and fibroblasts. Other plasminogen activator inhibitors have been identified: PAI-3 and protease nexin 1. Several protease inhibitors, such as

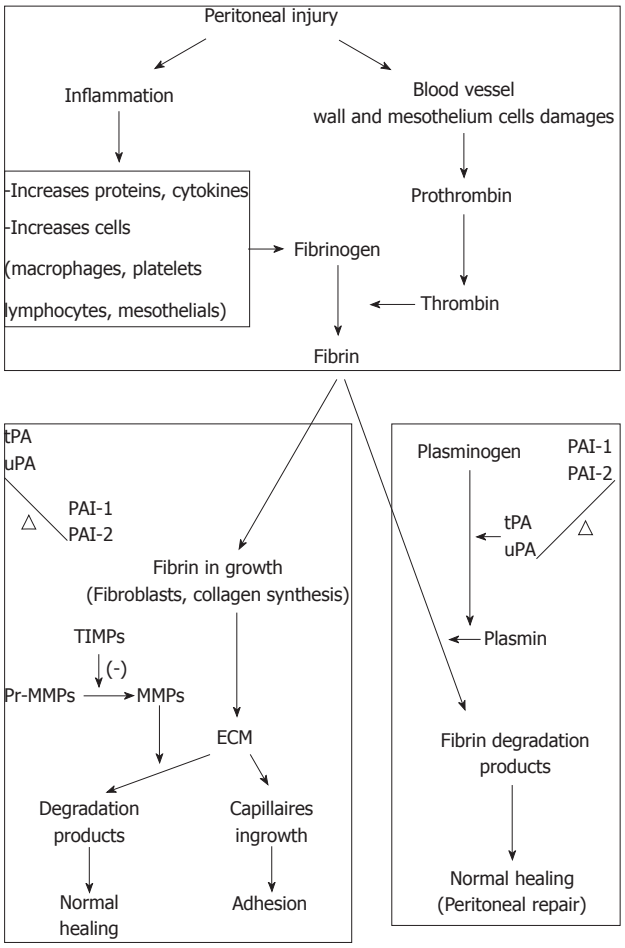

Figure 1 Balance between plasminogen activators and plasminogen inhibitors. TIMP: Tissue inhibitors of metalloproteinases; MMP: Matrix metalloprotease; ECM: Extracellular matrix; tPA: Tissue-type plasminogen activator; uPA: Urokinase-type plasminogen; PAI: Plasminogen-activating inhibitor.

α2-macroglobulin, α1-antitrypsin and α2-antiplasmin, inhibit plasmin directly. However, their roles in peritoneal fibrinolysis are not well defined[42]. The balance between plasminogen activators and plasminogen inhibitors is crucial in determining normal healing or adhesion formation (Figure 1). Therefore, PAI-1 is considered to be an important factor in the development of adhesions and high PAI concentrations are found in adhesions and peritoneal tissue of patients with extensive adhesions[43,44].

Prevention

Several preventive agents against postoperative peritoneal adhesions have been investigated. Their roles are in activating fibrinolysis, hampering coagulation, diminishing the inflammatory response, inhibiting collagen synthesis, or creating a barrier between adjacent wound surfaces. These prevention strategies can be grouped into four categories: general principles, surgical techniques, mechanical barriers, and chemical agents[3].

General principles and surgical techniques: Some basic principles should be respected during all abdominal surgical procedures. These principles are close to the "Halstedian principles" (W.S. Halsted 1852-1922), the first surgeon who recognized the importance of these measures[45]. Peritoneal damage should be avoided by careful tissue handling, meticulous hemostasis, continuous irrigation and avoiding unnecessary drying, ineffective use of foreign bodies, and suturing or clamping of tissue. The use of fine and biocompatible suture materials, atraumatic instruments and starch-free gloves is also recommended. Starched gloves are a significant risk factor for postoperative adhesions. Several experimental studies have shown that the use of starch-powdered gloves during laparotomy is associated with an increased risk of extensive postoperative peritoneal adhesions[46]. Foreign bodies most frequently found in postoperative adhesions are: surface powders from surgical gloves; lint from packs, drapes, or gowns; wood fibers from disposable paper items; and suture materials. However, recent data have suggested that, in the absence of an additional peritoneal injury, foreign bodies are an infrequent cause of adhesion induction[9,47]. Ordonez *et al*[48] have evaluated the effect of training on postoperative adhesion formation in a rabbit model. The training effect was evaluated by duration of surgery and amount of bleeding. This study has shown that there is a significant effect of experience on duration of surgery. With experience, duration of surgery progressively decreases, and postoperative adhesions also decrease in extent, tenacity, type and total score. According to these findings, surgical training and the respect of some basic principles ("Halstedian principles") are important for adhesion prevention.

Some intraoperative techniques, such as avoiding unnecessary peritoneal dissection or avoiding closure of the peritoneum, should be applied. Many experimental studies have shown that non-closure of the peritoneum is associated with decreased peritoneal adhesion formation[49-51]. However, some studies have reported no difference[52,53] or even decreased peritoneal adhesion[54] with peritoneal closure. However, grafting or suturing peritoneal defects may increase peritoneal ischemia, devascularization, and necrosis, predisposing the site to decreased fibrinolytic activity and increased adhesion formation[55].

Furthermore, surgical trauma should be reduced as much as possible. The surgical approach (open *vs* laparoscopic) could play an important role in the development of adhesions. In most abdominal procedures, the laparoscopic approach is associated with a significantly lower incidence of postoperative peritoneal adhesions or adhesion-related re-admissions. Brokelman *et al*[56] have shown in a prospective trial that there is no difference in tPA antigen, tPA-activity, uPA antigen, or PAI-1 antigen concentrations in peritoneal biopsies taken at the beginning compared to the end of the laparoscopic procedure, irrespective of the intra-abdominal pressure or light activity. In contrast, some studies have reported no difference between both surgical approaches. A role for CO_2 pneumoperitoneum in adhesion formation after laparoscopic surgery has been reported[48,57].

During laparoscopic surgery, CO_2 pneumoperitoneum by itself has a real impact on abdominal adhesions. It has

been demonstrated that adhesion formation increases with the duration of CO_2 pneumoperitoneum and insufflation pressure[48,57]. Indeed, prolonged laparoscopic surgery requires long duration and large volume gas insufflations, which raise concerns about the adverse effects of prolonged gas insufflations[58]. The standard CO_2 used in current laparoscopic practice is cold dry CO_2, which is not physiological to the normal conditions of the peritoneal cavity[57]. Many studies have shown that short-duration laparoscopy, < 3 h, with cold dry CO_2 insufflation can cause peritoneal alterations and result in numerous detrimental outcomes, including postoperative peritoneal adhesion formation[48,58]. The benefits of heated humidified CO_2 insufflation (37 ℃ and 95% relative humidity, physiological conditions) have been reported to include less hypothermia, less postoperative pains, shortened recovery room stay, better convalescence, less tumor spread and growth[48,58], and less adhesion formation[35]. Furthermore, Molinas *et al*[59] have demonstrated that CO_2 pneumoperitoneum increases postoperative peritoneal adhesions in a time- and pressure-dependent relationship, and that this increase is reduced by the addition of 2%-4% oxygen, suggesting peritoneal hypoxia as the driving mechanism. It supposes that when fibrinolytic activity decreases, the process of adhesion formation does not depend anymore on the surgical approach, but evolves on its own account.

Mechanical barriers: Liquid or solid mechanical barriers may prevent postoperative peritoneal adhesion formation by keeping peritoneal surfaces separate during the 5-7 d required for peritoneal re-epithelialization. They prevent contact between the damaged serosal surfaces for the first few critical days. An ideal barrier should be biodegradable, safe, non-inflammatory, non-immunogenic, persist during the critical re- mesothelialization phase, stay in place without sutures or staples, remain active in the presence of blood, and be rapidly and easily applied[60,61]. Also, it should not interfere with healing, promote infection, or cause adhesions. Barriers are currently considered the most useful adjuncts that may reduce postoperative peritoneal adhesion formation. Various solid or fluid barrier agents have been tested experimentally and in clinical trials.

Liquids such as crystalloids, dextran, hyaluronic acid, cross-linked hyaluronic acid and icodextrin have been used to prevent adhesion. They separate injured surfaces by "hydroflatation" but their effectiveness is controversial. Crystalloids, such as saline and Ringer's lactate, are used in large amounts but they are rapidly absorbed. The most commonly used hypertonic solution was 32% dextran 70, but it was abandoned because of serious complications[61]. Other liquid barriers that have the advantage of a longer residence time in the abdominal cavity, such as hyaluronic acid (Sepracoat®, Genzyme Corporation, Cambridge, MA, United States), cross-linked hyaluronic acid (Intergel® Hyalobarrier gel; Baxter, Pisa, Italy), and icodextrin (Adept®, Baxter Healthcare Corporation, Deerfield, IL, United States) have shown promising results in experimental and clinical studies[61]. Brown *et al*[62] have demonstrated that Adept is a safe and effective adhesion reduction agent in laparoscopy.

There are non-absorbable and bio-absorbable films, gels or solid membranes. The most commonly used mechanical barriers are oxidized regenerated cellulose (Interceed®; Johnson & Johnson Medical, Arlington, TX, United States), expanded polytetrafluoroethylene (Preclude Peritoneal Membrane®; W.L. Gore and Associates Inc., Flagstaff, AZ, United States), hyaluronic acid-carboxymethylcellulose (Seprafilm®; Genzyme Biosurgery, Cambridge, MA, United States) and polyethylenglycol (SprayGel®; Confluent Surgical Inc., Waltham, MA, United States). Preclude is non-degradable and requires a second operation for removal. The most extensively studied bioabsorbable films are Seprafilm and Interceed. Seprafilm is absorbed within 7 d and excreted from the body within 28 d[63,64]. Prospective randomized controlled trials have shown the efficacy of Seprafilm in reducing the incidence and extent of postoperative adhesions[65-68]. However, Seprafilm may cause a significant impairment of anastomoses, and should not be applied to anastomosis cases[69]. Other experimental studies have demonstrated that covering lesions of the parietal peritoneum with microsurgically applied autologous peritoneal transplants can completely prevent severe peritoneal adhesion formation. However, the advantage of a synthetic barrier is that the material does not need to be obtained surgically and can be cut to size outside of the abdomen and then applied without sutures[70].

Chemical agents: Chemical agents generally prevent the organization of the persisting fibrin, by fibroblastic proliferation inhibition. Many agents are used to inhibit this proliferation such as, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, calcium channel blockers, histamine antagonists, antibiotics, fibrinolytic agents, anticoagulants, antioxidants, hormones, vitamins, colchicines and selective immunosuppressors[60].

NSAIDs reduce peritoneal adhesions in some animal models by prostaglandin and thromboxane synthesis inhibition[9]. They decrease vascular permeability, plasmin inhibitors, platelet aggregation, and coagulation and also enhance macrophage function[9]. Rodgers *et al*[71] have shown that postoperative administration of anti-inflammatory drugs to the site of injury reduced the formation of postoperative adhesions in two animal models. A rat model has been used to investigate the efficacy of nimesulide, a selective cyclooxygenase-2 inhibitor, in the prevention of adhesion formation. This study has shown that preoperative intramuscular or postoperative intraperitoneal administration of nimesulide to the site of injury reduced the formation of postoperative adhesion in this rat model[72]. Generally, some anti-inflammatory drugs may be effective in preventing adhesions, but there is no clinical significant evidence from any published study to recommend their use in humans for this purpose, and several side effects still have to be ascertained[73].

Corticosteroid therapy reduces vascular permeability

and liberation of cytokines and chemotactic factors and has reduced peritoneal adhesion formation in some animal models[70]. However, corticosteroids have side effects, such as immunosuppression and delayed wound healing[60,74]. Kirdak *et al*[75] have investigated the effectiveness of different doses of methylprednisolone in preventing experimentally induced peritoneal adhesions in rats. They have found that there was no difference in the effectiveness of different methylprednisolone doses, administered topically, in preventing peritoneal adhesion formation, and furthermore, steroids did not prevent peritoneal adhesion development[75].

In animal models, these hormones may prevent adhesion formation, but some studies have not confirmed this effectiveness in humans[74]. Progesterone has been reported to have an anti-inflammatory as well as immunosuppressive effect, and may prevent adhesion formation[73]. However, Confino *et al*[76] have shown that there was no significant difference overall in the incidence of adhesion formation between progesterone-treated and control rabbits. They have revealed a beneficial effect of progesterone in the reduction of only minor adhesion formation formed after minor peritoneal damage[76]. Furthermore, it has been shown that neither estrogen nor gonadotropin-releasing hormone prevented adhesion formation, but there were fewer adhesions formed in estrogen-treated than untreated animals[77].

The use of anticoagulants to prevent the formation of peritoneal adhesions has been enthusiastically reported in the literature[78]. Many molecules have been used, such as heparin or dicumarol, which prevents adhesion by increasing the fibrinolysis due to serine esterase activity[79]. Heparin is the most widely investigated anticoagulant used for prevention of adhesions. However, its efficacy in reducing adhesion formation whether administered alone or in combination with interceed barrier has not been demonstrated in clinical trials[78].

Fibrinolytic agents such as recombinant tPA, when applied locally, have reduced adhesions in animal models[73]. However, these fibrinolytic agents may cause hemorrhagic complications[73]. Three different drugs, tPA (Actilyse®; Boehringer Ingelheim International GmbH, Ingelheim am Rhein, Germany), fondaparinux (Arixtra®; GlaxoSmithKline, France), and activated drotrecogin alfa (Xigris®; Elli Lilly and Co., DSM Pharmaceuticals, Inc. Greenville, NC, United States), which affect the coagulation process at various stages, have been studied for their effectiveness in preventing intraperitoneal adhesion formation in rats[80]. All three agents were effective in preventing adhesions when compared to the control group. Nevertheless, activated drotrecogin alfa seemed the most effective except when considering clinical applicability, in which case fondaparinux seemed to offer the greatest advantage[80]. However, further studies have suggested that all these approaches may have only limited success, impeded lack of safety, efficacy and many adverse effects without eliminating the problem of postoperative peritoneal adhesion formation[81,82].

Some antibiotics are commonly used for prophylaxis against postoperative infections and adhesion formation. Less peritoneal infection may lead to less peritoneal adhesion formation. Linezolid (Zyvox®; Pfizer, New York, NY, United States) has been found to reduce intraperitoneal adhesion formation in a rat uterine horn model[83]. However, other studies have shown that intra-abdominal application itself causes adhesion formation[73]. Sortini *et al*[84] have shown that antibiotics led to greater adhesion formation by Zühlke score as compared to saline, whereas no difference was observed between antiseptics and saline. Indeed, antibiotics in intraperitoneal irrigation solutions have been demonstrated to increase peritoneal adhesion formation in rat models, and thus, are not recommended as a single agent for adhesion prevention[79].

Vitamin E is the most studied vitamin in adhesion prevention. *In vitro* studies have demonstrated that vitamin E has antioxidant, anti-inflammatory, anticoagulant and antifibroblastic effects, and decreases collagen production. It has been found to be effective for reducing adhesion formation by some authors[85]. Corrales *et al*[86] have shown that vitamin E, administered intraperitoneally, is as effective as carboxymethylcellulose membrane in preventing postoperative adhesions. By contrast, the same effect has not been achieved after intramuscular administration[87]. A significant difference has been found between intraperitoneal and intramuscular vitamin E administration[87]. Thus, intraperitoneal administration of vitamin E might be recommended to prevent adhesion formation. However, according to our literature review, there have been no human studies that have recommended the use of vitamin E for postoperative adhesion prevention.

One study has been carried out to elucidate the effects of different concentrations of methylene blue on the process of peritoneal adhesion formation and to define its minimum dose that can effectively prevent the formation of such adhesions in a rat model[88]. It could be concluded that 1% methylene blue had the best anti-adhesion potential[88]. If methylene blue prevents peritoneal adhesions, it can cause significant impairment of anastomotic bursting pressure during the early phase of the wound healing process by its transient inhibitory effect on the nitric oxide pathway[89].

Adhesions are a result of the inflammatory response to tissue injury in the peritoneal space. Although the mechanism is unclear, local anesthetics are reported to have some anti-inflammatory effects, as shown in some animal studies[90]. These anti-inflammatory effects are related to the inhibition of neutrophils. It has also been shown that local anesthetics activate the fibrinolytic system, reduce factor Ⅷ, plasminogen and α2-antiplasmin concentration, and inhibit platelet aggregation[91,92]. Thus, besides the accelerative effect of a mixture of 2.5% lidocaine and 2.5% prilocaine in the wound healing process, some studies have demonstrated that intraperitoneal lidocaine and prilocaine inhibit the formation of postoperative peritoneal adhesions without compromising wound healing in a bacterial peritonitis rat model[93].

Hepatocyte growth factor (HGF) can inhibit collagen deposition and has fibrinolytic capacity[94,95]. Liu *et al*[96] have demonstrated that local application of recombinant adenovirus carrying the *HGF* gene reduced adhesion formation in a rat model. Other studies have investigated the use of gene therapy to manage postoperative adhesions. Smad7, a protein that occupies a strategic position in fibrinogenesis, inhibits transforming growth factor-β and has the potential to attenuate postoperative adhesion. Guo *et al*[97] have investigated in an experimental model the therapeutic potential of exogenous Smad7 to prevent fibrinogenesis in postoperative intra-abdominal adhesion. In this rat model, ultrasound-microbubble-mediated Smad7 transfection significantly decreased the incidence and severity of peritoneal adhesions, but the use of targeted gene therapy as a preventive agent against ASBO still needs extensive evaluation before any clinical trial.

CONCLUSION

Postoperative peritoneal adhesions are a major health problem with a significant economic impact. Fibrinolysis seems to be a key factor in determining the pathogenesis of adhesion formation and in its prevention. Several studies on this problem have been conducted. Their results are encouraging, but most of them are contradictory and have been conducted in animal models. Until additional findings from future clinical studies, only meticulous surgery can be recommended to reduce unnecessary morbidity and mortality rates from these untoward effects of surgery. In the current state of knowledge, preclinical or clinical studies are still necessary to evaluate the effectiveness of the several proposed prevention strategies for postoperative peritoneal adhesions.

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REVIEW

Endoscopic management of chronic radiation proctitis

Tarun Rustagi, Hiroshi Mashimo

Tarun Rustagi, Department of Internal Medicine, University of Connecticut, Farmington, CT 06030, United States
Hiroshi Mashimo, Gastroenterology Section 111, VA Boston Healthcare System, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02130, United States
Author contributions: Rustagi T and Mashimo H contributed equally to this work and wrote the paper with final editing and approval.
Correspondence to: Hiroshi Mashimo, MD, PhD, Gastroenterology Section 111, VA Boston Healthcare System, Brigham and Women's Hospital, Harvard Medical School, 150 South Huntington Ave., Boston, MA 02130, United States. hmashimo@hms.harvard.edu
Telephone: +1-857-2035640 Fax: +1-857-2035666
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Abstract

Chronic radiation proctopathy occurs in 5%-20% of patients following pelvic radiotherapy. Although many cases resolve spontaneously, some lead to chronic symptoms including diarrhea, tenesmus, urgency and persistent rectal bleeding with iron deficiency anemia requiring blood transfusions. Treatments for chronic radiation proctitis remain unsatisfactory and the basis of evidence for various therapies is generally insufficient. There are very few controlled or prospective trials, and comparisons between therapies are limited because of different evaluation methods. Medical treatments, including formalin, topical sucralfate, 5-amino salicylic acid enemas, and short chain fatty acids have been used with limited success. Surgical management is associated with high morbidity and mortality. Endoscopic therapy using modalities such as the heater probe, neodymium:yttrium-aluminium-garnet laser, potassium titanyl phosphate laser and bipolar electrocoagulation has been reported to be of some benefit, but with frequent complications. Argon plasma coagulation is touted to be the preferred endoscopic therapy due to its efficacy and safety profile. Newer methods of endoscopic ablation such as radiofrequency ablation and cryotherapy have been recently described which may afford broader areas of treatment per application, with lower rate of complications. This review will focus on endoscopic ablation therapies, including such newer modalities, for chronic radiation proctitis.

Key words: Chronic; Radiation proctitis; Endoscopic; Argon plasma coagulation; Radiofrequency; Cryoablation

Peer reviewers: Dr. Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria; Dr. Andrew McCulloch Veitch, BSc, MD, Consultant Gastroenterologist, New Cross Hospital, Wolverhampton, WV10 0QP, United Kingdom; Grigoriy E Gurvits, MD, Department of Gastroenterology, St. Vincent's Hospital and Medical Center, New York Medical College, 153 West 11th Street, Smith 2, New York, NY 10011, United States

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INTRODUCTION

Chronic radiation proctopathy (CRP) is a troublesome complication occurring in 5%-20% of patients following pelvic radiotherapy for carcinoma of the prostate, rectum, urinary bladder, cervix, uterus and testes[1-6]. Radiation-induced mucosal damage results in endothelial dysfunction, microvascular injury with intimal fibrosis, and fibrin thrombi of small arteries and arterioles leading to ischemia, fibrosis and the development of neovascular lesions[1,2]. CRP resolves spontaneously in many cases, but in some can lead to persistent rectal bleeding and iron deficiency anemia requiring blood transfusion[3]. Other symptoms of CRP include diarrhea, mucoid discharge, urgency, tenesmus, rectal pain and fecal incontinence. These symptoms interfere with daily activities and have an adverse effect on quality of life[4]. Treatment for CRP remains unsatisfactory. Medical measures, including formalin application[5], topical sucralfate[6], 5-amino salicylic acid enemas[7], short chain fatty acids[8] and antioxidants

such as vitamin E[9] and pentoxifylline[10] have been used with limited success. Surgical management is associated with high morbidity and mortality[11].

The basis of evidence for the therapy of CRP is generally insufficient. There are very few high-quality trials and comparisons between therapies are limited because of different evaluation methods. Most data are from case series of a single treatment from a single center. Therefore, a degree of pragmatism needs to be shown on the basis of these data and local availability of therapy. Sucralfate enemas seem to be the best available "medical" therapy and are safe and well tolerated. Additional use of oral metronidazole may enhance this effect[12]. Steroid enemas may have some effect, but are less well tolerated and probably have lower efficacy. Formalin therapy is effective in up to 48% of patients with CRP[13,14]. However, high rates of complications have been reported including rectal pain, incontinence, diarrhea, formalin-induced colitis, anal and rectal strictures, rectal ulcerations, and rectal perforation[13,14]. Also, the technique of application, concentration of formalin and the success rates reported in different studies highly vary[15]. The duration of effect based largely on anecdotal reports remains unclear, but appears to be around 3 mo[15]. Another described therapeutic modality is hyperbaric oxygen therapy, which is purported to have an angiogenic effect and stimulate collagen formation and re-epithelialization[16]. Aural barotrauma is the most common side effect reported, although it appears to be largely transient and minor[15,17]. The equipment needed is expensive and not readily available. Thus, at the present time, it is not a practical means of treating CRP outside of specialized centers and is usually reserved for cases refractory to more readily available forms of therapy.

The goal of endoscopic treatments of CRP is to achieve control of bleeding. Attaining this goal improves the patient's quality of life by reducing the need for iron replacement, blood transfusion and hospital admissions, resolving symptoms of anemia, and symptoms of hematochezia. Endoscopic therapy using modalities such as the heater probe[18], neodymium:yttrium-aluminium-garnet (Nd:YAG) laser[19,20], potassium titanyl phosphate (KTP) laser[21] and bipolar electrocoagulation[22] has been reported to be of some benefit, but at the expense of a high level of complications[23]. Of ablative therapies, thermal methods seem to be effective and safe. Simple heater probe treatment or argon plasma coagulation (APC) is the preferred method for their better safety profile. Intra-rectal formalin seems to be effective, but possibly has a higher rate of complications[14]. Newer methods of endoscopic ablation such as radiofrequency ablation and cryotherapy have been recently described which may afford broader areas of treatment per application. This review shall focus on endoscopic ablation therapies used in management of CRP.

CONTACT PROBE THERAPY: HEATER AND BIPOLAR PROBE

The heater probe has a teflon-coated heating element at its tip that delivers standardized energy over set times. Bipolar electrocautery probe has a pair of electrodes at its tip through which current is passed using the tissue for conduction[24]. Both devices are contact probes, making them useful for directed therapy in the setting of active bleeding. The disadvantage is char formation on the tip of the probe, leading to decreased treatment efficiency and requiring repeated cleaning. Fuentes *et al*[25] treated 8 patients with the heater probe for rectal bleeding, which required one to four treatment sessions for complete cessation or significant reduction in bleeding. In a randomized prospective trial by Jensen *et al*[18], a total of 21 patients were treated either by a heater probe (n = 9) or a bipolar electrocoagulation probe (n = 12). A mean of four sessions were required for either probe. In the 12 mo of endoscopic treatment *vs* 12 mo medical therapy, the severe bleeding episodes diminished significantly for the bipolar probe (75% *vs* 33%) and heater probe (67% *vs* 11%). No side effects were reported in any of the studies using these modalities (Table 1).

LASER THERAPY

Nd:YAG

Nd:YAG laser was one of the first endoscopic laser modalities used in the treatment of CRP. Leuchter *et al*[26] reported successful treatment of rectal hemorrhage in a patient after four applications. The laser uses a 1.06 nm wavelength and penetrates to a depth of up to 5 mm[27]. Nd:YAG laser has a low affinity for hemoglobin and H_2O but is well absorbed by tissue protein, thus making it ideal for deeper vessel coagulation[28]. Initially, a setting of 40 W and pulse duration of 1/2 s maximum is used with the tip at approximately less than 1 cm from the mucosal surface. The desired effect in treating telangiectasias is attained with the formation of white coagulum. The study by Barbatzos *et al*[20] involved nine patients who underwent a mean of three treatments. There were no complications, and bleeding was decreased to occasional spotting. Ventrucci *et al*[29] also reported successful treatment in nine patients. The median number of treatments required per patient was three to achieve cessation of bleeding in four patients and occasional spotting in four others. One patient still required transfusions at completion of the study. Transmural necrosis, fibrosis, stricture formation and recto-vaginal fistula are some of the complications reported with use of Nd:YAG. Nd:YAG use for CRP has declined because of its cost, the need to aim directly at telangiectasias, and the possibility of severe endoscopic damage if the laser strikes the endoscope in retroflexion (Table 2).

Potassium titanyl phosphate

The KTP laser uses the beam from the Nd:YAG laser that is passed through a KTP crystal, reducing the wavelength by half (532 nm)[30]. At this wavelength, the energy is absorbed by hemoglobin and the depth of penetration is more shallow (1-2 mm) compared to Nd:YAG. This affinity for hemoglobin permits selective coagulation, thus making it quite useful in the treatment of superficial vascular lesions. The use of KTP for CRP has been limited. Taylor *et al*[21] treated 26 patients with bleeding secondary

Table 1 Literature on contact probe therapy use in chronic radiation proctopathy

Authors	Modality	*n*	No. of treatment	Power settings	Response rate	Duration of study	Side effects
Jensen *et al*[18], 1997	Heater probe	12	4 (mean)	10-15 W, 1 s pulses	12/12 (100%)	24/12	None
Fuentes *et al*[25], 1993	Heater probe	8	1-4	20 J/pulse	8/8 (100%)	N/A	None
Jensen *et al*[18], 1997	Bipolar	9	4 (mean)	10-15 J	9/9 (100%)	24/12	None
Haulk *et al*[69], 1996	Bipolar	8		2-5 W or 11-25 W	8/8 (100%)	4/12	None
Mannoury *et al*[22],1991	Bipolar	4		Setting 5, 2 s pulses	4/4 (100%)	9/12	None

N/A: Not available.

Table 2 Literature on neodymium:yttrium-aluminium-garnet laser therapy use in chronic radiation proctopathy

Author	*n*	Power settings	Mean no. of sessions	Response rate	Duration (mo)	Side effects
Ventrucci *et al*[29], 2001	9		3	4/9 (44% CR), 4/9 (44% PR)	N/A	None
Taylor *et al*[21], 2000	23	4-10 W		15/23 (65%)	6	2 rectal ulcers
Barbatzos *et al*[20], 1996	9	20-30 W	3	6/9 (66% PR)	24	None
Chapuis *et al*[70], 1996	34	40 W		30/34 (88%)	6-64	4 mucous discharge, 1 acute prostatitis, 1 rectal stricture
Lucarotti *et al*[11], 1991	5	80 W		5/5 (100%)	18	NA
Jacobs[71], 1989	2	NA		2/2 (100%)	12	NA
Alexander *et al*[72], 1988	8	80-90 W		6/8 (75%)	21	3 ileus, 1 abdominal pain
Alqhuist *et al*[73], 1986	4	30-40 W		2/4 (50% CR)	12	1 tenesmus
Leuchter *et al*[26], 1982	1	60 W	4	1/1 (100% CR)	24	None

CR: Complete remission; PR: Partial remission; NA: Not available.

to CRP using 4-10 W and a median of two sessions. They reported a symptomatic improvement in 65% patients, while there was no change in seven (30%) and there was an increase in hematochezia in one (5%). No perforations or fistula formation were reported in the study.

Argon laser

The argon laser is functionally similar to KTP with similar wavelength, resulting in tissue heat penetration of 1-2 mm depth, and is also useful in superficial blood vessel photocoagulation. O'Conner[31] treated five patients using the argon laser at 1.5 W and reported cessation of bleeding after two to four treatment sessions with no complications. Buchi and Dixon[32] treated three patients successfully, with only one patient reporting cramps. Similarly, Taylor *et al*[33] reported control of bleeding achieved after a median of three sessions in 14 patients. Power was set between 3.5 and 8 W with a flow rate of 1.5-2.5 mL/s and no complication was encountered.

ARGON PLASMA COAGULATION

Laser therapy for hemorrhagic CRP was largely supplanted by argon plasma coagulation (APC), which is less expensive, easier, safer and more widely available. This involves the application of bipolar diathermy current using inert argon gas as a conducting medium, delivered *via* a through-the-scope catheter. Unlike traditional bipolar devices, the current jumps from the probe to the target lesion, with the arc being broken once the tissue is desiccated. The theoretical advantage is a uniform, more predictable and limited depth of coagulation (0.5-3 mm)[34], to minimize the risks of perforation, stenosis and fistulization. APC can be applied axially and radially, allowing tangential coagulation of lesions around rectal bends[35-37]. Also, the APC generator is mobile and can be used quickly and at any place or time[35-37]. Given all these benefits, APC has rapidly become the preferred, first-line endoscopic therapy for hemorrhagic CRP (Table 3).

Most studies on the use of APC in the management of CRP have demonstrated benefit (Table 3). APC ameliorates rectal bleeding associated with mild to moderate hemorrhagic CRP in 80%-90% of cases, and improves symptoms of diarrhea, urgency and tenesmus in 60%-75% of cases[38-41]. Ten studies also reported an increase in the mean hemoglobin levels after APC in almost all patients after the treatment, suggesting the effective control of rectal bleeding. Cumulative average increase in mean hemoglobin levels is around 2.26 gm% (range, 1.1-3.8 gm%). Relief of blood transfusion dependency has also been reported in almost all patients treated with APC (57 of 60 patients, 95%) in one series (Table 3).

However, APC has inherent limitations especially in very severe, extensive CRP, e.g., with greater than half of the rectal surface area involved or with fresh surface bleeding[38,41,42]. More diffuse lesions usually require repeated applications per session and multiple treatment sessions (ranging from one to five sessions). A few studies report up to 8 sessions needed to achieve complete resolution of symptoms, endoscopic disappearance of all telangiectasias, and complete cessation of bleeding[41,43]. The mean number of sessions per patient reported varies from 1 to 3.6 with a calculated overall cumulative mean of 2.13 sessions per patient (calculated median: 2) (Table 3). Mean interval between sessions usually ranges from 4 to 8 wk. Follow-up ranges from 1 to 48 mo with a mean of 3-31 mo across different studies (calculated overall mean: 15 mo). Recurrent proctopathy has been reported

Table 3 Literature on argon plasma coagulation therapy use in chronic radiation proctopathy

Study Ref.	*n*	Mean age (yr) (range)	Settings -flow rate- power	Mean No. of sessions per patient	Response rate	Improvement in anemia (% patients), mean increase in Hgb (gm%)	Relief of transfusion dependency	Follow-up duration mean (mo)	Complications/Side effects	% requiring transfusion
Swan *et al*[49], 2010	50	72.1 (51-87)	1.4-2 L/min, 50 W	1.36 (1-3)	96%	1.9 gm% mean increase		20.6 (6-48)	Short-term: 17 (34%) patients (proctalgia in 13, rectal mucous discharge in 4, incontinence in 1, fever in 1, and bleeding in 1); long-term: 1 (2%) asymptomatic rectal stricture	
Karamanolis *et al*[46], 2009	56	68.4 (45–86)	2.0 L/min, 40W	2 (1-8)	Mild (100%), severe (79%), total (89%)	N/A	7/9	17.9 (6-33)	one case of colonic explosion without perforation; No strictures or persistent ulcers; 2 on anticoagulation with recurrence	9/56 (16%)
Tormo *et al*[74], 2009	22	74.3	2 L/min, 50 W	2.58 (1-7), median-2	100%	N/A	N/A	N/A	None	2/22 (9%)
Alfadhli *et al*[14], 2008	14	74.7	1.2-2 L/min, 45-50 W	1.78	78.5%	2 gm% mean increase	N/A	3	2/14 (33.3%) mild	N/A
Latorre *et al*[75], 2008	38	70.9		3.6 ± 2.7		2.7 gm% mean increase		28		
Dees *et al*[76],2006	48		2 L/min, 50 W	Median-3	98%				Two patients-recurrent blood loss on anticoagulation; 1-ulcer	6/48 (12.5%)
Ben-Soussan *et al*[53], 2004	27	73.1 (53-86)	0.8-1.0 L/min, 40-50 W	2.66 (1-7)	92%			13.6 (3-31)	Side effects-anal/rectal pain (n = 3), vagal symptoms (n = 2), 3 colonic explosions-1 with perforation requiring surgery, no stricture	8/27 (30%)
Higuera *et al*[77], 2004	10		1.5-2.0 L/min, 60 W	1.9 (1-4), median (2)	100%	1.5-1.9 gm% mean increase	1/1 (100%)	31.1 (10-45)	No ulcers/strictures, 1 (10%)-tenesmus	1/10 (10%)
Sebastian *et al*[39], 2004	25	69 (53–77)	1.5 L/min, 30 W (25-40 W)	median-1	21/25 (76%, 81% or 84%)	2.4 gm% mean increase		Median 14-	1-rectal pain	
Urban *et al*[78], 2004	8			1-4	100%					
Ravizza *et al*[51], 2003	27	72 (62-83)	3 L/min + 60 W (n = 17) reduced to 2 L/min and 40 W (n = 10)	2 (1-5)	85% marked improvement, 10/27 only had minor bleeding, 48% complete resolution	3.2 g/dL mean increase	6/6 (100%) transfusion relief	11.5 (1-24)	Short term-2/27 (7%), 1-transient anal/rectal pain, 1-fever; long-14/27 (52%)-asymptomatic rectal ulcers	6/27
Gheorghe *et al*[58], 2003	42		60 W (23), 50 W (19)	1.34, 1.9						
Canard *et al*[48], 2003	30	70.7 (58-85)	0.8-2 L/min, 30-80 W	2.3 (1-5)	(87%)			20 (3-35)	Overall-47%; 3 severe (10%): 1 severe bleeding, 1 extensive necrosis of lower part of rectum, 1 perforation. 3 microrectitis and 2 asymptomatic rectal stenosis. Post-Rx pain in 6 patients (20%)	17%
Venkatesh *et al*[79], 2002	40	64-83	1-1.5 L/min, 40-60 W	Mean-1.35 median-1 (1-2)	97.5%	- 97.5% patients	20/21 (95.2%)	NR 3-30	1-urinary retention, 2-fever requiring antibiotics	21/40 (52.5%)
Taieb *et al*[80], 2001	11	73 (54-86)	0.8-2 L/min, 50W	3.2 (1-5)	82% CR, 18% PR	3.8 gm% mean increase	7/7 (100%)	19 (7-30)		7/11 (63.6%)
Tjandra *et al*[41], 2001	12		1.5L/min, 40 W	2 (1-3)	50% CR, 50% PR, 83% Signi	1.1 gm% mean increase	4/4 (100%)	11 (4-17)	None	4/12 (33%)
Smith *et al*[81], 2001	7		1.6 L/min, 40-45 W	1-3	71% CR, 29% PR			4-13	None	
Rolachon *et al*[82], 2000	12	70.3	1.0 L/min, 50 W	Mean (2.8 ± 0.8)	66% CR, 83% PR	1.8 gm% mean increase		6	3/12 (25%), 2-chronic rectal ulcerations, 1-asymptomatic rectal stenosis	
Kaassis *et al*[44], 2000	16	73.5 (62-80)	0.6 L/min, 40 W	Mean-3.7 (2-8)	44% CR, 56% PR		3/3 (100%)	10.7 (8-28)	No	3/16 (18.75%)
Tam *et al*[40], 2000	15		2 L/min, 60 W	Median-2 (1-4)	100%	2.5 gm% mean increase	3/3 (100%)	Median-24 (8-35)	2-asymptomatic rectal strictures requiring dilation	3/15 (20%)
Silva *et al*[45], 1999	28	65 (42-77)	1.5 L/min, 50 W	2.9 (1-8)	93%	1.2 gm% mean increase	-	10 (1-15)	No, 3-transient anal pain	15/28 (53%)
Fantin *et al*[62], 1999	7		3 L/min, 60 W	2 (2-4)	100%			Median 24 (18-24)	No	
Chutkan *et al*[83], 1997	12			1	92%			6.6	No	3/12 (25%)
Villavicencio *et al*[50], 2002	21	Median 72.6 (58-86)	1.2-2.0 L/min, 45-50 W	1.7 median (1-4)	95%	100% patients	4/4 (100%)	10.5 median (1-29)	4-rectal pain, tenesmus, diarrhea	4/21 (19%)
Rotondano *et al*[84], 2003	24		0.8-1.2 L/min, 40 W	Median 2.5	100%				1-RV Fistula	
Zinicola *et al*[42], 2003	14		2 L/min, 65 W	2 (1-4)	86%%		3/3 (100%)	19 (5-41)	1-asymptomatic recto-sigmoid stenosis	3/14 (21%)

to respond to additional rounds of APC therapy[44,45].

Patients on anticoagulants or aspirin demonstrate higher recurrence[46]. Kaassis *et al*[44] found that patients who were receiving anticoagulation therapy may require more APC sessions, but can achieve an equivalent clinical response as those who are not on anticoagulation. Rectosigmoid lesions are also more difficult to treat due to the tortuosity that often accompanies radiation injury in this region. When rectal lesions are very distant from the anus, application of APC with a rigid probe through an operating sigmoidoscope may be easier than through a flexible endoscope. Lesions located immediately above the dentate line in the upper part of the anal canal are also difficult to treat. These may require retroflexion of the scope with higher risk of rectal scarring, limited mobility of the endoscope, and greater patient discomfort. One technique described by Coriat *et al*[47], using a transparent cap attached to the tip of the colonoscope, allowed better visualization of low rectal lesions and of the upper part of the anal canal without retroflexion and proper distance for effective and safe APC delivery. Notwithstanding, APC may be avoided in the presence of radiation-induced rectal strictures and fistulae, which may worsen as the treated area heals.

Overall, the reported complication rate with APC has been variable (Table 3). Canard *et al*[48] reported an overall morbidity of 47%: post-treatment pain in 20% and severe complications in 3 (10%), including a patient with severe bleeding, extensive necrosis of lower part of the rectum, and perforation. Alfadhli *et al*[14] and Swan *et al*[49] reported complications in 30%-35%. On the other hand, the experiences of Villavincencio *et al*[50] were better, with a 19% incidence of both short-term (such as tenesmus, anismus) and long-term (including diarrhea, rectal pain) complications. The commonest procedure-related complication reported is anal or rectal pain with or without tenesmus, which is most likely to occur following treatment near the dentate line[50,51], and usually resolves spontaneously within few days or with standard analgesics[45,48,50,51]. Abdominal bloating and cramping, and vagal symptoms related to colonic distension have also been reported. One potential drawback of using APC is the possibility of excessive luminal distention from the rapid instillation of argon gas that occurs during treatment. It is recommended that, when possible, a two-channel endoscope should be used so that the insufflated argon gas can be removed periodically during the procedure[52]. Several authors have reported colonic explosion [1 of 56 (1.8%)[46] to 3 of 27 (11.1%)[53]] with or without perforation (Table 3) when the bowel has not been formally cleansed, and adequate colonic lavage is therefore a mandatory requirement[38,46,53,54]. Rare complications reported include arteriovenous fistula, urinary retention and necrosis of lower part of the rectum. Although life-threatening gas embolism has been reported during bronchoscopic application of APC, no such complication has been reported during gastrointestinal endoscopic application[55].

Rectal ulcers are common following APC treatment. Severe ulceration may result in "painting" of the rectal wall. Therefore, brief pulse treatment of targeted lesions is recommended[50]. Ravizza *et al*[51] reported asymptomatic rectal ulcers in 14 (52%) of 27 patients, a frequency that is relatively high in comparison with the reported overall frequency of about 3%-16% (Table 3) in other series, despite similar gas flow rate and power settings compared to the other studies. Furthermore, this data may underestimate the true frequency of rectal ulcer, as 41% of the patients in this study did not undergo endoscopy after the last APC session. However, no strictures were observed after ulcer healing[51]. Rectal ulcers developing during APC can be considered a consequence of thermal injury to already damaged and vascularly compromised tissue that is thus more fragile and has poorer healing[56]. Incidence of ulcers may be affected by the flow rate of the argon gas and power settings, the method of application, the interval between sessions, and the number of sessions subsequent to ulcer development which may delay ulcer healing due to repeated thermal injury[51]. The fact that rectal ulcers are not clinically troublesome means they should not be considered an absolute contraindication to APC, nor do they necessarily require any additional endoscopic follow-up[51].

Compared to ulcers, the occurrence of strictures is less common. The frequency of this complication varies among different studies, many studies describing no occurrence of rectal strictures while few studies reporting such complication in 2%[49]-13.3%[40] (Table 3). A review of literature by Ravizza *et al*[51] reported 9 cases of asymptomatic rectal strictures in 207 treated patients, with an overall frequency of 4.3%. However, given the fact that most of the rectal strictures are asymptomatic, their true incidence is difficult to estimate and theoretically would be higher than reported by several studies.

The studies involving APC are not uniform in method. The power settings range from 30 to 60 W (median 40-50 W), with an argon flow rate from 0.8 to 2 L/min (median 1.5-2 L/min) (Table 3). Lower power settings have been subscribed for lower complication rate and decreased number of treatment sessions required for complete coagulation, with almost all complications occurring at power settings above 45 W[48]. Duration of burn and power settings have also been correlated with depth of injury to the muscularis propria in swine colon[57]. Thus lower power settings appear to cause less injury while coagulating just as well as at higher settings. Unfortunately, most of the studies do not report the success of individual settings. Only few studies have compared APC at different settings. One small study of 42 patients compared 50 and 60 W therapies, but reported no statistical difference between the two[58]. Ravizza *et al*[51] found a higher rate of rectal ulceration with higher settings; 59% with flow of 3 L/min and a power of 60 W compared to 40% with a 2 L/min flow and a power of 40 W, albeit without statistical significance ($P = 0.4$) in the limited study.

No prospective comparative trials of the APC with other endoscopically directed treatment modalities exist, nor is there any experience on the role of adjuvant medical therapy such as the use of steroids, sucralfate or 5-aminosalicylic acid enemas between APC sessions. Most importantly, there are no control or crossover studies.

However, in many of the studies involving APC, most of the patients had unsuccessful results with medical therapy before undergoing APC. For example, in the study by Ravizza *et al*[51], 17 of their 27 patients had been treated unsuccessfully with corticosteroid or salicylate enemas. Tjandra *et al*[41,43] also found APC to be effective in 11 patients with CRP refractory to formalin therapy. Similarly in the study by Villavicencio *et al*[50], 12 of their 21 patients had been treated unsuccessfully with various pharmacologic agents including oral and rectal mesalamine, and rectal corticosteroids. Other forms of endoscopic treatment (laser photocoagulation, multicolor coagulation) had been performed in 5 of their patients, all failed in achieving control of bleeding[50]. In a study by Zinicola *et al*[42], 6 (42.8%) patients had previously failed treatment with steroid enemas or 5-aminosalicylic acid enemas. In a recent study by Swan *et al*[49], 16 patients who failed in previous treatments for CRP all responded to endoscopic APC therapy. Alfadhli *et al*[14] retrospectively compared the APC with topical formalin, and found APC to be more effective (79% *vs* 27% responders) and safer (14.3% *vs* 81.8% adverse effects) than topical formalin in controlling hematochezia. The rate of single-session APC responders (63.6%) was almost double that of the formalin-treated group (33.3%)[14].

RADIOFREQUENCY ABLATION

Radiofrequency ablation (RFA) with the BARRx Halo90 system has achieved superficial and broad fields of ablation in the esophagus[59,60] suggesting that similar benefits could be achieved in the colon and rectum. Zhou *et al*[61] have reported successful use of RFA with the BARRx Halo90 system in treating three patients with lower gastrointestinal bleeding from CRP, including two who failed in conventional therapy. In all cases, the procedure was well tolerated and hemostasis was effectively achieved after 1 or 2 RFA sessions. Re-epithelialization by neosquamous mucosa was observed over areas of prior hemorrhage above the prior dentate line. No stricturing or ulceration was seen on follow-up up to 19 mo after RFA treatment. In this report, real-time *in vivo* endoscopic optical coherence tomography (EOCT) was also used to assess the treatment efficacy. EOCT could visualize epithelialization and subsurface tissue microvasculature before and after treatment, demonstrating its potential for follow-up assessment of endoscopic therapies and directing areas for retreatment, without the need for excisional biopsy. This is particularly important for patients with radiation proctitis since biopsy is relatively contraindicated due to the high risk of rebleeding.

Several benefits of RFA have been found compared with other endoscopic treatments for radiation proctitis. These include squamous re-epithelialization seen after RFA with prevention of rebleeding and the relative lack of stricturing and ulceration that is seen often after other thermal ablative procedures. The tightly spaced bipolar array of the RFA catheter limits the radiofrequency energy penetration, restricting the RFA treatment to the superficial mucosa, thereby avoiding deep tissue injury in relatively ischemic mucosa and resulting in post-treatment ulceration and structuring, as commonly noted following conventional endoscopic therapies. Finally, RFA allows much broader areas of tissue to be treated simultaneously compared to the point-by-point approach required with heater or bipolar probes[18,22], or APC[44,62]. As with APC, the unit is mobile and can be used in different rooms of an endoscopic suite. The BARRx unit also delivers a consistent amount of energy to the surface using well-defined and reproducible ramp-up of energy. This minimizes the possibility of operator-dependence and over-treatment that may lead to perforations or ulcerations.

Nikfarjam *et al*[63] recently reported another case with extensive CRP that had continued bleeding despite APC. The HALO90 radiofrequency system was used for treating regions of proctitis at an energy density of 12 J/cm^2. At monthly intervals, over 3 mo, RFA was performed with a mean of 7 regions ablated at a time. The mean treatment time was 29 min. There was no significant bleeding after the first treatment session. The patient was symptom free at 6 mo follow-up with minimal evidence of residual mucosal abnormalities

CRYOABLATION

Cryoablation, similar to APC, is a noncontact method of therapeutic tissue destruction *via* application of extreme cold temperatures to a targeted area. Cryoablation has the benefit of uniform treatment of larger surface areas and ease of targeted application. Cryoablation works through immediate and delayed effects. Delayed effects are related to induction of ischemic necrosis.

Kantsevoy *et al*[64] reported the successful use of experimental endoscopic cryotherapy in patients with radiation proctitis, as a part of a pilot study that was conducted to evaluate the safety and efficacy of endoscopic cryotherapy for bleeding mucosal vascular lesions. They used a Prototype Ⅱ device to spray nitrous oxide through the accessory channel of an upper endoscope[65]. Complete cessation of bleeding was achieved in all 7 (100%) patients who underwent cryoablation therapy for radiation proctitis. A major advantage of the cryotherapy technique identified was the ability to treat large areas of mucosa relatively quickly. The only adverse effect reported was transient abdominal pain with spontaneous resolution in one out of a total of 26 patients treated for various gastrointestinal mucosal bleeding lesions.

Shaib *et al*[66] reported the first case of mucosal healing and symptomatic resolution of radiation proctitis using low-pressure cryoablation (CryoSpray, CSA Medical) in a patient who previously did not respond to medical therapy with steroid suppositories. Cryoablation was performed using a liquid nitrogen spray injected through the cryoablation catheter passed through an endoscopic channel. A total of four 10-s applications were used for each area of proctitis. During cryoablation, a decompression tube was placed in the rectum to prevent over-insufflation. No adverse effects after cryoablation were seen. Hemoglobin was reported to increase from 9.4 g/dL to 11.7 g/dL over the 15-wk follow-up period with

sigmoidoscopic resolution.

Battish *et al*[67] also reported similar results in small case series of 2 patients with established radiation proctitis who underwent cryoablation using liquid nitrogen (CryoSpray). Each patient underwent 4 applications of 10 s each with complete resolution of mucosal bleeding and telangiectasias on follow-up endoscopy. The only post-procedure adverse effect reported was transient abdominal distention in one patient.

Most recently, Hou *et al*[68] reported a prospective case series of 10 patients with hemorrhagic CRP with a mean follow-up of 3.3 mo. All patients underwent a single endoscopic session of cryotherapy, consisting of three 5-s applications per involved area of mucosa, performed with a 9F cryoablation catheter (formerly CryMed, now CSA Medical)[68]. Endoscopic improvement was reported in 70% of patients, with an overall 37% decrease in rectal telangiectasia density from a mean of 2.7 to 1.7 ($P = 0.02$). Symptomatic improvement was observed in 80% of patients with an overall 51% reduction in Radiation Proctitis Severity Assessment Scale score from a mean of 27.7 to 13.6 ($P = 0.009$)[68]. Severe complication included one (10%) patient with cecal perforation secondary to over-insufflation likely caused by a failure of the decompression tube. Subsequently, the protocol was adapted to reduce treatment duration and perform full colonoscopy after treatment for colonic decompression. One case (10%) of rectal ulcer was also reported[68].

Reports using cryoablation for CRP remain experimental and anecdotal. These early case reports support the use of cryoablation therapy in management of CRP. However, there has been no prospective study comparing cryoablation with other treatment modalities such as APC, with regards to efficacy, side effects and durability of results. Larger studies or case series are required to confirm the utility or superiority of cryoablation.

The current commercially available cryotherapy apparatus is less mobile and somewhat more cumbersome than most APC and the BARRX units, and requires maintaining a supply of liquid nitrogen which lasts approximately 2 wk in the current holding tank. Thus treatments for incidental findings, particularly in a lower volume endoscopy unit, may be more difficult. In our view, a major advantage of cryotherapy over the other heat-generating ablative methods is that colonic lavage to minimize the possibility of gas ignition is not necessary. However, drawing from the animal studies, the depth of tissue destruction may be deeper by CSA cryotherapy than that achieved by BARRx radiofrequency ablation, and it is unclear whether this could lead to greater strictures, abscess and fistulas, or whether cryotherapy is inherently less prone to such complications. Moreover, the rapidly expanding gas would require adequate venting which may be more difficult for lesions higher in the sigmoid colon.

CONCLUSION

Endoscopic therapies have become the treatment of choice in patients with troublesome bleeding due to CRP, and may be used in conjunction with medical therapies. The ability to safely treat these patients in an outpatient setting is extremely attractive. Endoscopic therapy has proven successful in stopping bleeding from CRP, in addition to providing symptomatic relief by reducing urgency, tenesmus, and the frequency of hematochezia and transfusion requirements. Initially, endoscopists had used the heater and bipolar probes[9,10], then the neodymium/yttrium aluminum garnet[11,12] and potassium titanyl phosphate lasers[13,14], which were each effective. Formalin administration through a rigid scope also proved effective[15,16]. The use of APC by endoscopy has become an attractive treatment option, because it is a noncontact approach that is efficient, effective, relatively safe and well tolerated.

While focal ablative tools such as lasers, contact probes and APC may be helpful when bleeding occurs from limited number of identifiable ectatic vessels, a larger field of arteriovenous malformations (AVMs) or oozing may be more difficult to control. Moreover, poor healing and subsequent ulcerations can exacerbate bleeding in this CRP field, which is vascularly compromised. Therefore methods allowing for broader field of treatment such as formalin instillation, or the newer methods of RFA and cryotherapy may be theoretically advantageous in this setting. In particular, the unexpected finding of neosquamous epithelialization with RFA may have further advantages in preventing rebleed.

Future comparison of these treatment modalities would be enhanced using the uniquely-suited EOCT as an imaging tool, since this allows broad areas of scan with subsurface near-microscopic visualization for vessel features and density.

Present evidence for endoscopic therapy of CRP remains largely anecdotal, and future studies to demonstrate efficacy need to adopt a standard scoring system for CRP. Denton *et al*[16] suggested possible scoring systems and outcome measures (including quality-of-life scores) that seem sensible in this disease. Adoption of such scoring system may allow better comparison of different studies and different modes of treatment. Moreover, bleeding from CRP often resolves spontaneously, and there needs to be larger randomized controlled studies for the treatment of CRP. Given such limitations and differences in availability of equipment and expertise, it is difficult to recommend a truly evidence-based algorithm for management of CRP. However, we recommend a trial of medical therapy such as sucralfate enemas with oral metronidazole for mild cases. Severe cases, particularly hemorrhagic CRP and those refractory to medical treatment, should be promptly offered endoscopic therapy. Currently, APC is the preferred first-line endoscopic modality given the vast experience and availability. Refractory cases should be referred to centers for hyperbaric oxygen therapy or centers performing newer endoscopic therapies such as radiofrequency and cryoablation, which may become the standard of care in the future particularly for more extensive lesions.

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ORIGINAL ARTICLE

Elevated serum alpha fetoprotein levels promote pathological progression of hepatocellular carcinoma

Peng Li, Shan-Shan Wang, Hui Liu, Ning Li, Michael A McNutt, Gang Li, Hui-Guo Ding

Peng Li, Ning Li, Hui-Guo Ding, Department of Hepatology and Gastroenterology, Beijing You'an Hospital, Beijing 100069, China
Shan-Shan Wang, Gang Li, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, China
Hui Liu, Department of Pathology, Beijing You'an Hospital, Beijing 100069, China
Michael A McNutt, Department of Pathology, Peking University Health Science Center, Beijing 100191, China
Author contributions: Li P and Wang SS contributed equally to this study; Liu H did immunohistochemical analysis; Li N performed the statistical analysis; McNutt MA revised the manuscript; Li G and Ding HG conceived and designed the study.
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Correspondence to: Gang Li, MD, Professor, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, China. ligang55@bjmu.edu.cn
Telephone: +86-10-82802891 Fax: +86-10-82802891
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Abstract

AIM: To investigate the biological role of alpha fetoprotein (AFP) and its clinical significance in carcinogenesis of hepatocellular carcinoma (HCC).

METHODS: Clinical analysis of HCC patients and immunohistochemical examination were conducted to evaluate the relationship between serum AFP level and patient mortality. Confocal microscopy, Western blotting, dimethylthiahzolyl-2,5-diphenyl-tetrazolium bromide, Cell Counting Kit-8 assays and flow cytometry were performed to explore the possible mechanism.

RESULTS: Among the 160 HCC patients enrolled in this study, 130 patients survived 2 years (81.25%), with a survival rate of 86.8% in AFP < 2 0 μg/L group, 88.9% in AFP 20-250 μg/L group, and 69.6% in AFP > 250 μg/L group, demonstrating a higher mortality rate in HCC patients with higher AFP levels. Surgical treatment was beneficial only in patients with low AFP levels. The mortality rate of HCC patients with high AFP levels who were treated surgically was apparently higher than those treated with conservative management. The results of immunohistochemistry showed that AFP and AFP receptor were merely expressed in tissues of HCC patients with positive serum AFP. Consistently, *in vitro* analysis showed that AFP and AFPS were expressed in HepG2 but not in HLE cells. AFP showed a capability to promote cell growth, and this was more apparent in HepG2 cells, in which the proliferation was increased by 3.5 folds. Cell cycle analysis showed that the percentage of HepG2 cells in S phase after exposure to AFP was modestly increased.

CONCLUSION: HCC patients with higher AFP levels show a higher mortality rate, which appears to be attributable to the growth promoting properties of AFP.

Key words: Alpha fetoprotein; Receptor; Hepatocellular carcinoma; Mortality; Survival

Peer reviewers: Michele Milella, MD, Division of Medical Oncology A, Regina Elena National Cancer Institute, Via Elio Chianesi, n. 53, 00144 Rome, Italy; George Sgourakis, MD, PhD, FACS, 2nd Surgical Department and Surgical Oncology Unit, Red Cross Hospital, 11 Mantzarou Str, Neo Psychiko, Athens 15451, Greece; Ingmar Königsrainer, MD, Department of General, Visceral and Transplant Surgery, Hoppe Seyler Str. 3, 72076 Tübingen, Germany

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide, and accounts for 5.6% of all human cancers[1-3]. HCC in China has an increasing incidence, and it has become the second most frequent cause of estimated cancer-related death in the country[4]. Approximately 75%-80% of primary liver cancers are attributable to persistent viral infections with hepatitis B virus (HBV) or hepatitis C virus (HCV)[5]. HBV is the major etiologic factor of HCC in China[6]. Alpha fetoprotein (AFP) has served as a useful biomarker for diagnosis of HCC since the 1970s, when most patients with HCC were diagnosed at an advanced stage and had clinical symptoms. Serum levels of AFP above the reference value of 10 μg/L occur in approximately 75% of HCC cases[7]. Although AFP has a relatively low specificity in some patients with chronic non-neoplastic liver diseases, and it is non-diagnostic in some cases of small HCCs, AFP is still viewed as an important biomarker for the diagnosis of HCC[5,8,9].

AFP is an oncofetal protein normally produced in the fetal liver and yolk sac, and it is undetectable or found in trace amounts only in adults. In addition to its use in clinical diagnosis of liver cancer, the biological and pathophysiological functions of AFP have generated considerable interest. There has been compelling progress in these areas, particularly in regard to the role of this protein in cell growth and apoptosis. A recent study showed that cytoplasmic AFP may function as a regulator by interacting with phosphatase and tensin homolog deleted on chromosome ten protein and stimulating cell growth through the PI3K/AKT signal pathway[10]. Moreover, AFP co-localizes and interacts with retinoic acid receptors-β (RAR-β) in the cytoplasm, and plays a role in inhibiting translocation of RAR-β into the nucleus *via* competitive binding to RAR-β with all trans retinoic acid (ATRA)[11,12]. Thus, cytoplasmic AFP serves as an inhibitor in the retinoic acid-retinoic acid receptor signaling pathway and is most likely at least in part responsible for retinoid resistance in tumor chemotherapy[10]. By counteracting the effect of AFP, it may be possible to increase the sensitivity of tumor cells to ATRA. AFP also interacts with caspase-3 in the cytoplasm and blocks onward transmission of signaling from caspase-8[13]. Knockdown of AFP increases the sensitivity of hepatoma cells to tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and thereby triggers caspase-3 signaling. Therefore, it is possible that the combination of *AFP* gene silencing together with ATRA/TRAIL treatment will enhance the chemotherapeutic efficiency of these agents.

AFP therefore should be regarded not only as a marker for diagnosis of HCC, but also as a factor involved in ontogenetic and oncogenic growth. AFP receptors have been identified in various cell lines and tissues[14-20], and these receptors have been isolated and characterized[21-23]. Moreover, the intracellular events triggered by the binding of AFP to its receptor are being studied[24-27]. High mortality rates and poor clinical outcome are typically observed in patients with primary liver cancer as well as in other AFP-producing cancers. However, the relationship of AFP and its receptor with the mortality and patient outcome has not been fully clarified. Thus, understanding of the mechanism underlying this phenomenon may benefit the development of new clinical therapeutic strategies.

In the current study, we retrospectively analyzed the relationship between serum AFP levels and the mortality in 160 HCC patients and examined the *in vitro* effects of AFP on cell growth in order to support the assumption that elevated AFP is a significant risk factor in HCC mortality due to its capability of promoting growth of tumor cells.

MATERIALS AND METHODS

Subjects

One hundred and sixty HCC patients hospitalized in Beijing You'an Hospital between January 2006 and June 2009 were recruited to this study and the relationship between serum AFP levels and mortality was retrospectively assessed. In each case, a preliminary diagnosis of HCC was made based on the guidelines for clinical diagnosis and staging of primary HCC published by the Chinese Society of Liver Cancer (2001), fulfilling at least one of the following criteria[28]: (1) a hepatic space-occupying lesion with serum AFP ⩾ 400 μg/L; (2) serum AFP < 400 μg/L but with a new hepatic space-occupying lesion, with arterial phase enhancement on computed tomography or magnetic resonance imaging. All the patients selected were confirmed by histopathological evaluation. Patient demographics and clinicopathological data are summarized in Table 1. The AFP cut-off value used in this study has been proved to be sensitive and specific, and defined by receiver operator characteristic curve as described before[29]. This study protocol (2006-LINSHEN-3) was approved by the Ethical Committee of Beijing You'an Hospital, Capital Medical University. Informed consent was obtained from all patients.

Of the 160 HCC patients, 88 underwent surgical resection and 72 were treated conservatively with only heteropathy. All the patients were followed up for 2 years at an interval of 6 mo. The survival rates of the patients with surgical and non-surgical management were retrospectively analyzed.

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm formalin-fixed, paraffin-embedded tissue blocks. Tissue sections were deparaffinized and rehydrated, and heat-induced epitope retrieval was carried out in a 10 mmol/L citrate buffer (pH 6.0). After endogenous peroxidase was

blocked with 3% H_2O_2, sections were incubated with primary antibodies against AFP (Santa Cruze Inc., United States, sc-8399) and AFPR (Santa Cruze Inc., United States, sc-51751) overnight at 4 ℃. A biotin-free horseradish peroxidase-labeled secondary antibody (Zhongshan Golden Bridge, Beijing, China) was used for 60 min at room temperature. Coloration was performed with 3,3'-diaminobenzidin.

Cell lines

HepG2 cells, an AFP-producing HCC cell line, were cultured in Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal calf serum (FCS). The HLE hepatoma cell line, which is an AFP non-producer showed no detectable amount of AFP, and was maintained in DMEM medium supplemented with 10% FCS.

Western blotting

Western blotting was performed for analysis of expression of AFP and AFP receptor (AFPR) in HepG2 and HLE cells as previously described[30]. Briefly, cell lysate (40 μg) from each sample was subjected to 10% sodium dodecyl sulfate-Polyacrylamide gel electrophoresis. Electrophoretic transfer of proteins from gels onto nitrocellulose membranes (Amersham, United Kingdom) was carried out in transblotting cells. Membranes were blocked by immersing in 5% nonfat milk (w/v)/PBS for 1 h, and then incubated with anti-AFP, AFPR and β-actin mAbs (Santa Cruz Biotech Inc, United States) at room temperature for 2 h. After rinsing with PBS/0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (Zhongshan Boil Tech Co, Beijing) IgG secondary Ab. Immunocomplexes were visualized by incubation of the filters with the Enhanced Chemiluninescence kit (Zhongshan Boil Tech Co, Beijing) and exposure of X-ray film.

Intracellular localization of AFP and AFPR

Localization of AFP and AFPR in HepG 2 and HLE cells was analyzed with confocal microscopy as previously described[13]. Mouse anti-human AFP and mouse anti-human AFPR antibodies were purchased from Santa Cruz Biotech Inc, United States. Secondary goat anti-mouse IgG antibodies conjugated with rhodamine (Tetramethylrhodamineisothiocyanate) were purchased from Jackson Immun Res Lab, Inc, United States. Images of HepG 2 and HLE cells were captured with a confocal laser microscope (Leica TCS-NT SP2, Germany).

Determination of viability

To assess the effect of AFP on cell proliferation, the dimethylthiahzolyl-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay and analysis were performed with the Cell Counting Kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan) as described previously[31]. The CCK-8 assay was used to verify the effect of AFP seen with the MTT assay[32]. Briefly, 5×10^3 HepG 2 and human lens epithelial (HLE) cells were pipetted into 96-well microplates with various concentrations of AFP (0, 0.01, 0.1, 1, 10 and 100 mg/L) for the MTT assay, and were further subdivided into 0, 50, 100, 200 and 400 μg/L concentrations for both the MTT and CCK-8 assays. The absorbencies were measured at a wavelength of 570 nm for MTT assay and 450 nm for CCK-8 assay on a Universal Microplate Reader (EL × 800). The percentage of cell proliferation for each treatment was calculated as % cell proliferation = [(A_{570} or 450sample-background)/(A_{570} or 450 control-background)] × 100%.

Cell cycle analysis

Cell cycle measurement was performed by flow cytometry of DNA following propidium iodide (PI) staining[33]. Briefly, HepG 2 and HLE cells were cultured in serum-free medium for 12 h to arrest the cell cycle. The supernatant was then replaced by fresh medium containing 10% FCS and cells were transferred into 6-well plates (3×10^5 cells/ well). Cells were then treated with AFP (400 μg/L) for 24 h and stained by addition of 10 mg/mL PI at a final concentration of 50 mg/L. DNA content was analyzed with a FACScan-420 flow cytometer (Becton-Dickinso) as described previously. The distribution of cells in different cell cycle stages was determined according to the DNA content.

Statistical analysis

All data were statistically analyzed using χ^2 test and the t test and SPSS16 software, and expressed as mean ± SD.

RESULTS

Relationship between serum AFP level and mortality of HCC patients

HCC patient survival was analyzed retrospectively. Table 2 shows the relationship between the serum AFP level and mortality among three groups of HCC patients. There were no significant differences in mortality among these groups in the first 6 mo of evaluation. However, after one year, a higher mortality rate was observed in the high AFP group (> 250 μg/L) as shown in Table 2. The mortality rate in these groups of patients showed a direct correlation with serum AFP level. At the end of 2 years, 130/160 (81.25%) patients were alive with a survival rate of 86.8% in AFP < 20 μg/L group, 88.9% in AFP 20-250 μg/L group, and 69.6% in AFP > 250 μg/L group. These results demonstrate that the HCC patients with AFP levels higher than 250 μg/L had a higher mortality rate.

Further analysis showed that the overall survival rate (88.9%, 48/54) in HCC patients with lower AFP levels (AFP < 20 μg/L and 20-250 μg/L) who were treated surgically, was apparently higher than in those with high AFP levels in both groups treated surgically (61.8%, 13/21) ($P < 0.05$) and nonsurgically (80.0%, 40/50) at 24 mo ($P < 0.05$) (Figure 1A). There were no significant differences in the survival rates between patients managed surgically and conservatively in the two groups with lower

Table 1 Clinical features of hepatocellular carcinoma patient cohort (n = 160) (mean ± SD)

	Concentration of AFP (μg/L)		
	< 20	20-250	⩾ 250
Age (yr)	51.6 ± 9.9	54.6 ± 10.3	51.2 ± 9.8
Sex (male/female)	56/12	29/7	43 /13
ALT (U/L)	58.0 ± 60.0	55.8 ± 48.2	58.7 ± 58.5
Child			
A	52	0	47
B	12	5	7
C	4	1	2
HBsAg (+)	56	28	50
HCV (+)	1	1	1
Non-viral	11	7	5

AFP: Alpha fetoprotein; ALT: Alanine transaminase; HBsAg(+): Hepatitis B virus antigen positive; HCV: Hepatitis C virus; Non-viral: Non-viral etiology.

AFP levels. It is noteworthy that the survival rates of HCC patients with high AFP levels (> 250 μg/L) treated surgically (61.8%, 13/21) were lower than those treated conservatively (86.4%, 19/22) at 24 mo (P < 0.05). This suggests that surgical management confers survival benefit to HCC patients with lower AFP levels, but may be detrimental to the clinical course of HCC patients with AFP levels higher than 250 μg/L.

Expression of AFP and AFPR in liver tissues of HCC patients

We performed immunohistochemistry to confirm the existence of AFP/AFPR in tumor tissues of HCC patients. As shown in Figure 1B, neither normal liver tissues nor negative serum AFP showed any detectable AFP and AFPR. Interestingly, AFPR was expressed in AFP positive tissues but not in AFP negative tissues. The co-expression of AFPR and AFP in HCC patients indicated the functional relationship between these two proteins. Specific stronger staining of AFP and AFPR appeared in the cytosol and membrane, but not in the nucleus of tumor cells (Figure 1B). AFPR was not detected in fibrocytes surrounding the tumor cells. There was no qualitative relationship between serum AFP levels and the AFPR levels. In HCC patients with low serum AFP levels, immunohistochemistry still exhibited stronger AFPR staining. It is thus far unknown whether there is a clinical significance associated with expression of AFPR. Further clinical studies are needed to explore and establish the importance of AFPR in occurrence and progression of HCC.

Confirmation of existence of AFP and AFPR in HepG2 and HLE cells

To further evaluate the role of AFP in cell growth, cultured HepG2 and HLE hepatoma cell lines were used. Data from Western blotting showed that both AFP and AFPR were expressed in HepG2 but not in HLE cells (Figure 2A). Morphologic images under confocal microscope further confirmed that AFP and AFPR were present and scattered throughout the cytoplasm in HepG2 cells (Figure 2B and C). Although variable expressions

Table 2 Survival of hepatocellular carcinoma patients with different alpha fetoprotein levels over two years

Time (mo)	Survival rates (%) (dead/alive cases)		
	< 20 μg/L	20-250 μg/L	⩾ 250 μg/L
0	100.0 (0/68)	100.0 (0/36)	100.0 (0/56)
6	95.7 (3/65)	97.2 (1/35)	91.1 (5/51)
12	94.1 (4/64)	97.2 (1/35)	80.4 (11/45)
18	91.2 (6/62)	91.7 (3/33)	73.2 (15/41)
24	86.8 (9/59)	88.9 (4/32)	69.6 (17/39)[a]

[a]P < 0.05 *vs* serum alpha fetoprotein < 20 μg/L and 20-250 μg/L groups.

of AFP in different cell lines have been reported previously, there has been no data regarding the presence and distribution of AFPR. It is of interest to note that AFPR is present only in cells with AFP. Consistent with the immunohistochemical observations noted above, HepG2 cells produced both AFP and AFPR, while HLE cells produced neither of these proteins. This suggests the possibility of co-transcription and functional interrelation of these molecules.

Effect of AFP on cell proliferation

To verify the effect of AFP on tumor growth, MTT and CCK-8 cell proliferation assays were performed. The MTT assay showed that the maximum effect of AFP in promoting growth of both HepG2 and HLE cells was achieved at a 24 h incubation, and the most effective AFP dosage was about 1 mg/L (Figure 3A). More significantly proliferative effect was found in HepG2 cells, which showed a 3.5-fold increase in proliferation as compared with HLE cells at an AFP concentration of 1 mg/L. HepG2 cells are AFP and AFPR producing cells, and these cells showed a higher sensitivity in their response to AFP. When the AFP concentration range was limited to 0-400 μg/L, dose-dependent growth was observed in both cell lines (Figure 3C), however, HepG2 cells showed greater proliferation, similar to that shown in Figure 3A and B. The CCK-8 assay was used to further confirm the results obtained with MTT using AFP at a concentration range of 0-400 μg/L, which demonstrated a similar trend of growth (Figure 3D).

Cell cycle analysis

Flow cytometry was used to examine the effect of AFP on the cell cycle. Separation of cells in G0/G1, S phase and G2/M was based upon linear fluorescence intensity after staining with PI. Representative profiles are shown in Figure 4. The large initial peak (left) represents cells in G0/G1, the intervening area represents cells in S phase, and the final tail/small peak (right) represents cells in G2/M. These results showed that when HepG2 cells were exposed to AFP (400 μg/L), the percentage of cells in S phase was modestly increased (19.4%, P < 0.01) (Figure 4A and B). In contrast to HepG2 cells, the profile of the cell cycle in HLE cells did not show any significant change (Figure 4C and D).

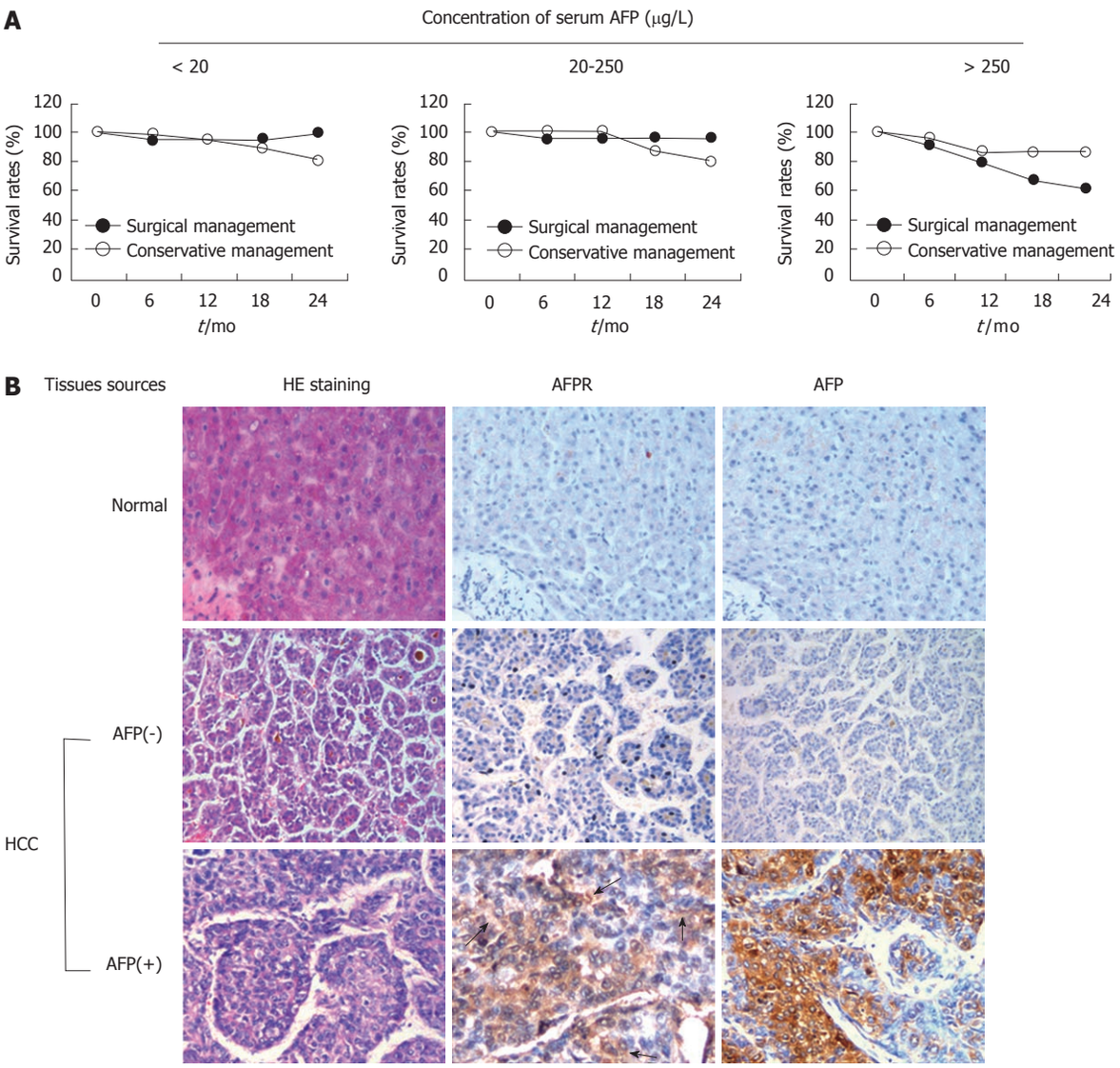

Figure 1 Survival rates of hepatocellular carcinoma patients and expression of alpha fetoprotein and alpha fetoprotein receptor in tumor tissues. A: Comparison of survival rates in hepatocellular carcinoma (HCC) patients with various alpha fetoprotein (AFP) levels who were treated surgically or non-surgically. Serum AFP levels of these HCC patients were divided into three groups: < 20 μg/L, 20-250 μg/L and > 250 μg/L; B: Immunohistochemical analysis of AFP and AFP receptor (AFPR) expressions in HCC tumor tissues. Membranous expression of AFPR is indicated with arrows. HE: Hematoxylin-eosin staining; AFP: Alpha fetoprotein; AFPR: Alpha fetoprotein receptor; HCC: Hepatocellular carcinoma.

DISCUSSION

HCC is one of the most common cancers worldwide, with a high mortality and prevalence rate in some countries, including China. Clinical studies have shown a potential relationship between AFP level and progression of HCC, and AFP may be used as a marker for monitoring treatment response in HCC patients[34-36]. As demonstrated in the retrospective analysis of 160 HCC patients in this study, patient mortality was apparently related to AFP levels. Patients with lower AFP levels (< 250 μg/L) showed a higher survival rate. Moreover, although surgical treatment has long been considered to be the primary therapeutic option for HCC[37], in this study surgery conferred survival advantage only in patients with lower AFP levels (< 250 μg/L). Surgical intervention in this study was associated with acceleration of death in patients with higher AFP levels (> 250 μg/L). These results may play a pathological role in carcinogenesis and progression of HCC.

There is extensive evidence showing that AFP is functionally an embryonic and fetal carrier/transport molecule for a multitude of ligands including fatty acids, bilirubin, heavy metals, steroids, retinoids, drugs, dyes and antibiotics[38]. However, the biological and pathophysiological roles of AFP associated with the occurrence and high mortality of HCC are still under study[22,39]. Silencing AFP expression by knockdown of its gene may play a role in growth arrest and apoptosis in human HCC cells[10,11,13,40]. Although AFP is widely used as a marker for diagnosis of HCC, some basic researches imply that AFP may be applicable in the treatment and prognostic monitoring of HCC patients[27,33]. Nevertheless, clinical observations supporting the biological role of AFP is still far from sufficient and additional data remains to be accumulated and evaluated.

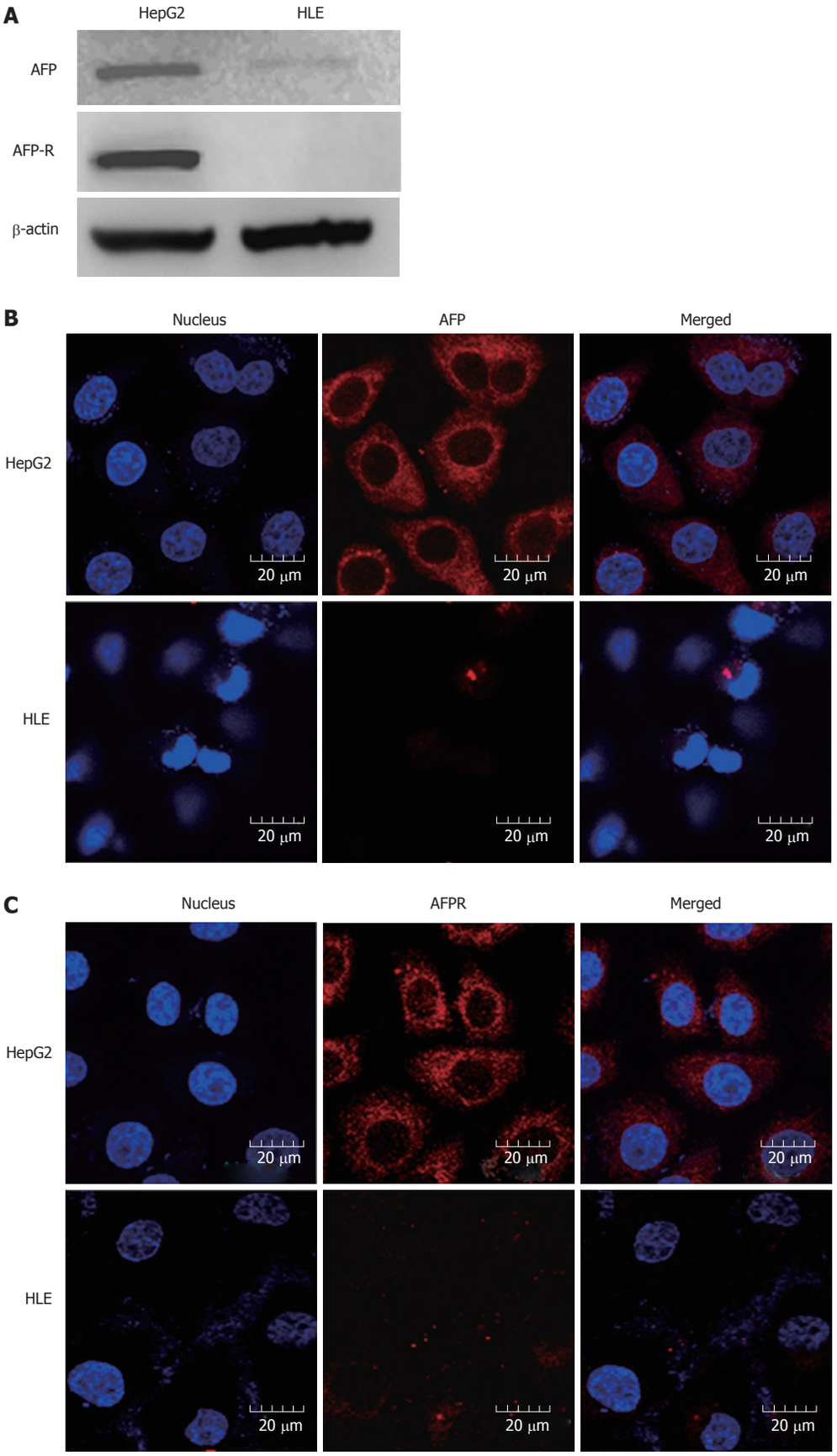

Figure 2 Expression of alpha fetoprotein and alpha fetoprotein receptor in HepG2 and HLE cells. A: Western blotting; B and C: Confocal microscopy. The immunoblots and images captured by cofocal microscopy are representative of experiments that were repeated at least three times. AFP: Alpha fetoprotein; AFPR: Alpha fetoprotein receptor.

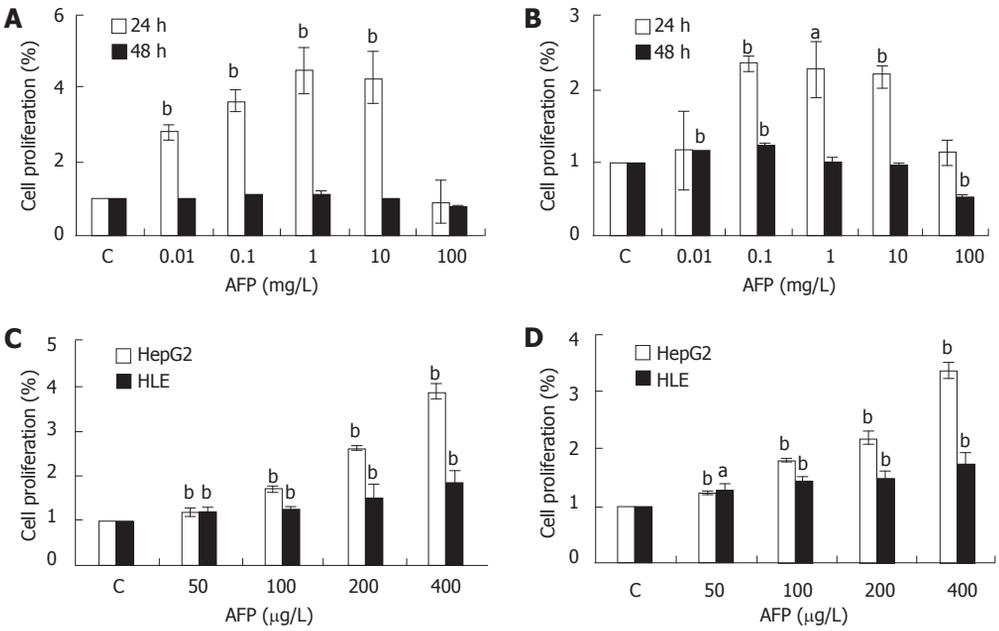

Figure 3 Effects of alpha fetoprotein in proliferation of HepG2 and HLE cells. Different concentrations (0, 0.01, 0.1, 1, 10 and 100 mg/L) of alpha fetoprotein (AFP) were tested in cell culture. The proliferation of HepG2 (A) and HLE (B) cells was evaluated with the dimethylthiahzolyl-2,5-diphenyl-tetrazolium bromide (MTT) assay at 24 and 48 h. HepG2 and HLE cells were further tested with 0, 50, 100, 200 and 400 μg/L AFP and proliferation was evaluated by MTT (C) and a cell counting kit (cck)-8 assay (D) at 24 h. The differences in proliferation between HepG2 and HLE cells were statistically analyzed with SPSS16 software. Data are representative of experiments that were repeated three times and are presented as mean ± SD for 6-9 samples.

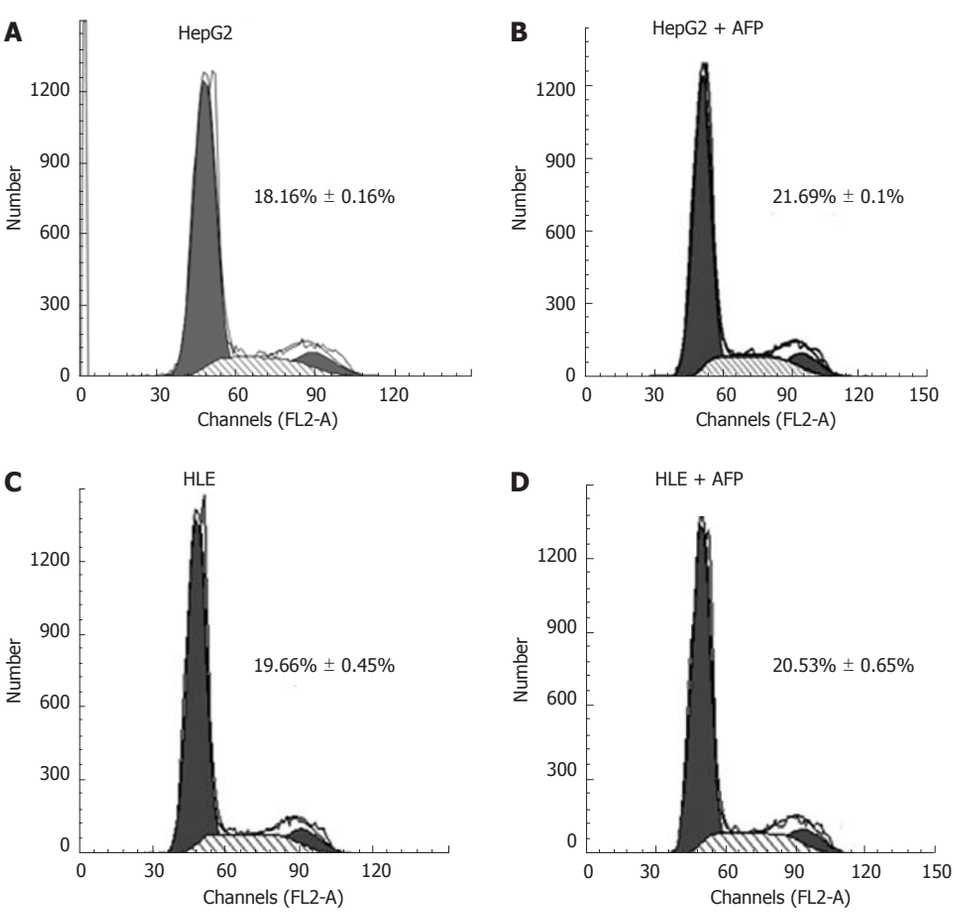

Figure 4 Effects of alpha fetoprotein on cell cycle progression of HepG2 and HLE cells. The distribution of cells in different cell cycle phases was determined according to DNA content. The data are representative of 3 independent experiments. Each panel represents a different treatment group. A: HepG2 cells without treatment; B: HepG2 cells treated with 400 μg/L alpha fetoprotein (AFP) for 24 h; C: HLE cells without treatment; D: HLE cells treated with 400 μg/L AFP for 24 h. In each panel, the number represents cell percentage in S phase. Data are presented as mean ± SD of 3 samples. AFP: Alpha fetoprotein.

The significance of AFPR in cell proliferation is of particular concern. However, until recently there has been little work involving AFPR in its clinical relation to HCC. Given the fact that AFP and its receptor are co-expressed in serum AFP positive but not in AFP negative tissues or cells as shown in this study, it is conceivable that cells expressing AFPR are more sensitive to AFP, thereby exhibiting greater proliferation and an associated increase in the proportion of cells in S phase. It has been inferred that AFP is secreted from hepatoma cells and acted on these cells through an autocrine mechanism. However, this does not occur in AFP negative tissues or cells as shown in this study. It is of particular interest that observations in HCC patients were consistent with findings from laboratory research, in that about one-third of HCC patients had similar AFP levels to healthy subjects, leading to a low mortality[41]. Thus, based on these results and findings in cultured cell assays, the intrinsic mechanism underlying the role of AFP in the mortality of HCC patients might be implicated.

Taken together, these results emphasize the significance and importance of serum AFP level in HCC patient survival. It appears that AFP is not simply a marker for diagnosis, it is also a growth factor in tumor progression. This concept of the function of AFP is consistent with the observations in this study in that HCC patients with higher serum AFP levels appear to have a higher mortality. Incorporation of serum AFP level into the criteria for evaluating prognosis and determining therapeutic options in HCC patients will likely be of significant benefit in patient management. Nonetheless, further clinical studies are needed to confirm this conclusion.

COMMENTS

Background

Although alpha fetoprotein (AFP) has been used as a serum marker for the clinical diagnosis of hepatocellular carcinoma (HCC), the biological role of this molecule in relation to its clinical significance is still unclear. The goal of this study is to evaluate the relationship between elevated serum AFP levels and survival of HCC patients and possible underlying mechanisms.

Research frontiers

AFP is an oncofetal protein and widely used as a marker in clinical diagnosis of HCC. In the last decade, compelling progress has been made in the two related research areas. First, AFP excreted into the circulation is able to promote cell growth and has been defined as a growth regulator in ontogenic growth and tumor progression. Second, it was recently demonstrated that intracellular AFP may function as a signal molecule through binding key proteins involved in growth or apoptosis signal pathways.

Innovations and breakthroughs

This study showed that AFP and AFPR were merely expressed in tissues of HCC patients with positive serum AFP. HCC patients with higher AFP levels had a higher mortality rate, which appears to be attributable to the growth promoting properties of AFP.

Applications

The findings in this study emphasize the significance and importance of serum AFP level in HCC patient survival. It appears that AFP is not simply a marker for diagnosis, it is also a growth factor in tumor progression. Incorporation of serum AFP level into the criteria for evaluating prognosis and determining therapeutic options in HCC patients may significantly benefit the management of HCC patients.

Peer review

This is an interesting study which underlines the importance of AFP in hepatocellular carcinoma. The paper is well written. This is another study which shows that AFP must play a key role in the decision making in operative treatment for HCC patients. A prospective study with comparable groups of HCC patients (size and number of tumor nodules, differentiation, clinical parameters of patients) either with low or high AFP would be ideal in the future.

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ORIGINAL ARTICLE

Effects of cyclooxygenase-2 on human esophageal squamous cell carcinoma

Li Zhang, Yong-Dong Wu, Peng Li, Jun Tu, Ying-Lin Niu, Cai-Min Xu, Shu-Tian Zhang

Li Zhang, Yong-Dong Wu, Peng Li, Ying-Lin Niu, Shu-Tian Zhang, Department of Gastroenterology, Beijing Friendship Hospital Affiliated with the Capital Medical University, Beijing 100050, China
Jun Tu, Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249, United States
Cai-Min Xu, Department of Biochemistry and Molecular Biology, National Laboratory of Medical Molecular Biology, Institute of Basic Medicine, Chinese Academy of Medical Sciences, Beijing 100005, China
Author contributions: Zhang ST designed the research; Wu YD, Li P and Tu J performed the research; Niu YL and Xu CM analyzed the data; Zhang L wrote the paper.
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Correspondence to: Shu-Tian Zhang, PhD, MD, Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, 95 Yong'an Road, Beijing 100050, China. niuziyue1@hotmail.com
Telephone: +86-10-63014411 Fax: +86-10-63020006
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Abstract

AIM: To study the relationship between the *cyclooxygenase (COX)-2* gene and the proliferation and apoptosis of esophageal squamous carcinoma EC109 cells.

METHODS: The techniques of RNA interference (RNAi) and cell transfection, as well as the levels of oncogenicity in nude mice, were used to study the role of COX-2 in the esophageal squamous carcinoma cell (ESCC) line EC109. Following RNAi and transfection, Western blotting analysis was used to determine the expression of the COX-2 protein. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay was used to evaluate cell growth, and flow cytometry was used to detect cell apoptosis.

RESULTS: Western blotting analysis demonstrated that *COX-2* expression was significantly reduced in EC109 cells treated with *COX-2*-specific short interfering RNA (siRNA) but was increased in EC109 cells transfected with *COX-2*. Furthermore, *COX-2* siRNA treatment inhibited cell proliferation ($P < 0.01$) and induced apoptosis in EC109 cells, as determined by an MTT assay and by flow cytometry, respectively. In contrast, transfected *COX-2* led to increased cell proliferation ($P < 0.05$) and decreased apoptosis in EC109 cells. In addition, combination treatment of cells with *COX-2* siRNA and aspirin had a synergistic effect ($P < 0.01$). For experiments measuring tumorigenicity, xenograft tumors of a greater volume and weight were found in the *COX-2* group compared with other groups ($P < 0.05$). A large dose of aspirin inhibited tumor growth in nude mice effectively ($P < 0.05$), and the rate of tumor suppression was 51.8% in the high-dose aspirin group.

CONCLUSION: *COX-2* plays a very critical role in ESCC carcinogenesis, and *COX-2* siRNA combined with aspirin has the potential to be an anticancer therapy for the treatment of ESCC.

Key words: Esophageal squamous cell carcinoma; Cyclooxygenase-2; Aspirin; Cell proliferation; Apoptosis; Synergismt; Transfection; RNA interference

Peer reviewer: Piero Marco Fisichella, MD, Assistant Professor of Surgery, Medical Director, Swallowing Center, Loyola University Medical Center, Department of Surgery, Stritch School of Medicine, 2160 South First Avenue, Room 3226, Maywood, IL 60153, United States

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INTRODUCTION

Esophageal carcinoma is a common malignant neoplasm worldwide with high fatality rates[1]. This condition can be classified as esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma. Cancers arising from esophageal tissue, especially ESCC, have high incidence rates in China. Despite surgical treatment and chemotherapy, the prognosis of ESCC had until recently been very poor. Thus, early identification and effective treatment are highly desirable for an improved prognosis.

Cyclooxygenase is an enzyme responsible for the synthesis of prostaglandins, and it has two isoforms, COX-1 and COX-2. COX-1 is a constitutive enzyme and is found in most mammalian cells. COX-2, on the other hand, is undetectable in most normal tissues but can be induced by tumor-promoting agents, growth factors and inflammation.

To date, many studies have shown *COX-2* to play a very important role in carcinogenesis. *COX-2* has been reported to be over-expressed in many malignant tumors, such as those in breast, lung, stomach, colon and pancreatic cancer[2-6], and levels of *COX-2* expression are associated with poor prognosis of some cancers[7]. Non-steroidal anti-inflammatory drugs (NSAIDs) and *COX-2* inhibitors have been shown to effectively suppress tumor development[8]. For instance, recent studies have indicated that the regular use of aspirin can reduce the risk of esophageal cancer by as much as 90%[9,10]. Due to the inhibitory effect of aspirin on COX activity, we hypothesized that *COX-2* is involved in the development of esophageal cancer.

In fact, the association between *COX-2* and ESCC has previously been examined. In these studies, the common findings were that *COX-2* was over-expressed in ESCC and that it contributed to carcinogenesis. However, the molecular mechanism by which *COX-2* promotes carcinogenesis in squamous cells remained unclear.

Previous research has shown that the mechanisms behind *COX-2* gene expression differed by cell type and the cell growth conditions. The pleiotropic effects of COX-2 on carcinogenesis include increased cellular proliferation, inhibition of apoptosis, increased angiogenesis, impaired cell adhesion and increased invasion of malignant cells[11-13].

In the present study, we have delineated the effects of increased or decreased levels of *COX-2* on human ESCC proliferation and apoptosis. Specifically, we have investigated the effect of *COX-2* overexpression on ESCC cell proliferation and apoptosis. We have also analyzed the effects of aspirin, a nonspecific *COX-2* inhibitor and the specific depletion of COX-2 by short interfering RNA (siRNA) in ESCC. The results showed that *COX-2* overexpression induced antiapoptotic activity and promoted tumorigenesis, while the inhibition of COX-2 effectively suppressed the proliferation of cancer cells and tumorigenesis in nude mice.

A recent study by Yang GZ *et al*[14] found that *COX-2* expression was upregulated during an early stage of ESCC, especially in more fully differentiated carcinomas. Therefore, the inhibition of *COX-2* by RNAi or aspirin treatment could be an effective strategy for the prevention and treatment of early stages of ESCC.

MATERIALS AND METHODS

Cell lines

EC109 is a cell line that was derived from a patient with a well-differentiated ESCC, and the line was obtained from the Cancer Institute at the Chinese Academy of Medical Sciences. EC109 cells were maintained in RPMI 1640 culture medium (Invitrogen, United States) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 2% L-glutamine. The cells were grown in a humidified 37 ℃ incubator with 5% CO_2. They were fed every 3 d with complete medium and were subcultured when confluent.

Construction of hCOX-2 expression vectors and transient transfections

The modified pOSML-PGHS-2 plasmid, kindly provided by Dr. Smith WL (University of Michigan, United States), contains the full-length *hCOX-2* gene. After the sequence of full-length *hCOX-2* cDNA had been confirmed by sequence analysis, *COX-2* cDNA (approximately 1.9 kb) was cloned into the pcDNA3.1/V5HisA expression vector. One day before transfection, EC109 cells were seeded at 2.5 × 10^5/plate in 6 cm dishes in RPMI 1640 antibiotic-free medium containing 10% fetal bovine serum (FBS) until they were 80%-90% confluent. After 24 h, 800 μL of RPMI 1640 medium without FBS or antibiotics was added to each well, and the cells were transfected with either the *COX-2* expression plasmid (pcDNA3.1V5HisA/*hCOX-2*) or with an empty control vector (pcDNA3.1V5HisA) using Lipofectamine™2000 (Invitrogene, Carlsbad, CA) according to the manufacturer's protocol. In brief, 5 μg of *COX-2* plasmid DNA was diluted in serum- and antibiotic-free RPMI 1640 medium to a total volume of 100 μL. In addition, 5 μL of Lipofectamine™2000 was diluted with serum- and antibiotic-free RPMI 1640 medium to a total volume of 100 μL. The diluted plasmid DNA was combined with the diluted Lipofectamine™2000. Following incubation for 20 min at room temperature, 200 μL of the mixture was added to each well, and the cells were incubated at 37 ℃ in a CO_2 incubator for 5 h. The RPMI 1640 medium containing 10% FBS was changed after 5 h, and the cells were incubated for an additional 24 h at 37℃ in a CO_2 incubator. For the combined treatment of *COX-2* transfection and aspirin, 8 mmol/L aspirin was added 24 h after the *COX-2* plasmid (pcDNA3.1V5HisA/*hCOX-2*) was transfected into the EC109 cells, and the cells were treated with aspirin for 48 h.

COX-2 siRNA synthesis and transfection

COX-2 siRNA was synthesized by GeneChem (Shanghai, China). The *COX-2*-specific siRNA was designed to target positions 293-311 (5'-CUGCUCAACACCGGAAU-

UUtt-3') of the *COX-2* transcript (GenBank Accession No: NM_000963). We also used a scrambled siRNA as a negative control. This negative control had no significant homology to any known human, mouse or rat gene sequence (non-silencing-FITC: 5'-UUCUCCGAACGUGUCACGUtt-3'). EC109 cells were plated in 6-well plates at 2×10^5 cells per well, and RPMI 1640 medium without antibiotics was added to each well. After 24 h, the cells were grown to reach 40%-50% confluence and were transfected with *COX-2* siRNA using the Lipofectamine ™2000 reagent according to the manufacturer's instructions. The original stock of siRNA was resuspended in siRNA suspension buffer provided by the manufacturer. From this stock (20 μmol/L), 15 μL was diluted with 250 μL of Opti-MEM, and 5 μL Lipofectamine™2000 was diluted with 250 μL of Opti-MEM. After 5 min at room temperature, these were combined and incubated for 20 min. The siRNA-Lipofectamine complexes were then added to the plated cells and mixed gently. The cells were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 for 84 h. For combination treatment of *COX-2* siRNA with aspirin (Sigma, United States), 8 mmol/L aspirin was added 48 h after the *COX-2* siRNA was transfected into the EC109 cells, and the cells were treated with aspirin for 36 h.

Western blotting

Cells were harvested and lysed in lysis buffer, and Western blotting analysis was performed using conventional protocols. Briefly, the protein concentration of the extracts was determined using a bicinchoninic acid kit (Pierce, United States) with bovine serum albumin as the standard. Total protein samples (30 μg) were loaded and separated by 10% SDS-PAGE gels and then transferred to protran nitrocellulose membranes (Whatman, United Kingdom). The membranes were incubated with an anti-COX-2 antibody (1:200; Santa Cruz, United States) and, after extensive washing, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000; Santa Cruz) for 1 h at room temperature. The signal was detected by chemiluminescence using an ECL Detection Kit (Amersham, United States). The membranes that were probed for *COX-2* were re-probed for β-actin (1:10 000; Santa Cruz, United States) to normalize for loading and/or quantification errors and to allow for the direct comparison of protein expression. The bands were quantified using a Gel EDAS analysis system (Cold Spring United States Corporation) and Gel-Pro Analyzer 3.1 software (Media Cybernetics, United States).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay

To evaluate the effects of COX-2 overexpression and aspirin treatment on the proliferation of the EC109 cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. In brief, EC109 cells were seeded in 96-well plates at a density of 2×10^5 cells/well and cultured for 24 h. The cells were then transfected with the *COX-2* expression plasmid (pcDNA3.1V5HisA/hCOX-2) or the empty control vector (pcDNA3.1V5HisA) for 72 h. At 24 h post-transfection, the co-treated cells were treated with aspirin (8 mmol/L) for an additional 48 h. Then, 20 μL of 5 mg/mL MTT solution (Gibco, Grand Island, United States) was added to 200 μL of the media in each well. After an additional incubation for 4 h, the media was discarded, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density at 492 nm was read using an automated microplate reader. The experiments were conducted in triplicate, and the results are shown as the mean ± standard deviation (SD) of three independent experiments.

Detection of the rate of apoptosis by flow cytometry

To determine the effects of *COX-2* overexpression and aspirin treatment on apoptosis, EC109 cells were transfected with either an empty control vector, *hCOX-2* cDNA alone or with *hCOX-2* cDNA plus aspirin treatment. The cells were collected by centrifugation at 200 *g* for 5 min and stained with propidium iodide (PI; R&D Company, Minneapolis, United States). The pellets were washed twice with ice-cold phosphate-buffered saline (PBS), fixed overnight at 4 ℃ in 70% ethanol and stored at -20 ℃. The pellets were washed twice with PBS, and the cells were incubated with 5 μg/mL PI and 50 μg/mL RNase A in PBS for 1 h at room temperature in the dark. Flow cytometry was conducted using a FACSCalibur flow cytometer (BectonDickson, Mountain View, CA). Ten thousand events were collected per sample, and the cells were analyzed for the rate of apoptosis using CELLQUEST software. This experiment was performed in triplicate.

Tumorigenicity in nude mice

To generate stably transfected cell lines, cells transfected with pcDNA3.1V5HisA/*hCOX-2* as well as cells transfected with the empty control vector (pcDNA3.1V5HisA) were selected with 500 μg/mL G418. After 30 d of selection with G418, a G418-resistant stably transfected cell line was created. EC109 parent cells and stably transfected cells were trypsinized and collected. The cell viability was > 95% as determined by trypan blue staining. Cells (4×10^6) in 0.1 mL PBS were inoculated subcutaneously onto the backs of 4- to 6-wk-old male BALB/c nude mice (five per group). Male BALB/c nu/nu mice weighing (15.24 ± 0.57) g were purchased from the Experimental Animal Center at the Chinese Academy of Medical Sciences and Peking Union Medical College. The mice were housed under sterile conditions. For mice given the combination of *COX-2* transfection and aspirin treatment, a high dose (0.315 mg/dL^{-1}) or a low dose (0.016 mg/dL^{-1}) of aspirin was orally administrated once per day starting one day before the inoculation of *COX-2*-transfected EC109 cells. The aspirin administered to experimental animals was provided by the Bayer Animal Health Company. The tumor size was monitored weekly

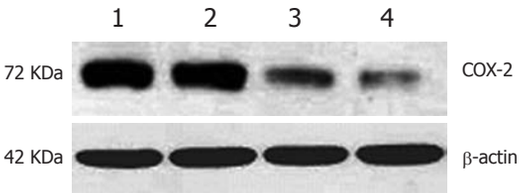

Figure 1 Western blotting for cyclooxygenase-2 protein expression after transfection of EC109 cells. 1: Parental cells; 2: *hCOX-2*-transfected cells; 3: Vector control cells; 4: Cells treated with aspirin for 48 h after transfection with *hCOX-2*.

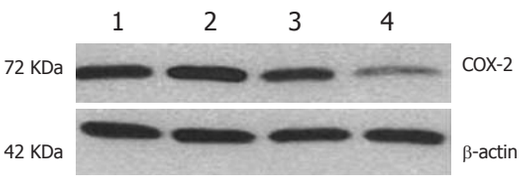

Figure 2 Western blotting for cyclooxygenase-2 protein expression after RNA interference treatment of EC109 cells. 1: Parental cells; 2: Negative control siRNA; 3: *Cyclooxygenase-2 (COX-2)* short interfering RNA (siRNA); 4: Cells treated with aspirin for 48 h after transfection with *COX-2* siRNA.

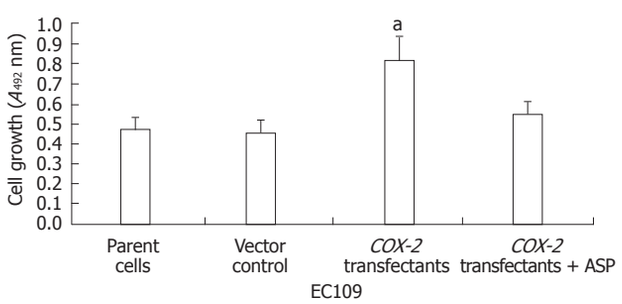

Figure 3 Inhibition of cell proliferation in EC109 cells by *hCOX-2* transfection and aspirin treatment (*n* = 3, mean ± SD). [a]*P* < 0.05. ASP: Aspirin.

using calipers, and the tumor volume was calculated using the following formula: volume = 0.5 × length × width[2]. At the end of 4 wk, all the mice were sacrificed and their weight was recorded. The tumors were excised and kept in 4% formalin for immunohistochemical analysis. The percentage of cells expressing nuclear Ki67 was used for the assessment of cellular proliferation rate. The nuclear Ki67 expression and the nuclear COX-2 protein expression was detected by immunohistochemistry. The Ki67 and COX-2 antibodies for immunohistochemistry were purchased from the Fuzhou Mai Xin Company (Fuzhou, China). PV6001 Kits were purchased from Zhong Shan Golden Bridge Biological Technology (Beijing, China). All procedures using animal experimentation were approved by the local animal research authorities, and animal care was conducted in accordance with institutional guidelines.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS 11.0, United States). The differences between the groups were assessed using the analysis of variance test. The results were considered statistically significant when the *P* value was < 0.05 or < 0.01.

RESULTS

Expression of COX-2 in EC109 cells transfected with pcDNA3.1V5HisA/hCOX-2 and treated with aspirin

The expression of *COX-2* was evaluated in EC109 cells transfected with either the pcDNA3.1V5HisA/*hCOX-2* or pcDNA3.1V5HisA vector. For the combined treatment group, 8 mmol/L aspirin was added to the cell culture 24 h after transfection with the *COX-2* plasmid (pcDNA3.1V5HisA/*hCOX-2*) for a period of 48 h. COX-2 protein expression was evaluated by Western blotting analysis. In comparison to the parent cell line or the control vector transfectants, a slight increase in *COX-2* expression was observed in the *COX-2*-transfected EC109 cells. However, the expression of *COX-2* was suppressed after aspirin was added to the *COX-2*-transfected EC109 cells. For Western blotting analysis, the *COX-2* expression was normalized to β-actin expression by band intensity (Figure 1).

Inhibition of COX-2 protein expression by RNAi and/or aspirin treatment

Given the importance of *COX-2* in ESCC carcinogenesis, we used an *in vitro* experimental model that used ESCC cell lines to determine whether *COX-2*-specific siRNA was able to downregulate *COX-2* expression. From a panel of ESCC cell lines, we choose a well-differentiated line (EC109) that had a moderate level of *COX-2* expression. The effect of *COX-2* siRNA treatment on protein expression was assessed by Western blotting analysis. As shown in Figure 2, *COX-2* expression was reduced in EC109 cells 84 h after transfection with the *COX-2* siRNA (100 nmol/L), while the negative control siRNA had no effect on the expression of *COX-2*. *COX-2* expression was normalized to β-actin expression by band intensity. *COX-2* expression was significantly further decreased in the EC109 cells transfected with *COX-2* siRNA for 36 h and also in the EC109 cells treated with aspirin for 48 h (Figure 2). These results suggest that the co-administration of aspirin and siRNA had a synergistic effect on the inhibition of *COX-2* gene expression.

COX-2 overexpression and aspirin treatment increase EC109 cellular proliferation

The MTT assay was conducted to elucidate the effects of *COX-2* transfection and aspirin treatment on EC109 cell proliferation. The values are expressed as the mean ± SD of three independent experiments. The results from this assay indicated that *COX-2* transfection led to a marked increase in cell proliferation at 72 h (Figure 3). Statistical analysis found that cell proliferation was significantly increased in *COX-2*-transfected EC109 cells ($P < 0.05$). However, there was no significant increase in the proliferation of cells transfected with the empty vector, as compared to parent cells. Furthermore, cell proliferation was not significantly affected by the administration of aspirin

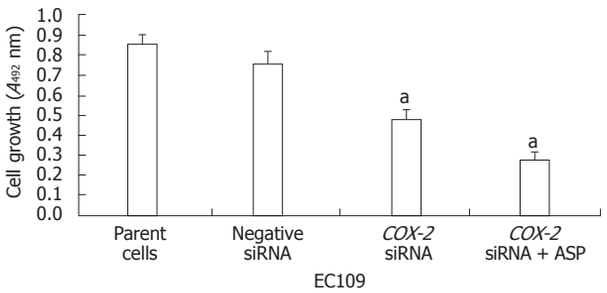

Figure 4 Inhibition of cell proliferation in EC109 cells by short interfering RNA and aspirin treatment (n = 3, mean ± SD). $^{a}P < 0.01$. siRNA: Short interfering RNA; ASP: Aspirin.

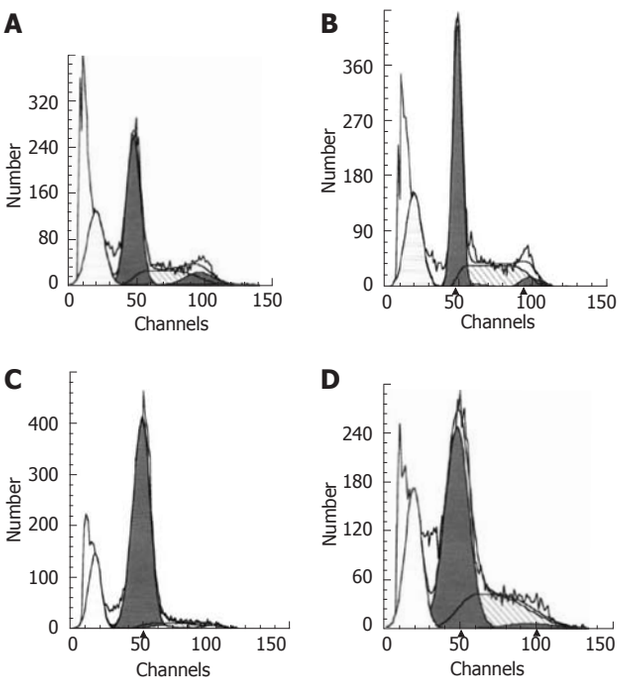

Figure 5 *hCOX-2* transfection and aspirin treatment induces apoptosis in EC109 cells. A: Parental cells; B: Samples transfected with the control pcDNA3.1V5HisA vector; C: Samples transfected with the *cyclooxygenase-2*-expressing plasmid pcDNA3.1V5HisA/hCOX-2; D: Samples transfected with the pcDNA3.1V5HisA/hCOX-2 plasmid for 24 h and treated with aspirin for an additional 48 h.

to *COX-2*-transfected cells as compared with control cells (Figure 3). These data demonstrate that cell proliferation was increased following *COX-2*-transfection in EC109 cells and decreased upon co-administration of aspirin. Therefore, the expression of *COX-2* was important for proliferation in EC109 cells. Moreover, *COX-2* overexpression by transfection promoted cell proliferation, and the inhibition of *COX-2* expression by aspirin treatment inhibited cell proliferation in EC109 cells.

COX-2 RNAi and aspirin treatment reduce EC109 cellular proliferation

To elucidate the effects of the siRNA and aspirin treatments on EC109 cell proliferation, the MTT assay was used, and cell proliferation was determined by counting the numbers of viable cells. The values are expressed as the mean ± SD of three independent experiments. There was no significant reduction in the number of viable EC109 cells at 24 h post-treatment. However, at 48 h, we detected a significant decrease in the number of viable cells. Furthermore, *COX-2* siRNA treatment resulted in a significant inhibition of cell proliferation in the EC109 cells ($P < 0.01$) at 84 h post-transfection (Figure 4). The proliferation was not affected by treatment with the control siRNA. Additionally, the treatment of siRNA-transfected cells with aspirin further increased the inhibition of cell proliferation ($P < 0.01$, Figure 4). These data demonstrate that *COX-2* expression is important for proliferation in EC109 cells and that the inhibition of *COX-2* by siRNA alone or combined with aspirin treatment can inhibit the proliferation of EC109 cells.

COX-2 overexpression and aspirin treatment reduce the rate of apoptosis in EC109 cells

To determine the effect of *COX-2* transfection and aspirin treatment on apoptosis in esophageal cancer EC109 cells, flow cytometry was performed on PI-stained cells. The results showed that EC109 cells transfected with *hCOX-2* were resistant to apoptosis. Comparing to the rate of apoptosis that was 28.7% in parent cells group and 26.96% in empty vector transfected cells group, the rate of apoptosis was decreased to 17.75% in COX-2-transfected EC109 cells (Figure 5). Apoptosis was not significantly influenced by transfection with the empty vector control. However, the rate of apoptosis increased to 24.97% when the transfected EC109 cells were treated with aspirin (Figure 5). These data indicate that apoptosis in EC109 cells was affected by *COX-2* transfection and aspirin treatment.

COX-2 RNAi and aspirin increase the rate of EC109 cellular apoptosis

To determine the effect of siRNA and aspirin treatment on the rate of apoptosis in EC109 cells, treated cells were labeled with PI and analyzed by flow cytometry. The inhibition of *COX-2* by siRNA alone or with aspirin treatment resulted in a significant decrease in the growth of the EC109 cells due to the induction of apoptosis. Apoptosis was not significantly influenced by treatment with the negative control siRNA. Comparing to the rate of apoptosis that was 1.35% in parent cells group and 1.72% in negative control siRNA-treated cells group respectively, the rate of apoptosis was increased to 4.33% in siRNA-treated EC109 cells (Figure 6) and was further increased to 11.66% when EC109 cells were treated with both the COX-2-specific siRNA and aspirin (Figure 6). These data indicate that the COX-2-specific siRNA alone or in combination with aspirin treatment induced the apoptosis of EC109 cells.

Tumorigenicity in nude mice

***COX-2* overexpression increases tumor growth:** Human *COX-2* cDNA was cloned into the pcDNA3.1V5HisA vector, which was stably transfected into the EC109 cells.

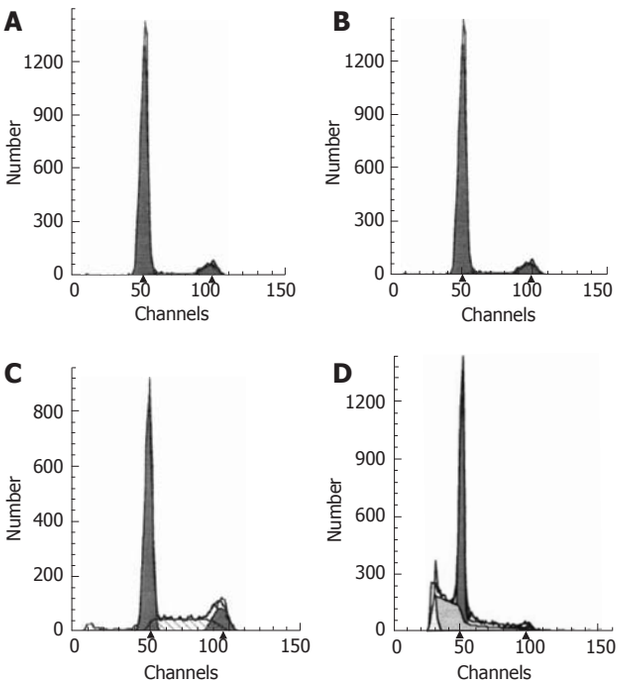

Figure 6 Short interfering RNA-mediated *cyclooxygenase-2* knockdown with aspirin treatment induces apoptosis in EC109 cells. A: Parental cells; B: Samples treated with the negative control short interfering RNA (siRNA); C: Samples treated with *cyclooxygenase-2 (COX-2)* siRNA; D: Samples treated with COX-2 siRNA for 48 h and treated with aspirin for an additional 36 h.

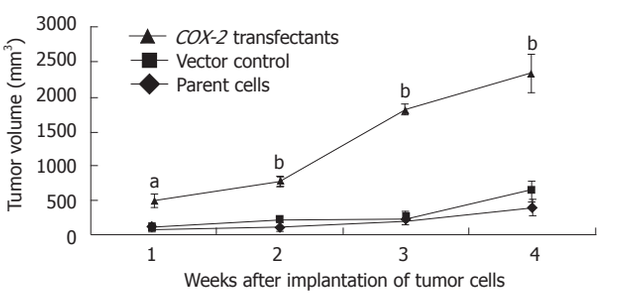

Figure 7 Effect of cyclooxygenase-2 on tumor growth in the nude mouse xenograft tumor model (n = 15, mean ± SD). $^{a}P < 0.05$, $^{b}P < 0.01$. COX-2: Cyclooxygenase-2.

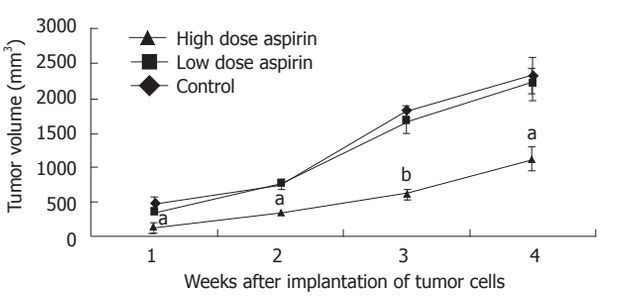

Figure 8 Effect of aspirin on tumor growth in the nude mouse xenograft tumor model (n = 15, mean ± SD). $^{a}P < 0.05$, $^{b}P < 0.01$.

To assess changes in cell biology after gene transfection, cell proliferation was measured by an MTT assay. *COX-2*-transfected cells appeared to have a markedly different growth pattern, as compared to the EC109 parent cells or the vector control-transfected cells (Figure 3). To investigate the role of *COX-2* in tumorigenicity, tumor progression was evaluated in nude mice. Following selection, G418-resistant stably transfected cells were inoculated into nude mice. The first xenografted tumor was found 3 d after the implantation of *COX-2*-transfected tumor cells, and tumor sizes were measured weekly. Four weeks after cell transfer, tumors established by EC109 parent cells or vector control (pcDNA3.1V5HisA) cells produced tumors of 393.43 mm^3 ± 118.48 mm^3 and 634.50 mm^3 ± 159.29 mm^3, respectively, while tumors from *COX-2*-transfected (pcDNA3.1V5HisA/*hCOX-2*) cells produced markedly larger tumors that were 2344.49 mm^3 ± 273.52 mm^3 in volume ($P < 0.01$ or $P < 0.05$, Figure 7). When mice were sacrificed at 28 d post-transfer, the EC109 parent cells and vector control cells had produced tumors that were 0.67 g ± 0.15 g and 0.89 g ± 0.29 g, respectively, while *COX-2*-transfected cells had produced larger tumors that were 2.58 g ± 0.26 g in weight ($P < 0.05$). Data are expressed as the mean ± SD of five independent samples (n = 15). These data show that *COX-2* gene expression increased tumor growth.

Aspirin treatment reduces tumor growth: Aspirin, an inhibitor of *COX-2*, was administered to nude mice starting one day before the implantation of *COX-2*-stably transfected EC109 cells. At day 28 after implantation, a high dose of aspirin (0.315 mg/dL^{-1}, once per day) had significantly reduced tumor volume ($P < 0.01$ or $P < 0.05$) and tumor weight ($P < 0.05$) in this xenograft model (Figure 8). However, there was no significant difference in tumor volume between the low-dose aspirin (0.016 mg/dL^{-1}, once per day) and control group. Date are expressed as the mean ± SD of five independent samples (n = 15). The tumor inhibition rate for the high-dose aspirin group was 51.80%, and the tumor inhibition rate for the low-dose aspirin group was 5.98%. No digestive hemorrhages or ulcers were found in the mice. Therefore, large doses of aspirin effectively inhibited the growth of these xenografted tumors in nude mice.

Evaluation of Ki67 immunostaining in cancer xenografts: The expression of Ki-67 protein is strictly associated with cell proliferation, which makes Ki-67 expression a biomarker and a strong predictor of cancer. We mainly observed Ki-67 protein expression in the nuclei of xenografted tumor cells (Figure 9). Immunohistochemical sections stained for Ki-67 were quantified with a computer-aided image analysis machine using the association between high gray-scale density and tumor cell proliferation. The quantification of staining intensity demonstrated that cell proliferation in tumor xenografts from cells transfected with *COX-2* was greater than in tumors from parent cells and vector control-transfected cells.

DISCUSSION

A large body of evidence suggests that *COX-2*-overexpressing cancer cells may possess a survival advantage that

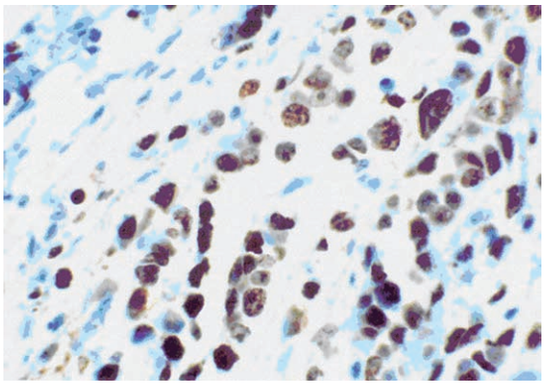

Figure 9 Ki-67 expression in the nuclei of cells in xenograft tumors from pcDNA3.1V5HisA/hCOX-2-transfected EC109 cells at 400 x magnification.

facilitates tumor development and progression[15,16]. In our study, RNAi was used as a potent tool to inhibit *COX-2* gene expression levels in human ESCC. *COX-2* RNAi resulted in significant suppression of cellular proliferation in EC109 cells. This result, together with the observation that aspirin treatment inhibited the growth of the EC109 cells, indicates that *COX-2* activity may be directly associated with proliferation in EC109 cells. To further investigate the role of *COX-2* in ESCC carcinogenesis, EC109 cells were transfected with recombinant *COX-2*-expressing vectors to establish stably transfected cell lines. The increased COX-2 protein expression observed in transfected cells suggests that *COX-2* transfection effectively increases *COX-2* levels and leads to the increased proliferation of these cells.

Other studies have found an association between *COX-2* overexpression and the antiapoptotic signature of cancer[17-19]. In this study, an antiapoptotic effect was observed in *COX-2*-transfected cells. Considering the proapoptotic effect of aspirin, our results suggest that *COX-2* overexpression contributed to the antiapoptotic signature of the EC109 cells. Furthermore, *COX-2* RNAi promoted apoptosis in these cells. Our data suggest that *COX-2*-specific siRNA treatment combined with the administration of aspirin is a promising treatment option for *COX-2*-expressing ESCCs. *COX-2* overexpression-mediated upregulation of the antiapoptotic protein Bcl-2 has been proposed to be a mechanism that leads to the antiapoptotic signature of cancer cells[20].

In light of these results obtained from *in vitro* experiments, we hypothesized that *COX-2* cDNA would enhance the *in vivo* tumorigenesis of EC109 cells. As expected, nude mice transfected with *COX-2* showed faster tumor growth and larger tumors, which is in contrast to mice transfected with the parental cell line or the vector control cells. Furthermore, the immunohistochemical data support these findings, as Ki-67, a marker of cell proliferation, was found at increased levels in the *COX-2*-tranfected tumor xenografts. In addition, the suppression of *COX-2* expression by high-dose aspirin treatment effectively attenuated the oncogenesis of EC109 cells *in vivo*.

Although transfection of *COX-2* into EC109 cells resulted in increased cellular proliferation, stronger antiapoptotic activity and enhanced tumorigenesis *in vivo,* the EC109 cell line itself had endogenous *COX-2* expression. Therefore, we deduce that *COX-2* function is correlated with the amount of its expression and enzyme activity.

NSAID-mediated growth inhibition of tumor cells involves COX-prostaglandin E_2 (PGE_2)-dependent and COX-PGE_2-independent pathways[21]. In COX-PGE_2-dependent pathways, COX-2 inhibitors suppress carcinogenesis *via* the inhibition of COX-2-derived PGE_2, which promotes cell proliferation and immunosuppression[22]. The elevated levels PGE_2 in *COX-2*-overexpressing cells are related to the metastatic potential of the cancer cells, which can be reduced in a dose-dependent manner by COX inhibitors[12]. Furthermore, the COX-2 enzyme itself can promote cancer development and progression[23]. In COX-PGE_2-independent pathways, the mechanism by which COX-2 inhibitors suppress carcinogenesis involves the reduced activation of Akt, inhibition of nuclear factor-κB activation, downregulation of the antiapoptotic protein Bcl-XL, inhibition of peroxisome proliferator-activated receptor (PPAR)-δ, activation of PPAR-γ and the activation of caspase family members. One or more of these COX-PGE_2-independent effects could contribute to the proapoptotic and antiproliferative effects of NSAID treatment[24]. The hypophosphorylation of the retinoblastoma tumor suppressor protein as well as the downregulation of multiple proliferation-promoting cyclins and cyclin-dependent kinases induced by NSAIDs has been reported in colon carcinoma cells[23]. The extent of the use of COX-PGE_2-dependent or -independent pathways following NSAID treatment may depend on the cell type.

Gene therapy and RNAi are the most exciting and promising technologies in biomedicine today. The goal of gene therapy is to introduce genetic material (DNA or RNA) encoding a protein, which is missing or defective, into a patient's cells or tissues. RNAi is the sequence-specific post-transcriptional silencing of gene expression mediated by small double-stranded RNA molecules. RNAi has become an exceptionally powerful method to inhibit the expression and function of disease-causing genes, and it performs better than all similar techniques previously tested in gene therapy. However, the use of RNAi is still in its infancy, and the safety of the delivery system and tissue-targeting and the potential for adverse effects or lifelong RNAi persistence should still be considered before RNAi becomes a feasible and safe therapy for patients. In fact, RNAi-mediated gene therapy remains the single most promising biomedical strategy for knocking down expression of the *COX-2* gene in human ESCC, and we believe that the successful clinical implementation of RNAi gene therapy and its eventual translation into clinical benefits are merely a question of time.

In this study, no gastric mucosal hemorrhages were found in mice, even after administration of large doses of aspirin. However, the gastric mucosa of nude mice is different from human gastric mucosa. In patients, larger

doses of aspirin can increase the risk of bleeding, including bleeding ulcers, and combined therapy with RNAi can allow for lower doses of aspirin. The risk for bleeding tends to decrease as the dose of aspirin is decreased. Further long-term animal experiments may be required to test the effectiveness of *COX-2* inhibitors and RNAi in the treatment of ESCC.

In conclusion, our present study has indicated that *COX-2* plays a crucial role in the carcinogenesis of human ESCC by increasing cell proliferation and resistance to apoptosis and subsequently enhancing tumorigenesis *in vivo*. These results suggest that *COX-2* may be a new gene target for ESCC treatment and that inhibition of *COX-2* expression by RNAi and aspirin treatment may serve as an early and effective prevention and treatment of early-stage, *COX-2*-expressing ESCC.

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COMMENTS

Background

Esophageal cancer can be divided into two major groups including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. Epidemiological studies indicate that ESCC is the dominant form in Asian countries, especially China, Japan and Korea. Some studies have shown that *COX-2* is overexpressed in ESCC and contributes to carcinogenesis. However, the molecular mechanisms by which *COX-2* promotes carcinogenesis in squamous cells remain unclear. Given the high fatality rate and the rapidly increasing incidence of ESCC, the definition of the role of *COX-2* in the pathogenesis and therapy of this cancer is highly desirable.

Research frontiers

In this study, RNAi was used as a potential tool to inhibit *COX-2* gene expression in human ESCC. *COX-2* RNA interference (RNAi) significantly suppressed the proliferation of EC109 cells in a manner similar to treatment with aspirin, a non-specific COX inhibitor. Moreover, *COX-2* transfection effectively increased COX-2 levels and increased the proliferation of EC109 cells. These results indicate that COX-2 may be directly associated with the proliferation of EC109 cells. Considering the proapoptotic effects of aspirin, our results favor the view that *COX-2* overexpression contributes to the acquisition of antiapoptotic activity in EC109 cells. Moreover, *COX-2* RNAi treatment was able to promote apoptosis. *COX-2* short interfering RNA (siRNA) combined with aspirin has the potential to be an effective anticancer therapy for the treatment of ESCC. Furthermore, the tumorigenicity assay in nude mice revealed a faster tumor growth and larger xenograft tumor size in the *COX-2*-transfected cell group. Alternatively, the suppression of *COX-2* expression by high-dose aspirin treatment effectively attenuated the oncogenesis of EC109 cells *in vivo*.

Innovations and breakthroughs

In the past few years, RNAi has become an important research tool to study and manipulate a particular gene and its function. Furthermore, the use of siRNA as a potent and specific inhibitor of a specific target gene provides a new therapeutic approach for many incurable diseases, particularly cancer. In addition, aspirin may boost the effectiveness of chemotherapy in the treatment of cancers. This is the first study to report that *COX-2* siRNA combined with aspirin treatment has the potential to be an effective anticancer therapy for ESCC.

Applications

Gene therapy and RNAi are the most exciting and promising technologies in biomedicine today. RNAi is an exceptionally powerful method to inhibit the expression and the function of disease-causing genes, and it has out-performed all similar methodologies previously tested in gene therapy. The data presented here suggest that the use of *COX-2*-specific siRNA combined with aspirin is a promising treatment for *COX-2*-expressing ESCCs. The present research believes that the successful clinical implementation of this combination therapy is merely a question of time.

Terminology

Non-steroidal antiinflammatory drug (NSAID): NSAIDs block the COX enzymes and reduce the levels of prostaglandins throughout the body. NSAIDs are used primarily to treat inflammation, mild to moderate pain, and fever. Aspirin is a unique NSAID.

Peer review

The authors aimed to study the relationship between the *COX-2* gene and the proliferation and apoptosis of esophageal squamous carcinoma EC109 cells. They have demonstrated that *COX-2* expression was significantly reduced in EC109 cells treated with *COX-2*-specific siRNA but was increased in cells transfected with *COX-2*. They have concluded that COX-2 plays a critical role in ESCC carcinogenesis, and that *COX-2* siRNA combination treatment with aspirin has the potential to be an effective anticancer therapy for ESCC. Overall, the manuscript is very interesting and adds to the body of literature on the positive effects of aspirin in carcinogenesis. It is also very well written and the conclusions are supported by the data.

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BRIEF ARTICLE

FibroSURE™ and FibroScan® in relation to treatment response in chronic hepatitis C virus

Keyur Patel, Mireen Friedrich-Rust, Yoav Lurie, Mircea Grigorescu, Carol Stanciu, Chuan-Mo Lee, Eugene R Schiff, Dieter Häussinger, Michael P Manns, Guido Gerken, Isabelle Colle, Michael Torbenson, Erik Pulkstenis, G Mani Subramanian, John G McHutchison, Stefan Zeuzem

Keyur Patel, John G McHutchison, Department of Gastroenterology and Hepatology, Duke Clinical Research Institute, Durham, NC 27715, United States
Mireen Friedrich-Rust, Stefan Zeuzem, Department of Gastroenterology and Hepatology, J.W. Goethe University Hospital, Frankfurt 60590, Germany
Yoav Lurie, Department of Gastroenterology and Hepatology, Tel Aviv Sourasky Medical Center, Tel Aviv 64239, Israel
Mircea Grigorescu, Department of Clinical Infectious Disease, Spitalul Clinic de Urgenta, Cluj-Napoca 400162, Romania
Carol Stanciu, Department of Gastroenterology and Hepatology, Institutul de Gastroenterologie si Hepatologie, Iasi 700111, Romania
Chuan-Mo Lee, Department of Gastroenterology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, 833 Taiwan, China
Eugene R Schiff, Departments of Gastroenterology and Internal Medicine, University of Miami, FL 33136, United States
Dieter Häussinger, Department of Gastroenterology and Hepatology, Universitätsklinik Düsseldorf 40255, Germany
Michael P Manns, Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover 30623, Germany
Guido Gerken, Department of Gastroenterology and Hepatology, Universitätsklinikum Essen 45122, Germany
Isabelle Colle, Department of Gastroenterology and Hepatology, Ghent University Hospital, Ghent 9000, Belgium
Michael Torbenson, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21231, United States
Erik Pulkstenis, Department of Biostatistics, Human Genome Sciences, Inc., Rockville, MD 20850, United States
G Mani Subramanian, Department of Clinical Research, Human Genome Sciences, Inc., Rockville, MD 20850, United States
Author contributions: Patel K was the primary author of the manuscript. Other authors contributed to the manuscript and the study (including manuscript review). Lurie Y, Grigorescu M, Stanciu C, Lee CM, Schiff ER, Häussinger D, Manns MP, Gerken G and Colle I enrolled patients in the study; Friedrich-Rust M, Pulkstenis E, Subramanian GM, McHutchison JG and Zeuzem S were responsible for study design, conduct and analysis.
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Correspondence to: Dr. Keyur Patel, Department of Gastroenterology and Hepatology, Duke Clinical Research Institute, PO Box 17969, Durham, NC 27715, United States. keyur.patel@duke.edu
Telephone: +1-919-6687193 Fax:+1-919-6687186
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Abstract

AIM: To compare histological endpoint assessment using noninvasive alternatives to biopsy during treatment in a chronic hepatitis C virus (HCV) cohort.

METHODS: Patients with chronic HCV were randomized to receive interferon-based therapy for 24 (genotypes 2/3) or 48 (genotype 1) wk. FibroSURE™ (FS) was assessed at baseline and at week-12 post-treatment follow-up. Baseline biopsy for METAVIR was assessed by a single pathologist. FibroScan® transient elastography (TE) was performed during treatment in a patient subset.

RESULTS: Two thousand and sixty patients (n = 253 in Asia) were classified as METAVIR F0-1 (n = 1682) or F2-4 (n = 378). For F2-4, FS (n = 2055) had sensitivity and specificity of 0.87 and 0.61, respectively, with area under the receiver-operating curve of 0.82; corresponding values for TE (n = 214) and combined FS/TE (n = 209) were 0.77, 0.88 and 0.88, and 0.93, 0.68 and 0.88. Overall FS/TE agreement for F2-4 was 71% (κ = 0.41) and higher in Asians *vs* non-Asians (κ = 0.86 *vs* 0.35; $P < 0.001$). Combined FS/TE had 97% accuracy in Asians (n = 33). Baseline FS (0.38 *vs* 0.51, $P < 0.001$) and TE (8.0 kPa *vs* 11.9 kPa, P = 0.006) scores were lower in patients with sustained virological response than in nonresponders, and were maintained through follow-up.

CONCLUSION: FS and TE may reliably differentiate mild from moderate-advanced disease, with a potential for high diagnostic accuracy in Asians with chronic HCV.

Key words: Albinterferon alfa-2b; FibroScan; FibroSURE; Hepatitis C virus; Interferon; Sustained virological response; Transient elastography

Peer reviewers: Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan; A Mithat Bzodayi, MD, PhD, Hepatology Institute, Department of Gastroenterology, Ankara Medical Faculty, Ankara University, 06100 Cebeci Ankara, Turkey

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INTRODUCTION

Complications of chronic hepatitis C virus (HCV) infection occur as a consequence of progressive liver fibrosis, leading to cirrhosis, liver failure, and hepatocellular carcinoma. Histological assessment of liver injury and fibrosis is important for making treatment decisions, as well as for predicting prognosis and therapeutic outcome, in chronic liver disease[1]. Liver biopsy is, however, an invasive procedure, limited by issues relating to sampling, cost and morbidity, and only provides a static measure of fibrosis[2]. Accurate noninvasive methods of monitoring changes in fibrosis would be helpful in following the natural history of the disease and monitoring potential antifibrotic responses to antiviral or other treatment modalities. The past decade has seen the development of several noninvasive predictive indices for hepatic fibrosis based on direct and indirect serum markers, as well as imaging modalities to measure liver stiffness, such as transient elastography (TE) (FibroScan®, Echosens, Paris, France)[3]. Serum HCV FibroSURE™ (FS) (Laboratory Corporation of America, Raritan, NJ, United States) combines α_2-macroglobulin, haptoglobin, γ-glutamyl transpeptidase, apolipoprotein A_1, alanine transaminase, and total bilirubin into a proprietary algorithm for fibrosis and inflammatory activity[4]. Both noninvasive modalities have been extensively evaluated in viral hepatitis and other chronic liver diseases[5]. The combination of serum tests, such as FS or FibroMeters, and TE appears to improve the cross-sectional diagnostic accuracy for advanced-stage disease in chronic HCV[6,7]. The French Haute Autorité de Santé has approved FS and TE as first-line tests to detect cirrhosis in chronic HCV[8]. Few studies, however, have determined the utility of either biomarkers or TE to accurately follow longitudinal changes in fibrosis both during and after antiviral therapy to better define long-term histological outcomes in chronic HCV[9-12]. Although there are emerging studies of TE in Asian patients with chronic liver disease (mostly due to chronic hepatitis B virus), no studies have evaluated the utility of both FS and TE in Asian patients with chronic HCV[13,14].

The aims of the present study were to: (1) compare the diagnostic utility of FS and TE for fibrosis at baseline in treatment-naïve patients with chronic HCV; (2) determine concurrent changes in both FS and TE with virological responses during and after albinterferon alfa-2b (albIFN) combination therapy; and (3) evaluate the performance of these noninvasive tests for the detection of significant fibrosis in an Asian cohort.

MATERIALS AND METHODS

Study population

Adult patients with chronic HCV genotype (Gt) 1 or 2/3 (n = 2225) who had not previously received interferon (IFN)-α therapy were enrolled in two global phase Ⅲ studies of albIFN conducted at 136 centers worldwide between December 2006 and October 2008 (ClinicalTrials. gov nos. NCT00402428 and NCT00411385)[15,16]. Patients were excluded if they had decompensated liver disease or other causes of chronic liver disease, including co-infection with hepatitis B virus or human immunodeficiency virus; a significant co-existing medical condition; Gilbert's disease; or alcohol or drug dependence. Patients were randomized in a 1:1:1 ratio to one of three open-label treatment groups: albIFN 900 or 1200 μg every 2 wk, or PEGinterferon alfa-2a (PEGASYS®, Hoffmann-La Roche Inc., Nutley, NJ) 180 μg once weekly. All patients also received oral ribavirin (RIBASPHERE®, 3 Rivers Pharmaceuticals®, Warrendale, PA, United States) 800 mg/d (Gt 2/3) or 1000-1200 mg/d (Gt 1) in two divided doses. Treatment duration was 24 (Gt 2/3) or 48 (Gt 1) wk, with follow-up at week 48 or 72, respectively, for sustained virological response (SVR) assessment. Patients with detectable HCV RNA at post-treatment week-12 follow-up were determined to be nonresponders (NRs) and were not required to complete final follow-up at post-treatment week 24. Serum HCV-RNA levels were measured by real-time polymerase chain reaction assay (COBAS® Ampliprep/COBAS® Taqman® HCV Test, Hoffman-La Roche): limit of detection was 15 IU/mL and lower limit of quantitation 43 IU/mL.

All patients provided written informed consent and the institutional review boards of all participating centers approved the studies, which were performed in accordance with the Helsinki Declaration of 1975. The authors accept full responsibility for the accuracy of the whole content, including findings, citations, and references contained in this manuscript.

Procedures for fibrosis assessment

Pretreatment liver biopsies were available in 2060 patients, and evaluated for METAVIR fibrosis stage and

Table 1 Baseline patient demographics *n* (%)

Host characteristics	All (*n* = 2060)	Patients with TE (*n* = 214)
Mean age ± SD (yr)	45.2 ± 11.4	45.7 ± 11.7
Race		
Caucasian	1599 (77.6)	169 (79.0)
Black	98 (4.8)	5 (2.3)
Asian	322 (15.6)	39 (18.2)
Other	41 (2.0)	1 (0.5)
Genotype		
1	1186 (57.6)	120 (56.1)
2/3	874 (42.4)	94 (43.9)
Male sex	1196 (58.1)	105 (49.1)
Mean BMI ± SD	26.6 ± 5.1	25.1 ± 4.2
ALT > 1.5 × ULN	911 (44.2)	98 (45.8)
Mean biopsy length ± SD (mm)	17.0 ± 9.1	15.8 ± 8.2
METAVIR fibrosis stage		
F0	740 (35.9)	80 (37.4)
F1	942 (45.7)	91 (42.5)
F2	159 (7.7)	16 (7.5)
F3	101 (4.9)	9 (4.2)
F4	118 (5.7)	18 (8.4)
METAVIR activity		
A0-1	1125 (54.6)	126 (58.9)
A2-3	935 (45.4)	88 (41.1)

ALT: Alanine transaminase; BMI: Body mass index; SD: Standard deviation; TE: Transient elastography; ULN: Upper limit of normal.

activity grade by a single pathologist (Torbenson M) who was blinded to study assignments or results. Adequate biopsy quality was based on assessment by the pathologist of specimens ⩾ 15 mm in length and/or including ⩾ 6 portal tracts. The METAVIR scoring system classifies fibrosis on a five-point scale: F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = few septa; F3 = numerous septa without cirrhosis; and F4 = cirrhosis; necro-inflammatory activity is graded on a 4-point scale: A0 = none; A1 = mild; A2 = moderate; and A3 = severe[17].

Fasting serum samples were frozen at -70 ℃ within 2 h of collection. Assessment with FibroSURE, a commercial serum marker panel assay, was performed independently, with blinding of clinical and pathologic assessments at a central laboratory (Laboratory Corporation of America), at baseline and 12 wk after the end of treatment. The TE measurements were obtained using FibroScan at baseline, weeks 12, 24 and 48, and 12 wk after the end of treatment in patients with HCV Gt 1, and weeks 12, 24 and 36 with Gt 2/3, at 40 study sites as part of a protocol-specified substudy. Results of TE with ⩾10 acquisitions, a success rate ⩾ 60%, and an interquartile range < 30% of the median value were considered valid measurements, as per manufacturer's recommendation and prior studies[3,18,19].

Statistical analysis

Patient demographic and clinical laboratory characteristics were descriptively summarized, and reported as mean ± SD and range. All tests were two-sided, and statistical significance was assessed at the 0.05 level. Performance characteristics differentiating mild (F0-1) from moderate-severe (F2-4) fibrosis at baseline were determined for FS. Performance of this assay for F2-4 was determined by area under the receiver-operating characteristic curve (AUROC) using the DeLong method[20]. Values for AUROC were standardized relative to a uniform prevalence distribution, and an adjusted AUROC was calculated to account for spectrum bias, using the difference between the mean stage of advanced fibrosis minus the mean stage of nonadvanced fibrosis[21]. The FS modality provides a continuous regression index with a corresponding predicted individual fibrosis stage[22]. An FS index < 0.32 was used for stage F0-1. For two-stage predictive indices with FS for F0-1, F1-2, and F3-4, the midpoint index value was used as a threshold for assignment of stage for analysis. The TE cut-off values were chosen *via* AUROC analysis as the point at which sensitivity and specificity were maximized. Recommended thresholds for TE in chronic HCV of > 7 kPa and > 12.5 kPa for F2 and F4, respectively, were also assessed[6]. For assessing the utility of combined FS and TE, prediction was based on a logistic-regression model containing both indices, as well as their pairwise interaction. The measure of agreement chosen was Cohen's κ. Differences between continuous variables were assessed by Student's *t* test, assuming unequal variance. All statistical analyses were performed using SAS® 9.2 (SAS Institute, Cary, NC).

RESULTS

Patient demographics

Baseline biopsy (mean length 17 mm ± 9 mm) results were available from 2060 patients with chronic HCV. Patients were mostly men (58.1%) and caucasian (77.6%), with a mean age of 45.2 ± 11.4 years and a prevalence of significant fibrosis of 18.3% (Table 1).

Baseline FibroSURE performance

Results for FS and biopsy were available in 2055 patients. For stages F2-4, FS had a sensitivity of 0.87, a specificity of 0.61, and an AUROC of 0.82 [95% confidence interval (CI) 0.80-0.84, Figure 1]; the corresponding adjusted AUROC relative to a uniform prevalence distribution was 0.84. For F4, sensitivity was 0.63, specificity was 0.85, and AUROC was 0.83 (95% CI 0.79-0.86). The misclassification rate for FS was 34% (*n* = 703/2055), and most of these patients (93%; *n* = 653) were false-positive F2-4 (Figure 2). For biopsy specimens > 15 mm and F2-4 (46.0%; *n* = 948/2055), sensitivity was 0.86, specificity was 0.61, and AUROC was 0.83 (95% CI 0.80-0.86). The FS misclassification rate, however, remained 34% in these patients with longer biopsy specimens. For biopsies > 15 mm and F4, sensitivity was 0.67, specificity was 0.84, and AUROC was 0.86 (95% CI 0.81-0.91). For moderate-severe necro-inflammatory activity of A2-3, sensitivity and specificity were both 0.66, and AUROC was 0.71 (95% CI 0.69-0.73).

Baseline TE

Results of TE and biopsy were available in 214 patients. For stage F2, TE > 10.1 kPa had a sensitivity of 0.77, a specificity of 0.88, an AUROC of 0.88 (95% CI 0.82-0.93),

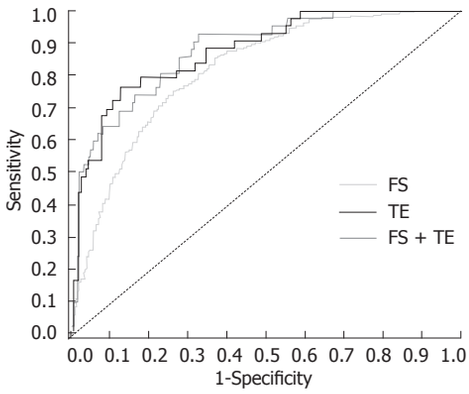

Figure 1 Baseline area-under-the-receiver-operating-curve analysis for stages F2-4 for FibroSURE and transient elastography. Of the overall cohort with baseline biopsy (n = 2060), FibroSURE (FS) was available in 2055 patients, transient elastography (TE) in 214, and both FS and TE in 209. Area-under-the-receiver-operating-curve values for stages F2-4 were 0.82 for FS, 0.88 for TE, and 0.88 for FS + TE. Differences between FS and TE were not significant. FS: FibroSURE; TE: Transient elastography.

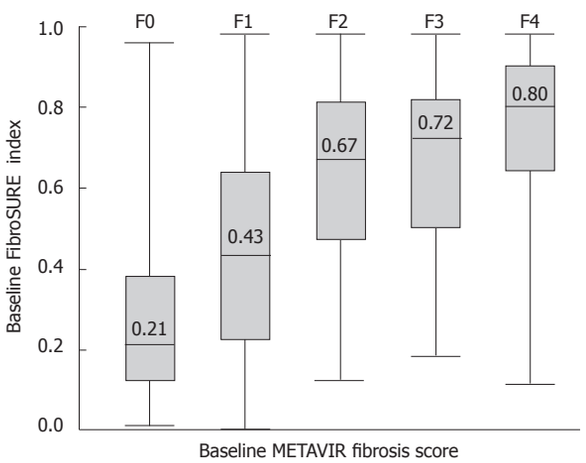

Figure 2 Boxplot distribution of FibroSURE index scores in all patients at baseline (n = 2055). Box parameters represent 25th and 75th percentile index scores for each fibrosis stage; median index values are shown within box, and upper and lower range limits are represented by vertical lines.

and an adjusted AUROC of 0.88 (Figure 1). For F4, TE > 11.7 kPa had a sensitivity of 0.94, a specificity of 0.88, and an AUROC of 0.93 (95% CI 0.88-0.98). The misclassification rate for TE was 14% (n = 31), with approximately two-thirds of these patients (68%; n = 21) classified as false-positive F2-4. For F2-4 with biopsy specimens > 15 mm (39.3%, n = 84; F2-4 prevalence 22.6%, n = 19), sensitivity was 0.63, specificity was 0.91, and AUROC was 0.83 (95% CI 0.72-0.93).

Performance characteristics of TE, using a previously recommended threshold of > 7 kPa for stages F2-4[6], indicated a higher sensitivity and lower specificity of 0.88 and 0.65, respectively, with a lower overall accuracy of 0.70. For stage F4 at a TE threshold of > 12.5 kPa, sensitivity was lower at 0.72, but with a similar specificity of 0.89.

Baseline comparison of combined FibroSURE and TE

Both FS and TE results were available in 209 patients before therapy. For this subset, prediction of stages F2-4 using FS and TE in combination had a sensitivity of 0.93, a specificity of 0.68, an AUROC of 0.88 (95% CI 0.82-0.94), and an adjusted AUROC of 0.88 (Figure 1). Agreement between FS and TE, however, for F2-4 was 0.71 (95% CI 0.65-0.77), with a Cohen's κ of 0.41 (95% CI 0.30-0.52). Among 61 patients with nonconcordance for FS and TE, 88% (n = 54) were F2-4 by FS and F0-1 by TE; biopsy indicated mild-stage disease in most of these 54 patients [F0-1 in 88.9% (n = 48); F2-4 in 11.1% (n = 6)]. Conversely, only seven of the 61 patients were F0-1 by FS and F2-4 by TE; four of these patients were F0-1 by biopsy.

For the 148 patients with agreement between FS and TE, 68% (n = 101) were stages F0-1 and 32% (n = 47) were F2-4 by both noninvasive tests. Biopsy results, however, indicated agreement with both FS and TE in 86% (n = 128), with 3% and 11% misclassified by both noninvasive tests as F0-1 and F2-4, respectively. Biopsies were > 15 mm in 56 of the patients for which there was agreement between FS and TE, and concordance results for the noninvasive tests were compared with the biopsy results: there was a slight reduction in the proportion of patients misclassified by the combination of FS and TE (from 14.0% to 10.7%), although the sample size was relatively small. Agreement between FS and TE for stage F4 increased to 0.85 (95% CI 0.80-0.90; κ = 0.53), and compared with biopsy, misclassification rates (biopsy < F4) for both FS and TE were 7.3%, with all cases being false positives.

Changes in transient elastography during therapy

Results for TE were available in a subset of 217 patients who completed treatment (HCV Gt 1, n = 122; Gt 2/3, n = 95). Mean TE scores were lower at baseline in patients who achieved an SVR compared with NRs (8.0 *vs* 11.9 kPa; P = 0.006). Further multivariate modeling showed no association with Gt, race, or body mass index, but significant increases in liver stiffness in older patients (P < 0.001) and men (P = 0.03). In addition, TE scores were higher in patients with METAVIR grades 2-3 inflammatory activity score at baseline (11.8 *vs* 7.3; P < 0.001), with further minimal declines in TE measurements during therapy for patients with an SVR and NRs. The difference at baseline remained significant only at week 12 (P = 0.03) and lost significance at later on-treatment time points (Figure 3). At the follow-up visit, overall mean changes in TE from baseline were -1.3 kPa (P < 0.001) and -2.7 (P = 0.04) for patients with an SVR and NRs, respectively, reflecting declines to levels that remained different (6.9 *vs* 10.1; P = 0.049). There was no correlation between changes in TE and alanine transaminase during or after therapy.

Changes in FibroSURE after antiviral therapy

Baseline FS scores were available in 2082 patients (HCV Gt 1, n = 1200; Gt 2/3, n = 882), with 1731 also available at post-treatment follow-up. Patients who achieved an SVR (n = 1305) had lower mean baseline FS fibrosis index scores compared with NRs (n = 777; 0.38 *vs* 0.51;

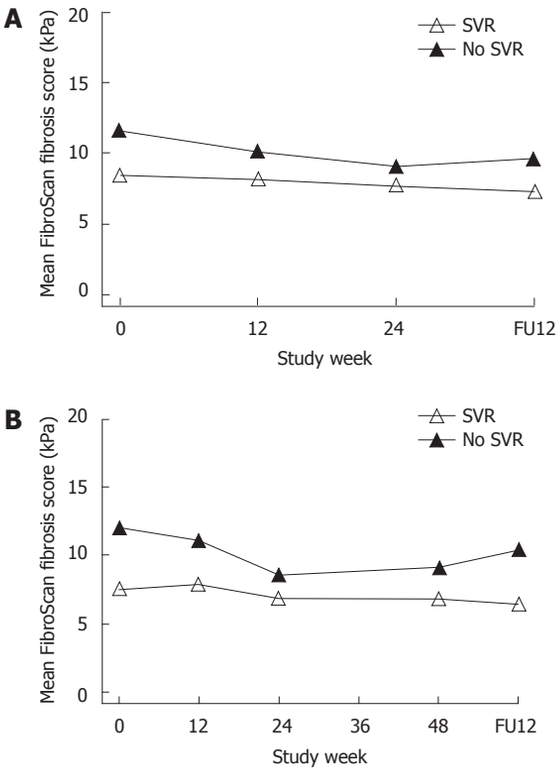

Figure 3 Changes in transient elastography with therapy according to sustained virological response. Mean FibroScan fibrosis scores over time by sustained virological response status in patients with hepatitis C virus (A) genotypes 2/3 and (B) genotype 1. FU: Follow-up.

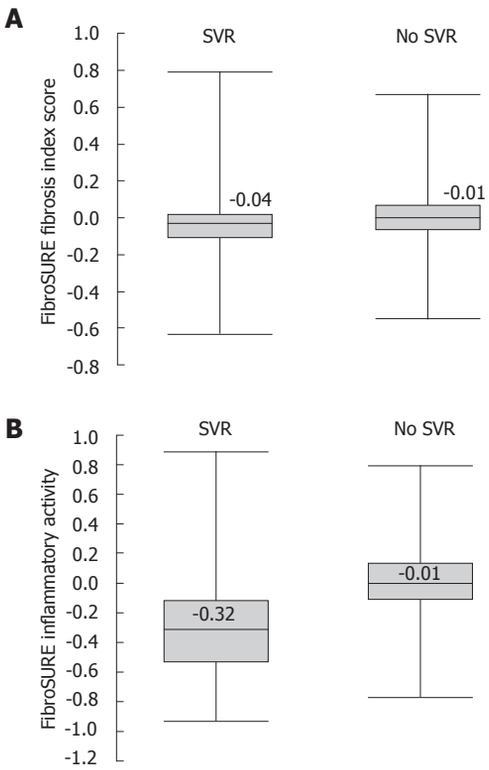

Figure 4 Changes in FibroSURE with therapy according to sustained virological response. Changes in FibroSURE (A) fibrosis index and (B) inflammatory index at 12 wk post-treatment compared with baseline values by sustained virological response status.

Table 2 Performance of FibroSURE and transient elastography in patients from Asia *vs* non-Asian regions

Test	Region (*n*)	F2-4 (%)	Sens	Spec	PPV (%)	NPV (%)	Accuracy (%)	AUROC
FS	Asia (253)	24.9	0.90	0.60	42.9	95.0	67.6	0.83
	NAR (1802)	17.4	0.86	0.61	31.9	95.4	65.5	0.82
TE	Asia (33)	24.2	0.88	0.92	77.8	95.8	90.9	0.92
	NAR (181)	19.3	0.77	0.87	58.7	94.1	85.1	0.87
FS + TE	Asia (33)	24.2	0.88	1.00	100.0	96.2	97.0	0.97
	NAR (176)	19.3	0.91	0.68	40.8	97.0	72.7	0.88

AUROC: Area under the receiver operating curve; FS: FibroSURE; NAR: Non-Asian region; NPV: Negative predictive value; PPV: Positive predictive value; Sens: Sensitivity; Spec: Specificity; TE: Transient elastography.

$P < 0.001$). Baseline FS necro-inflammatory activity scores were not significantly lower in patients with an SVR (0.45 *vs* 0.47; $P = 0.06$). At post-treatment follow-up week 12, there was a significant reduction in FS fibrosis scores from baseline in patients with an SVR compared with NRs (Δ = -0.06 *vs* 0.0; $P < 0.001$; Figure 4). As expected with a biochemical response that accompanies viral clearance, there was a significant reduction in FS necro-inflammatory activity scores in patients with an SVR compared with NRs following antiviral therapy (Δ = -0.35 *vs* -0.02, $P < 0.001$) (Figure 4).

FibroSURE and transient elastography in Asian patients

There were 253 Asian patients with a baseline biopsy (mean length 13.5 ± 8.4 mm), classified as stages F0-1 in 75% (n = 190) and F2-F4 in 26% (n = 63). For F2-4 with FS (n = 253), sensitivity was 0.90, specificity was 0.60, and AUROC was 0.83. These results were comparable to those in patients from a non-Asian region (NAR; n = 1802), with a sensitivity of 0.86, a specificity of 0.61, and an AUROC of 0.82 (Table 2). In comparison to FS, TE had a similar sensitivity (0.88), but a higher specificity (0.92) and AUROC (0.92, $P = 0.11$); however, there were only 33 Asian patients with available TE. There was no difference in F2-4 prevalence between Asian patients with available TE (25%) and FS (24%). Despite the small cohort, TE results for F2-4 in Asian patients were comparable to those in NAR patients (n = 181, AUROC 0.92 *vs* 0.87, $P = 0.35$).

For patients with both noninvasive tests available (Asian, n = 33; NAR, n = 176), agreement between FS and TE for stages F2-4 was higher in Asian than in NAR patients [94%

(κ = 0.86) *vs* 67% (κ = 0.35); $P < 0.001$]. The combination of FS and TE improved the accuracy for F2-4 in both Asian (97.0%) and NAR patients (72.7%), with AUROC values of 0.97 and 0.88, respectively (Table 2).

Most patients from the Asian region with available FS or TE achieved an SVR (85%; $n = 216$) and thus comparisons with NRs were not feasible. Baseline FS fibrosis scores, however, were higher in Asian than in NAR patients with an SVR (0.45 *vs* 0.36; $P < 0.001$), likely reflecting differences in F2-4 prevalence between the two study populations. Changes in FS fibrosis scores at week-12 follow-up were also comparable between Asian and NAR patients with an SVR (Δ = -0.04 *vs* -0.06; $P = 0.09$). In addition, mean TE measurements were comparable at baseline between Asian and NAR patients with an SVR (8.8 *vs* 7.8 kPa; $P = 0.56$), with no significant differences in changes at week 12 (-0.14 kPa *vs* -0.25 kPa, $P = 0.91$) or after therapy (-1.4 kPa *vs* -1.3 kPa, $P = 0.98$).

DISCUSSION

This large prospective cohort study in patients with chronic HCV provides validation of the diagnostic utility of serum markers and TE in relation to biopsy and IFN-based therapy. Few studies have addressed longitudinal changes in either serum markers or TE with therapy and, importantly, the present global study also provides the first evaluation of both noninvasive tests in patients from the Asia-Pacific region with chronic HCV. One limitation of this study was that TE data could only be obtained at 40 non-US study sites, as this device is not yet approved for use in the United States. Despite the limited cohort size for TE ($n = 214$) compared with FS ($n = 2055$), in accordance with prior observations, the overall results of this study indicate that both FS and TE have potential utility in the detection of moderate-severe-stage disease. However, the performance characteristics of these noninvasive tests (particularly TE) may be somewhat better for exclusion of cirrhosis[6,23]. For stages F2-4, FS and TE were effective in both Asian and NAR patients, but the agreement and accuracy of combined FS and TE were higher in the limited cohort of Asian patients with both tests ($n = 33$). Changes in FS and TE in relation to SVR were similar for both Asian and NAR patients.

This study shows that both pretreatment TE ($n = 217$) and FS ($n = 2082$) scores were lower in patients who achieved an SVR than in NRs, and that these differences were maintained through week 12 of therapy. Multivariate modeling indicated that older age and male sex (both predictive of lower virological responses in chronic HCV) were also independently associated with higher TE measurements at baseline. Other smaller studies, however, have failed to demonstrate similar baseline associations. A recent study from France evaluated TE and FS in 112 patients with chronic HCV receiving antiviral therapy, but did not include baseline biopsy or evaluation during therapy[11]. That study did not find any significant differences at baseline between patients with an SVR and NRs. Another study assessed TE alone before and after therapy in a Japanese chronic HCV cohort of 145 patients, and noted no differences at baseline between patients with an SVR and NRs[12]. Similar findings from another small French cohort evaluating TE alone have also been reported[24]. A recent meta-analysis of longitudinal studies in viral hepatitis indicated that both FS and TE could estimate treatment effect on fibrosis progression, although TE appeared to have early variability on treatment due to possible changes in necro-inflammatory activity[25]. The present study suggests that FS and TE could provide useful adjunctive information for the prediction of virological response prior to IFN-based therapy for chronic HCV. These noninvasive tests, however, likely reflect baseline differences in inflammatory response, but could complement established host-viral predictors of virological response to IFN-based therapy, such as HCV-RNA levels, viral Gt, race, and host *IL28B* polymorphism[26,27].

At follow-up, both FS and TE declined in patients who achieved an SVR. This is in accordance with prior observations that successful treatment with a biochemical response was associated with a decline in serum fibrosis marker indices or TE measurements in patients with chronic HCV[9-12,28,29]. A limitation of this study is that post-treatment biopsies were not required as part of these two clinical registration trials, which would have allowed for correlation between the observed declines in noninvasive test scores and changes in fibrosis or necro-inflammation. TE measurements may vary significantly with immune-mediated inflammatory responses in patients with chronic hepatitis B virus[30]. In contrast to other studies in patients with chronic HCV[6], however, this study also demonstrated a significant association between TE and histological necro-inflammatory activity at baseline. This association may have implications for establishing TE thresholds for different fibrosis stages in chronic HCV and may also explain the decline in liver stiffness measurements in patients with an established SVR in the present cohort. In this study, 80% of patients had no or mild fibrosis (stages F0-1) prior to treatment and thus were incapable of achieving a significant regression in fibrosis.

Both Asian and NAR cohorts demonstrated comparable performance characteristics for FS and TE. The observed accuracy and specificity for prediction of stages F2-4 in Asians, however, was higher in the small TE cohort. The combination of FS and TE in Asian patients resulted in a high accuracy for prediction of F2-4, with no false-positive results. Thus, biopsies for staging F2-4 could have been avoided in almost all Asian patients in this small cohort of 33 patients. There was excellent agreement between FS and TE in Asian patients, and this may partly relate to a slightly higher prevalence of advanced-stage disease and lower body mass index in the Asian cohort. Furthermore, increased waist circumference appears to be a common reason for failure of TE in European cohorts[19]. Although there are potential issues in obtaining adequate TE measurements in Asian patients

due to a narrow intercostal space, this was not a limiting factor in the present study[13]. Thus, the combination of FS and TE in Asian patients with chronic HCV merits further evaluation.

One of the strengths of this study is that sample collections were standardized per protocol for the two phase Ⅲ clinical trials, laboratory assessments were performed centrally, and all biopsies were evaluated by a single experienced liver histopathologist. Standardization significantly reduced the heterogeneity observed in prior studies comparing results across different geographic populations[23]. Biopsy sampling and observer error, however, are inherent limitations to the development and validation of all fibrosis biomarkers[31]. The experience of the pathologist may be more important than biopsy characteristics[32]. Furthermore, prior studies have indicated that the accuracy of liver biopsy (and noninvasive tests) is dependent on sample size[9,33-35]. In contrast to these prior observations, no significant change in the diagnostic accuracy of FS for stages F2-4 in > 900 patients with biopsies > 15 mm was found in the present study. Of note, TE accuracy for F2-4 appeared to decline in > 80 patients with this optimal biopsy length, although only 19 with biopsy F2-4 were in this cohort.

Prior studies have suggested that noninvasive performance indices for stages F2-4 and F4 are improved using sequential algorithms of FS and TE[6], or aspartate aminotransferase-to-platelet ratio index and FS[36]. In the present study, the F2-4 results for combined FS and TE indicated a comparable AUROC and agreement (0.88 and 71%, respectively) to those observed in a recent study from France in 302 patients with chronic HCV (0.91 and 72%, respectively) with a higher prevalence of advanced-stage disease[37]. Although discordance rates between sequential FS and TE with biopsy were not reported in the French study, prior data from that cohort indicated 84% concordance between biopsy and FS/TE agreement. This observation is similar to the 86% agreement noted in the present study; however, in contrast to the French cohort, cases of discordance between biopsy and FS/TE agreement were due to false positives with the noninvasive tests. In practical terms, agreement between FS and TE regarding prediction of F2-4 in the present study cohort could have avoided 71% of biopsies, although 10% of patients would still have been misclassified as having significant fibrosis. The discordance rate between FS and TE was 29%, with biopsy and TE agreement in most of the cases that appeared to have mild-stage disease. As expected, misclassification rates and discordance between FS and TE with biopsy were significantly reduced for prediction of F4.

With a broader range of available therapeutic options for patients with chronic HCV in the future, noninvasive measures that can accurately exclude advanced-stage disease will likely assume a more significant clinical role in the treatment decision process. Recent mathematical modeling indicates that a perfect biomarker of stages F2-4 may not exceed an AUROC of 0.9[38], and thus various issues regarding biopsy sampling error and noninvasive test discordance should be individualized when using these tests to predict a threshold of F2 in clinical practice. The observed heterogeneity among studies (including the present one) for optimized TE cutoffs indicates that a range of liver stiffness measurements for each fibrosis threshold in patients with chronic HCV may be more appropriate[39]. Standardization of AUROC curves or other methods to reduce effects of spectrum bias in disease prevalence allows for comparison of FS across studies, including selected cohorts within studies, but not for TE due to variable optimal thresholds[21]. No significant differences between observed and standardized AUROC values were found in the present study for either noninvasive measure.

In summary, this study demonstrates that a combination of serum and imaging noninvasive tests can be used for prediction of at least moderate-stage disease in a global cohort of patients with chronic HCV, including the potential of higher accuracy for the combination of FS and TE in Asian patients. Furthermore, some baseline differences in index values for both FS and TE were dependent on virological response and merit further evaluation in the context of IFN-based therapy.

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ACHIEVE-1 investigators: Benhamou Y, Hôpital Pitié-Salpêtrière, Paris, France; Berg T, University Hospital Charite Berlin/Virchow Klinikum, Berlin, Germany; Bourliere M, Hopital Saint-Joseph, Marseille, France; Chang TT, National Cheng Kung University Hospital, Tainan, Taiwan, China; Couzigou P, Hopital Haut-Leveque, Pessac, France; Davis G, Baylor University Medical Center, Dallas, TX, United States; Encke J, Universitätsklinikum Heidelberg, Germany; Forns X, Hospital Clinic i Provincial, Barcelona, Spain; Gerken G, University Hospital of Essen, Germany; Ghalib R, Liver Institute at Methodist Dallas, Texas; Gladysz A, Przychodnia przy Łowieckiej, Wroclaw, Poland; Grigorescu M, Spitalul Clinic de Urgenta, Cluj-Napoca, Romania; Häussinger D, Universitätsklinik Düsseldorf, Germany; LurieY, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; Manns M, Medizinische Hochschule Hannover, Germany; Marcellin P, Hopital Beaujon, Clichy, France; Moreno R, Hospital Universitario La Princesa, Madrid, Spain; Pawlotsky JM, Hopital Henri Mondor, Creteil, France; Plisek S, Klinika Infekcních Nemocí, Hradec Kralove, Czech Republic; Poupon R, Hôpital Saint-Antoine, Paris, France; Rodriquez-Torres M, Fundacion de Investigacion de Diego, Santurce, Puerto Rico; Safadi R, Holy Family Hospital, Nazareth, Israel; Schiff E, University of Miami, Florida, United States; Stanciu C, Institutul de Gastroen-

terologie si Hepatologie, Iasi, Romania; Trepo C, CHU de Lyon, Hôpital de l'Hôtel Dieu, France; Zeuzem S, J.W. Goethe University Hospital, Frankfurt, Germany.

ACHIEVE-2/3 investigators: Baruch Y, Rambam Health Care Campus, Haifa, Israel; Benhamou Y, Hopital Pitie-Salpetriere, Paris, France; Berg T, University Hospital Charite Berlin/Virchow Klinikum, Germany; Bourliere M, Hopital Saint-Joseph, Marseille, France; Bronowiki JP, Hôpital de Brabois, Vandoeuvre, France; Lee CM, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan; Cho M, Pusan National University Hospital, Busan, South Korea; Colle I, Ghent University Hospital, Ghent, Belgium; Delwaide J, CHU Sart Tilman, Liège, Belgium; Encke J, Universitätsklinikum Heidelberg, Germany; Gerken G, University Hospital Essen, Germany; Han KH, Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea; Häussinger D, Universitätsklinik Düsseldorf, Germany; Lee KS, Yongdong Severance Hospital, Seoul, South Korea; Langlet P, CHU Brugmann ULB-Site Victor Horta, Brussels, Belgium; Manns M, Medizinische Hochschule Hannover, Germany; Marcellin P, Hopital Beaujon, Clichy, France; Michielsen P, University Hospital Antwerp, Edegem, Belgium; Pawlotsky JM, Hopital Henri Mondor, Creteil, France; Poupon R, Hôpital Saint-Antoine, Paris, France; Schiff E, University of Miami, Florida; Trepo C, CHU de Lyon, Hôpital de l'Hôtel Dieu, France; Tur Kaspa R, Rabin Medical Center Beilinson Hospital, Petach Tikva, Israel; Um SH, Korea University Medical Center, Anam Hospital, Seoul, South Korea; Zeuzem S, J.W. Goethe University Hospital, Frankfurt, Germany.

COMMENTS

Background

Liver biopsy is an invasive procedure associated with significant costs and risk of complications. Noninvasive alternatives to biopsy for the determination of fibrosis stage include serum [FibroSURE (FS)] or imaging-based [transient elastography (TE) FibroScan] tests. The combination of these two modalities appears to have a good predictive value for excluding cirrhosis. Fibrosis is a predictor of virological response to chronic hepatitis C virus (HCV) therapy, and noninvasive tests may also be useful in this regard. Test values may vary with antiviral therapy for chronic HCV, perhaps due to changes in hepatic inflammation or with body habitus. Few studies have evaluated the utility of both these noninvasive modalities in patients with chronic HCV during interferon-based therapy, and there are limited data for these tests in Asian patients, particularly in comparison with non-Asian cohorts.

Research frontiers

Both FS and TE are validated measures for the noninvasive assessment of fibrosis. One important research issue is the determination of their utility in following changes in fibrosis or inflammation, e.g., as part of the natural history of disease or during and after antiviral therapy.

Innovations and breakthroughs

In this study, both FS and TE demonstrated good potential utility in the detection of moderate-severe-stage disease, but the performance characteristics of these noninvasive tests (particularly TE) may be somewhat better for exclusion of cirrhosis. Agreement between these tests and their accuracy for predicting disease stage may be higher in Asian than in non-Asian patients with chronic HCV. Patients that achieved a sustained virological response with interferon therapy appeared to have lower noninvasive test values at baseline.

Applications

Both FS and TE appear to have good clinical utility in predicting moderate-severe fibrosis prior to therapy, in both Asian and non-Asian patients with chronic HCV. Lower index scores at baseline may signify a better chance of responding to antiviral therapy.

Terminology

FS comprises a combination of simple biochemical blood tests that predict fibrosis. TE is an ultrasound-based imaging method to measure liver stiffness, which also predicts fibrosis.

Peer review

The authors investigated FS *vs* Fibroscan in treatment-naive patients with HCV. This article is unique and interesting.

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BRIEF ARTICLE

Evaluation of Fujinon intelligent chromo endoscopy-assisted capsule endoscopy in patients with obscure gastroenterology bleeding

Tarun Gupta, Mostafa Ibrahim, Jacques Deviere, André Van Gossum

Tarun Gupta, Mostafa Ibrahim, Jacques Deviere, André Van Gossum, Department of Hepato-Gastroenterology, Erasme Hospital-Free University of Brussels, Route de Lennik, 808, 1070 Brussels, Belgium

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Correspondence to: André Van Gossum, MD, PhD, Department of Hepato-Gastroenterology, Erasme Hospital-Free University of Brussels, Route de Lennik, 808, 1070 Brussels, Belgium. andre.vangossum@erasme.ulb.ac.be

Telephone: +32-25-553712 Fax: +32-25-554697
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Abstract

AIM: To investigate the potential benefit of Fujinon intelligent chromo endoscopy (FICE)-assisted small bowel capsule endoscopy (SBCE) for detection and characterization of small bowel lesions in patients with obscure gastroenterology bleeding (OGIB).

METHODS: The SBCE examinations (Pillcam SB2, Given Imaging Ltd) were retrospectively analyzed by two GI fellows (observers) with and without FICE enhancement. Randomization was such that a fellow did not assess the same examination with and without FICE enhancement. The senior consultant described findings as P0, P1 and P2 lesions (non-pathological, intermediate bleed potential, high bleed potential), which were considered as reference findings. Main outcome measurements: Inter-observer correlation was calculated using kappa statistics. Sensitivity and specificity for P2 lesions was calculated for FICE and white light SBCE.

RESULTS: In 60 patients, the intra-class kappa correlations between the observers and reference findings were 0.88 and 0.92 (P2), 0.61 and 0.79 (P1), for SBCE using FICE and white light, respectively. Overall 157 lesions were diagnosed using FICE as compared to 114 with white light SBCE ($P = 0.15$). For P2 lesions, the sensitivity was 94% vs 97% and specificity was 95% *vs* 96% for FICE and white light, respectively. Five (P2 lesions) out of 55 arterio-venous malformations could be better characterized by FICE as compared to white light SBCE. Significantly more P0 lesions were diagnosed when FICE was used as compared to white light (39 *vs* 8, $P < 0.001$).

CONCLUSION: FICE was not better than white light for diagnosing and characterizing significant lesions on SBCE for OGIB. FICE detected significantly more non-pathological lesions. Nevertheless, some vascular lesions could be more accurately characterized with FICE as compared to white light SBCE.

Key words: Endoscopy; Video-capsule; Small bowel; Obscure gastrointestinal bleeding; Arterio-venous malformation; Fujinon intelligent chromo endoscopy

Peer reviewers: Roberto de Franchis, Professor of Medicine (Gastroenterology), Department of Medical Sciences, University of Milan, Head, Gastroenterology 3 Unit, IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena Foundation, Via Pace 9, 20122 Milano, Italy; Josep M Bordas, MD, Department of Gastroenterology IMD, Hospital Clinic, Llusanes 11-13 at, Barcelona 08022, Spain; John Marshall, MD, Professor of Medicine, Division of Gastroenterology, University of Missouri School of Medicine, Columbia, MO 65201, United States

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INTRODUCTION

Obscure gastroenterology (GI) bleeding (OGIB) is defined as bleeding of unknown origin that persists or recurs after a negative initial or primary endoscopy (colonoscopy or upper endoscopy) result[1]. OGIB is frequently caused by a lesion in the small bowel[2]. Small bowel capsule endoscopy (SBCE), which allows the non-invasive visualization of mucosa throughout the entire small bowel, has revolutionized the exploration of small bowel diseases, and particularly the evaluation of OGIB[3]. Several studies showed that a SBCE is highly effective in detecting small-bowel lesions, with an overall diagnostic yield superior to that of push enteroscopy or radiological imaging, and comparable to double balloon enteroscopy (DBE)[4-7]. In addition, the SBCE procedure is technically easy and well tolerated, and carries a low risk of complications[6]. SBCE is the first-line examination in OGIB after a negative upper and lower gastrointestinal endoscopy[8].

The Fujinon intelligent chromo endoscopy (FICE) system is a new, virtual chromoendoscopy technique that enhances mucosal visibility, wherein the bandwidth of the conventional endoscopic image is narrowed down arithmetically using computerized spectral estimation technology[9]. Pohl *et al*[10,11] have previously demonstrated that FICE is comparable to conventional chromoendoscopy for surveillance of Barrett's esophagitis and detection of colonic polyps. There are limited data regarding use of FICE for small bowel lesions. In a recent case series of 17 patients, Neumann *et al*[12] used DBE with FICE technology and demonstrated a benefit in characterizing angiodysplasias, in delineating the submucosal capillary network, and in the detection of small bowel polyps.

The positive yield of capsule endoscopy for evaluation of OGIB is around 60%. This value further decreases if the examination is delayed after the index bleed[6]. The addition of FICE technology to SBCE may improve the diagnostic yield.

The objective of our study was to assess the value of a FICE equipped small bowel (SB) capsule for evaluation of obscure GI bleeding and for the detection and characterization of SB lesions.

MATERIALS AND METHODS

This study was conducted in the endoscopy unit of a tertiary care referral centre and teaching hospital. Sixty consecutive SBCE examinations (Pillcam SB2; Given Imaging Ltd, Israel), which were performed for OGIB, were chosen for the study. These examinations were performed to analyze either obscure-overt or obscure-occult GI bleeding in the preceding year. All included patients had received a polyethylene glycol-based bowel preparation before the examination and were given metoclopramide (10 mg) as a prokinetic before swallowing the capsule. These examinations were already analyzed under white light only by the senior consultant, who has more than eight years experience in conducting capsule endoscopy and enteroscopy. Two GI fellows with similar experience (70 procedures) in conducting capsule endoscopy (observers 1 and 2) analyzed the examinations with and without FICE enhancement using Rapid Reader (Version 6: Given Imaging Ltd, Israel); their experience was considered as reaching a minimum threshold number to assess competency. The fellows were allowed to use the speed of their choice for reading. Randomization was such that a fellow did not assess the same examination with and without FICE enhancement. In practice, fellow 1 read the videos 1 to 30 without FICE and the following videos (31 to 60) with FICE, and vice-versa for fellow 2. The entire examination was conducted either under white light or FICE technology. Rapid Reader (Version 6) provides three channels for FICE technology (Table 1)[13]. Channel 1 was used for examination with FICE by both fellows, as the wavelength settings for red, green and blue light were found to be most suited to small bowel evaluation. This was determined by the consultant and the observers after a pilot study of 15 patients. No historical data for the patients were made available to the observers. The analysis was conducted by the GI fellows on the department's central computer. Individual findings were password protected. The boundary between the jejunum and the ileum was fixed empirically as the half time of the capsule small-bowel passage time. Both fellows used the common structured terminology as per the Given Capsule Endoscopy working group[14]. Both fellows were blinded to the findings (white light) of the senior consultant, which were taken as the reference. The senior consultant analyzed the results based on his reference findings. All findings were labeled as P0, P1 and P2 lesions (non-pathological, intermediate bleed potential-superficial erosions, red spots-or high potential bleed potential-hemorrhagic erosions, ulcer, arterio-venous malformations, or tumor) by the senior consultant[15]. Each time a lesion was seen in the FICE mode and not with white light, it was re-evaluated by the senior consultant. Comparison was performed for the detection of SB lesions, as well for characterization, i.e. vascular pattern, of a lesion that was considered as an arterio-venous malformation or a tumor as a Gastrointestinal stromal tumor (GIST) or an adenocarcinoma.

Institutional review board approval has been obtained from the local Ethical Committee for this retrospective study.

Table 1 Wavelengths in nm (Fujinon intelligent chromo endoscopy)[13]

	Red	Green	Blue
FICE channel 1	595	540	535
FICE channel 2	420	520	530
FICE channel 3	595	570	415

FICE: Fujinon intelligent chromo endoscopy.

Table 2 Number of lesions (P0, P1, P2) detected by the two observers and the senior consultant, respectively

	Observer 1	Observer 2	Consultant (reference findings)
Total	117	154	131
P0	20	27	15
P1	37	55	41
P2	60	72	75

Statistics analysis

Inter-observer correlation was calculated by using kappa statistics. A kappa value of 1 was considered perfect agreement and 0 was considered no agreement at all. Sensitivity and specificity for P2 lesions was calculated for FICE and white light SBCE. SPSS software [version 16, (SPSS Inc, Chicago, IL)] was used for the analysis. Statistical analysis of categorical values was conducted using the Fischer's exact test. Significance was accepted at a value of $P < 0$ .05.

RESULTS

Visualization of the entire small bowel was achieved in 56 patients; in the remaining four, the capsule reached the terminal ileum. All patients had excellent bowel preparation. In these 60 patients, observers 1 & 2 detected 117 [P0 (20), P1 (37), P2 (60)] and 154 [P0 (27), P1 (55), P2 (72)] lesions, respectively, as compared to 131 [P0 (15), P1 (41), P2 (75)] by the senior consultant (Table 2). The commonest P2 lesions were arterio-venous malformations (n = 43), followed by small bowel ulcerations (n = 27), small bowel tumors (GIST n = 2; adenocarcinoma n = 1), and Dieulafoy's lesions (n = 2). Intra-class correlation for P1 and P2 lesions was calculated as 0.69 and 0.89 respectively. Intra-class kappa correlations between the observers and reference findings were 0.88 (P2), 0.61 (P1) for SBCE using FICE, and 0.92 (P2), 0.79 (P1) for SBCE with white light respectively. Overall 153 lesions were diagnosed by the two observers with SBCE using FICE as compared to 118 by SBCE with white light (P = 0.15). Considering the senior consultant's findings as the gold standard, for P2 lesions, the sensitivity was 94% (0.87-1.02) *vs* 97% (0.92-1.02) and specificity was 95% (0.87-1.03) *vs* 96% (0.86-1.04) for FICE and white light respectively. In 5/55 arterio-venous malformations, analysis of SBCE with FICE did not help in detection, but did result in better characterization of these P2 lesions as compared to analysis with white light (Figures 1-3). The mean duration for analyzing SBCE with FICE was longer than when white light was used (75 min *vs* 55 min). Significantly more P0 lesions were diagnosed by the 2 observers when FICE was used as compared to white light (39 *vs* 8, $P < 0.01$) (Table 3).

DISCUSSION

Capsule endoscopy has become a cornerstone in the non-invasive evaluation of OGIB. The high diagnostic yield of intestinal SBCE has been proven in several studies, and ranges from 55% to 81%[4-7,16,17]. The rate of rebleeding in patients with OGIB and negative SBCE is significantly lower (4.6%) compared with those with a positive SBCE (48%)[18].

The FICE system is based on a computed spectral estimation technology that processes the reflected photons to reconstruct virtual images with a choice of different wavelengths. This leads to enhancement of the tissue microvasculature as a result of the differential optical absorption of light by hemoglobin in the mucosa. These abnormal areas can be defined by magnification[9,19,20].

Ours is the first study in the literature that has used the potential advantages of FICE to assist the detection and characterization of OGIB lesions by capsule endoscopy and using the new Rapid Reader 6 (Given Imaging Ltd, Israel). Our study suggests that addition of FICE technology does not help in identifying clinically significant lesions during analysis of SBCE. This contrasts with existing data on FICE. Ringold *et al*[21] described two cases in which high-contrast imaging with FICE improved the visibility of normal mucosal vessels and aided in the detection of vascular ectasias that were not easily seen by routine double-balloon enteroscopy. Similarly, improved detection rates for arterio-venous malformations and adenomatous polyps were noted by Neumann *et al*[12] with FICE aided enteroscopy in a series of 17 patients. Recently, Imagawa *et al*[22] reported that FICE may improve visibility of small bowel lesions that were detected under white light by video capsule. This series is not really comparable, in that 75 out of the 145 lesions were tumors. Moreover, the authors of the study did not report the detection rate or characterization of the lesions. Interestingly, they observed that setting 1 of the FICE system was the best one for improving visibility, confirming our selection of channel 1 as the most effective.

Arterio-venous malformations were also the predominant lesions in our study. Despite this, the number of lesions detected by FICE and white light capsule endoscopy were similar. In fact, FICE detected significantly more non-pathological (P0) lesions as compared to white light. There was similar concordance for FICE and white light SBCE for pathological lesions (P2) in our study. The mean duration for analyzing SBCE with FICE was longer than when white light was used, thus diminishing the appeal of FICE for SBCE.

There are a few reasons for the limited effectiveness of SBCE with FICE for identifying lesions in our study. All patients had excellent bowel preparation; hence, there was better visualization by white light alone. This is supported by a recent meta-analysis that showed that small-bowel purgative preparation (polyethylene glycol solution or sodium phosphate) improves the diagnostic yield of the examination[23]. We also found that white reflections tend to increase during the FICE mode, potentially interfering with the image quality. This may be particularly important for clearly discerning ulcerated lesions and may result in their erroneous reporting. In addition, channel 1

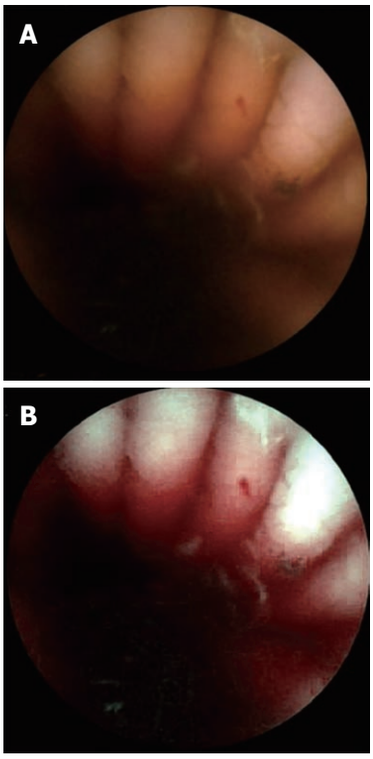

Figure 1 A non-pathological lesion (P0) with Fujinon intelligent chromo endoscopy.

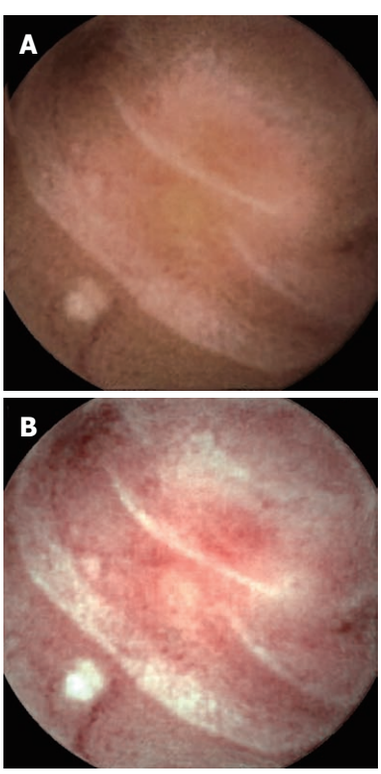

Figure 2 A non-pathological lesion (P1) with Fujinon intelligent chromo endoscopy.

of FICE had a prominent red hue; hence, small red spots and prominent folds appeared as angio-ectasias when

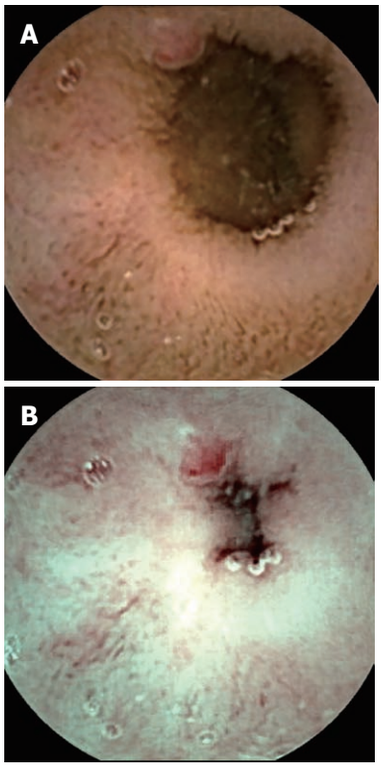

Figure 3 Vascular lesion (P2) with white light and Fujinon intelligent chromo endoscopy.

FICE was used. Several lymphangectasias, floating clots, and prominent veins were also found amongst the P0 lesions diagnosed by SBCE when FICE was used, thus reducing its utility.

We found that assessment of SBCE with FICE to be somewhat useful in the better characterization of vascular lesions. Arborization of the vascular network in the case of arterio-venous malformations was better assessed by FICE as compared to white light in 5 patients during this study, making their detection easier.

The strong points of the study were the use of the central workstation computer for capsule analysis, thereby enabling use of similar settings, image resolution and password protection of the reference and observers' findings. The common structured terminology, as per the Given Capsule Endoscopy working group, was used by both observers[14].

A first limitation of our study was the analysis of retrospective data and the relatively small patient numbers. A longer prospective study with patient follow up may be required to confirm our findings.

A second limitation is the absence of a reference standard for defining the SB lesions. Obviously, in several patients, lesions were confirmed by enteroscopy, biopsies, radiology, or surgical specimens, but not for all. The absence of a well-defined standard reference is a common problem in studies assessing the diagnostic yield of capsule endoscopy.

In conclusion, this is the first study to assess the ability of a FICE-equipped video capsule for detecting and

Table 3 Number of small bowel findings (patients) described by the two observers when using either white light or Fujinon intelligent chromo endoscopy compared to the senior consultant (white light)

	White light (two observers)	FICE (two observers)	*P* value (FICE *vs* WL for observers 1 and 2)	White light (senior consultant)
P0 (no patients)	8 (8)	39 (24)	< 0.01	15 (10)
P1 (no patients)	43 (33)	49 (32)	0.30	41 (29)
P2 (no patients)	67 (38)	65 (37)	1.00	75 (40)

FICE: Fujinon intelligent chromo endoscopy; WL: White light.

characterizing SB lesions in cases of OGIB. SBCE with FICE technology did not improve diagnostic yield, but did detect more P0 lesions. FICE may improve the characterization of vascular lesions in a limited number of cases. Further improvements in software technology for FICE may be required to enhance its clinical application in capsule endoscopy for OGIB.

In our study, FICE-assisted SBCE analysis was not better than white light for diagnosing and characterizing significant lesions in the evaluation of OGIB. FICE detected significantly more non-pathological lesions; however, some vascular lesions could be more easily characterized on FICE as compared to white light SBCE.

COMMENTS

Background

Obscure gastroenterology bleeding (OGIB) is frequently caused by a lesion in the small bowel. Capsule endoscopy that allows visualization of the small bowel is now recognized as the first step procedure in case of OGIB. Arteriovenous malformations are the most frequent lesions that are responsible for OGIB. Quality of the video images, as well as the experience of the reader, are mandatory for providing accurate results.

Research frontiers

The goals of video capsule endoscopy are the detection of lesions and their characterization. The reader is invited to qualify the lesion as clinically significant or not. This is important for defining further therapeutic strategy. Improvement of image quality may improve the diagnostic yield.

Innovations and breakthroughs

The Fujinon intelligent chromo endoscopy (FICE) system is a new, virtual chromoendoscopy technique that has been designed to enhance visibility of lesions. This technique, which is used for conventional endoscopy, has been adapted for video capsule endoscopy (given imaging). In this retrospective study, FICE did not improve the detection of small bowel lesions in comparison with the white light, but did allow better characterization of some vascular lesions.

Applications

FICE technology is now available in clinical practice. Its use should be reserved for the better characterization of lesions that were detected by white light. Some prospective studies should be performed for confirming the present data.

Terminology

OGIB is defined as digestive bleeding of unknown origin that persists or recurs after a negative endoscopy work-up. Video capsule endoscopy is a non-invasive method allowing a complete investigation of the small bowel. FICE is a new chromoendoscopic tool that has been designed for enhancing visibility of lesions in comparison to white light.

Peer review

This interesting study is well conducted and the results and conclusions are of practical application.

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BRIEF ARTICLE

Operative link for gastritis assessment *vs* operative link on intestinal metaplasia assessment

Massimo Rugge, Matteo Fassan, Marco Pizzi, Fabio Farinati, Giacomo Carlo Sturniolo, Mario Plebani, David Y Graham

Massimo Rugge, Matteo Fassan, Marco Pizzi, Surgical Pathology and Cytopathology Unit, Department of Diagnostic Medical Sciences and Special Therapies, University of Padova, 35100 Padova, Italy
Massimo Rugge, Istituto Oncologico del Veneto IOV-IRCCS, 35100 Padova, Italy
Fabio Farinati, Giacomo Carlo Sturniolo, Department of Gastroenterology and Surgical Sciences, Gastroenterology Unit, University of Padova, 35100 Padova, Italy
Mario Plebani, Department of Diagnostic Medical Sciences and Special Therapies, Clinical Chemistry Unit, University of Padova, 35100 Padova, Italy
David Y Graham, Digestive Disease Division, Baylor College of Medicine, Veterans Administration Hospital, Houston, TN 77030, United States
Author contributions: Rugge M, Fassan M, Farinati F, Sturniolo GC, Plebani M and Graham DY designed the research; Rugge M, Fassan M, Farinati F and Pizzi M collected the data; Rugge M, Fassan M, Pizzi M and Graham DY analyzed the data; Rugge M and Fassan M wrote the paper; Graham DY revised the final version of the manuscript.
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Correspondence to: Massimo Rugge, MD, FACG, Full Professor of Surgical Pathology, Chair of the Surgical Pathology and Cytopathology Unit, Department of Medical Diagnostic Sciences and Special Therapies, University of Padova, Istituto Oncologico del Veneto IOV-IRCCS, Via Aristide Gabelli 61, 35100 Padova, Italy. massimo.rugge@unipd.it
Telephone: +39-049-8218990 Fax: +39-049-8272277
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Abstract

AIM: To compare the reliability of gastritis staging systems in ranking gastritis-associated cancer risk in a large series of consecutive patients.

METHODS: Gastric mucosal atrophy is the precancerous condition in which intestinal-type gastric cancer (GC) most frequently develops. The operative link for gastritis assessment (OLGA) staging system ranks the GC risk according to both the topography and the severity of gastric atrophy (as assessed histologically on the basis of the Sydney protocol for gastric mucosal biopsy). Both cross-sectional and long-term follow-up trials have consistently associated OLGA stages Ⅲ-Ⅳ with a higher risk of GC. A recently-proposed modification of the OLGA staging system (OLGIM) basically incorporates the OLGA frame, but replaces the atrophy score with an assessment of intestinal metaplasia (IM) alone. A series of 4552 consecutive biopsy sets (2007-2009) was retrieved and reassessed according to both the OLGA and the OLGIM staging systems. A set of at least 5 biopsy samples was available for all the cases considered.

RESULTS: In 4460 of 4552 cases (98.0%), both the high-risk stages (Ⅲ + Ⅳ) and the low-risk stages (0 + Ⅰ + Ⅱ) were assessed applying the OLGA and OLGIM criteria. Among the 243 OLGA high-risk stages, 14 (5.8%) were down-staged to a low risk using OLGIM. The 67 (1.5%) incidentally-found neoplastic lesions (intraepithelial or invasive) were consistently associated with high-risk stages, as assessed by both OLGA and OLGIM ($P < 0.001$ for both). Two of 34 intestinal-type GCs coexisting with a high-risk OLGA stage (stage Ⅲ) were associated with a low-risk OLGIM stage (stage Ⅱ).

CONCLUSION: Gastritis staging systems (both OLGA and OLGIM) convey prognostically important information on the gastritis-associated cancer risk. Because of its clinical impact, the stage of gastritis should be included as a conclusive message in the gastritis histology report. Since it focuses on IM alone, OLGIM staging is less sensitive than OLGA staging in the identification of patients at high risk of gastric cancer.

Key words: Gastritis; Staging; Atrophic gastritis; Intestinal metaplasia; Operative link for gastritis assessment; Operative link on intestinal metaplasia assessment

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INTRODUCTION

Gastric mucosal atrophy is by far the greatest risk factor for non-hereditary, intestinal-type distal gastric cancer (GC)[1]. The gold standard for atrophy assessment is histology, but non-invasive tests (mainly pepsinogen serology) are also applied for this purpose[1-3].

According to the current international literature, atrophy is defined as the "loss of appropriate glands". This definition covers both the "loss" of native glands (replaced by fibrosis) and the metaplastic replacement of the appropriate (native) glands due to antral intestinalization, corpus antralization [i.e., spasmolytic polypeptide-expressing metaplasia (SPEM)] and/or intestinalization[2].

Consistent evidence correlates the extent/topography of atrophy with the risk of GC, and it is on these grounds that a system for staging gastritis [the operative link for gastritis assessment (OLGA) staging system] was proposed[4]. Gastritis stages (0 to Ⅳ) express increasing extents of atrophy, as assessed histologically on antral and corpus biopsies. In different epidemiological settings, both cross-sectional and long-term follow-up studies have consistently allocated a small minority of gastritis patients to stages Ⅲ-Ⅳ, associating only this population with a significantly higher GC risk (high-risk OLGA stages)[3,5-10]. OLGA stages Ⅲ-Ⅳ have also been consistently associated with molecular tissue markers of high-risk gastritis[11,12]. These correlations potentially support the advisability of endoscopic follow-up for such high-stage patients.

A significant correlation has been demonstrated between high-risk OLGA stages and pepsinogen serology; this correlation between organic and functional disease supports the rationale for implementing serology in GC secondary prevention programs[5].

A recently-proposed modification of the OLGA staging system (OLGIM)[10] basically incorporates the same staging frame, but replaces the "global" score for atrophy (in its different phenotypic variants) with the histological assessment of intestinal metaplasia (IM) alone. The rationale behind the OLGIM proposal lies in the fact that IM is easier to assess histologically than the "global" spectrum of the atrophic lesions (as in the OLGA approach).

This study compares the OLGA and OLGIM staging systems in the assessment of gastritis-associated gastric cancer risk (i.e., stages 0- Ⅰ-Ⅱ = low-risk stages *vs* Stages Ⅲ-Ⅳ = high-risk stages).

MATERIALS AND METHODS

Patients

All gastric biopsy sets recorded between January 2007 and December 2009 were retrieved from the archives of the Surgical Pathology and Cytopathology Unit at the Department of Diagnostic Medical Sciences and Special Therapies of Padova University. Case recruitment did not distinguish between initial or follow-up endoscopies; all the patients considered were natives of the Veneto region and underwent endoscopy at the same institution (Padova Teaching Hospital). The institute's ethical regulations on research conducted on human tissues were followed.

For all the cases considered, a set of at least 5 biopsy samples was available (2 samples from the antral mucosa, 1 from the mucosa of the incisura angularis, and 2 from the anterior and posterior walls of the oxyntic stomach). In accordance with the biopsy sampling protocol, additional specimens had been obtained from any focal lesions. Details were always available regarding the site of the biopsy.

For the purposes of the study, pediatric patients (under 18 years old), patients with a history of autoimmune gastritis, and those who had undergone esophageal or gastric surgery, esophagogastric endomucosal resection or submucosal dissection were excluded.

Original slides or serial sections (4-6 microns thick) obtained from archival paraffin-embedded tissue samples [hematoxylin and eosin, Alcian- Blue and Periodic Acid Schiff stain and Giemsa for *Helicobacter pylori* (*H. pylori*)] were histologically re-considered.

Histology

Three trained gastroenterology pathologists (Fassan M, Pizzi M and Rugge M), blinded to any endoscopic or clinical information, jointly examined all the histology specimens and reached a consensus on the score for each of the histological variables considered. For OLGA staging purposes, atrophy was defined as the loss of appropriate glands with or without epithelial metaplasia (i.e., IM in antral and/or oxyntic biopsy samples; pseudo-pyloric metaplasia in oxyntic biopsy samples)[2]. Glandular atrophy was scored according to the recommendations in the OLGA staging tutorial[4,13]. For OLGIM staging purposes, only IM was considered and scored according to the recommendations of the OLGIM proposers[10]. The inter-observer consistency in assessing the two staging systems was tested by means of *K* statistics in a randomly selected series of 100 cases and was ranked as "excellent" (k coefficient = 0.75 and 0.77 for OLGA and OLGIM, respectively).

Any incidentally-found neoplastic lesions were histologically assessed according to internationally validated criteria[14,15]. Within the spectrum of gastric intra-epithelial neoplasia (IEN), the categories considered were: low-grade

Table 1 Demographic and pathological features of the study population

	Total	Stage 0		Stage Ⅰ		Stage Ⅱ		Stage Ⅲ		Stage Ⅳ	
		OLGA	OLGIM	OLGA	OLGIM	OLGA	OLGIM	OLGA	OLGIM	OLGA	OLGIM
Cases	4552	2967	3018	951	927	391	378	199	188	44	41
Age (yr) mean ± SD (median)	55.1 ± 15.8 (57.0)	51.0 ± 15.9 (50.5)	51.1 ± 15.9 (50.6)	60.7 ± 13.1 (63.1)	61.1 ± 12.8 (63.3)	64.4 ± 10.9 (65.4)	64.7 ± 10.8 (66.3)	67.1 ± 9.6 (67.7)	67.0 ± 9.7 (67.7)	67.5 ± 13.1 (67.6)	67.4 ± 13.3 (68.4)
Sex (M/F)	2085/2467	1344/1623	1364/1 654	447/504	438/489	164/227	158/220	98/101	94/94	32/12	31/10
Hp + ve (%)	1698 (37.30)	1188 (40.00)	1198 (39.70)	322 (33.90)	318 (34.30)	130 (33.20)	126 (33.30)	47 (23.60)	45 (23.90)	11 (25.00)	11 (26.80)
Neoplastic lesions[1] (No. of cases)	67	3	3	2	3	3	4	39	38	20	19

[1]Including low- and high-grade intraepithelial neoplasia and invasive gastric cancers. SD: Standard deviation; M: Males; F: Females; *Hp*: *Helicobacter pylori*; OLGA: Operative link for gastritis assessment; OLGIM: Operative link on intestinal metaplasia assessment.

IEN (LG-IEN) and high-grade IEN (HG-IEN). The inter-observer consistency in assessing IEN lesions was tested by means of *K* statistics in a randomly selected series of 35 IEN/gastric cancer cases and was ranked as "fair to good" (k coefficient = 0.66). Gastric cancer was diagnosed in the presence of neoplastic epithelia infiltrating the lamina propria.

Statistical analysis

The strength of the association between the stage of gastritis and the demographic and pathological features was calculated using Wilcoxon's signed rank test (W), and the modified Kruskal-Wallis nonparametric test for trend (KW), as appropriate. The inter-observer consistency in classifying atrophic and IEN lesions was tested in two series of 100 and 35 randomly-selected biopsy sets, respectively, calculated as the overall proportion of agreement (the number of total paired observations in which the same result was obtained), and tested using Fleiss's kappa statistic[16]. Stata software (Stata Corporation, College Station, TX) was used for all the calculations. A P value < 0.05 was considered significant.

RESULTS

Overall, 4552 consecutive biopsy sets were considered. The male/female ratio was 1/1.18, and the patients' mean age was 55.1 years (median 57.0, range 20-89). For the males, the mean age was 55.0 years (range 20-88), while for the females it was 55.1 years (range 20-89).

Overall, 2967 biopsy sets (65.2%) showed no atrophic changes (i.e., stage 0 gastritis according to both OLGA & OLGIM) and the prevalence of the low-risk stages was 94.7% and 95.0% according to OLGA and OLGIM, respectively (Table 1).

In all, there were 67/4552 (1.5%) incidentally-found neoplastic lesions (either intraepithelial or invasive), including: 21 cases of LG-IEN; 6 cases of HG-IEN; 40 cases of GC (6 cases of diffuse-type proximal GC; 34 of intestinal-type distal GC). The M/F ratio among the neoplastic patients was 1.03/1 and their mean age (67.8 years; median 68; range 45-86) was significantly higher than that of the non-neoplastic patients (mean 54.8 years; median 57 years; range 20-89) (W; $P < 0.001$).

H. pylori was assessed histologically in 1698 patients (37.3%). Its prevalence among the neoplastic cases was 26 (38.8%), disregarding any previous eradication therapies. However, this study only focuses on the neoplastic risk associated with the stage of gastritis, without any specific reference to, or speculation about, the impact of the etiology on the morphogenesis of the gastric disease.

For 4460 out of 4552 cases (97.98%), low-risk stages (0 + Ⅰ + Ⅱ) and high-risk stages (Ⅲ + Ⅳ) were staged consistently using either OLGA or OLGIM criteria (Figure 1). For the 92 (2.0%) cases staged inconsistently, 14 were considered as low-risk using the OLGIM criteria and as high-risk according to OLGA. No cases staged as high-risk by OLGIM were down-staged when the OLGA criteria were applied.

The number of patients with high-risk stages (Ⅲ-Ⅳ) was 243 according to OLGA and 229 according to OLGIM, i.e., among the 243 OLGA high-risk stages, 16 (6.6%) were down-staged by OLGIM; in particular, 14 OLGA stages Ⅲ and Ⅳ were classified as stage Ⅱ according to OLGIM.

In all, 67 intraepithelial (i.e., non-invasive) or invasive neoplastic lesions were detected. All the 27 intraepithelial neoplasia coexisted with intestinalized glands. Among the 40 cases of invasive adenocarcinoma, 6 (15%) were located in the cranial stomach and histologically featured a solid/diffuse-type GC; the other 34 (85%) were cases of intestinal-type GC. After distinguishing between low- and high-risk stages, a significant association emerged between stages Ⅲ-Ⅳ and both intraepithelial and invasive neoplasia according to both the staging systems [W; $P < 0.001$ for both (Table 1)].

Fifty-nine of 67 (88.1%) and 57/67 (85.1%) intraepithelial or invasive neoplastic lesions were associated with high-risk OLGA and OLGIM stages, respectively. Six gastric cancers were detected in cases classified as OLGA/OLGIM low-risk gastritis: all 6 were diffuse-type gastric cancers (Figure 2). Two intestinal-type GCs coexisting with OLGA stage Ⅲ were associated with OLGIM stage Ⅱ gastritis (Figures 2 and 3).

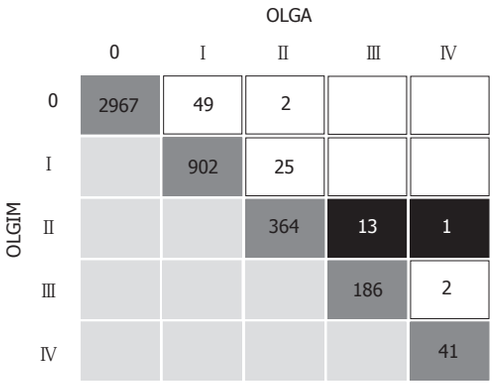

Figure 1 Distribution of patients by the two considered staging systems (operative link for gastritis assessment *vs* operative link on intestinal metaplasia). OLGA: Operative link for gastritis assessment; OLGIM: Operative link on intestinal metaplasia.

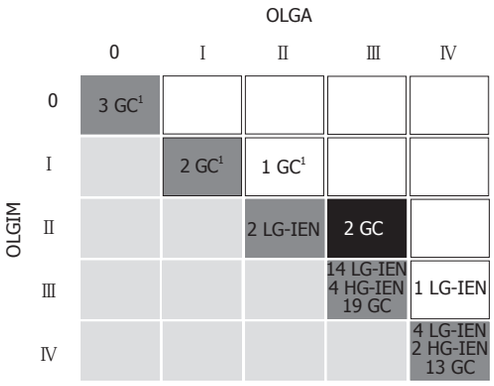

Figure 2 Distribution of preneoplastic/neoplastic lesions in the two considered staging-systems (i.e., operative link for gastritis assessment (OLGA)] and operative link on intestinal metaplasia assessment stages (OLGIM)]. [1]Diffuse-type (signet ring) gastric cancer. GC: Gastric cancer; LG-IEN: Low-grade intraepithelial neoplasia; HG-IEN: High-grade intraepithelial neoplasia.

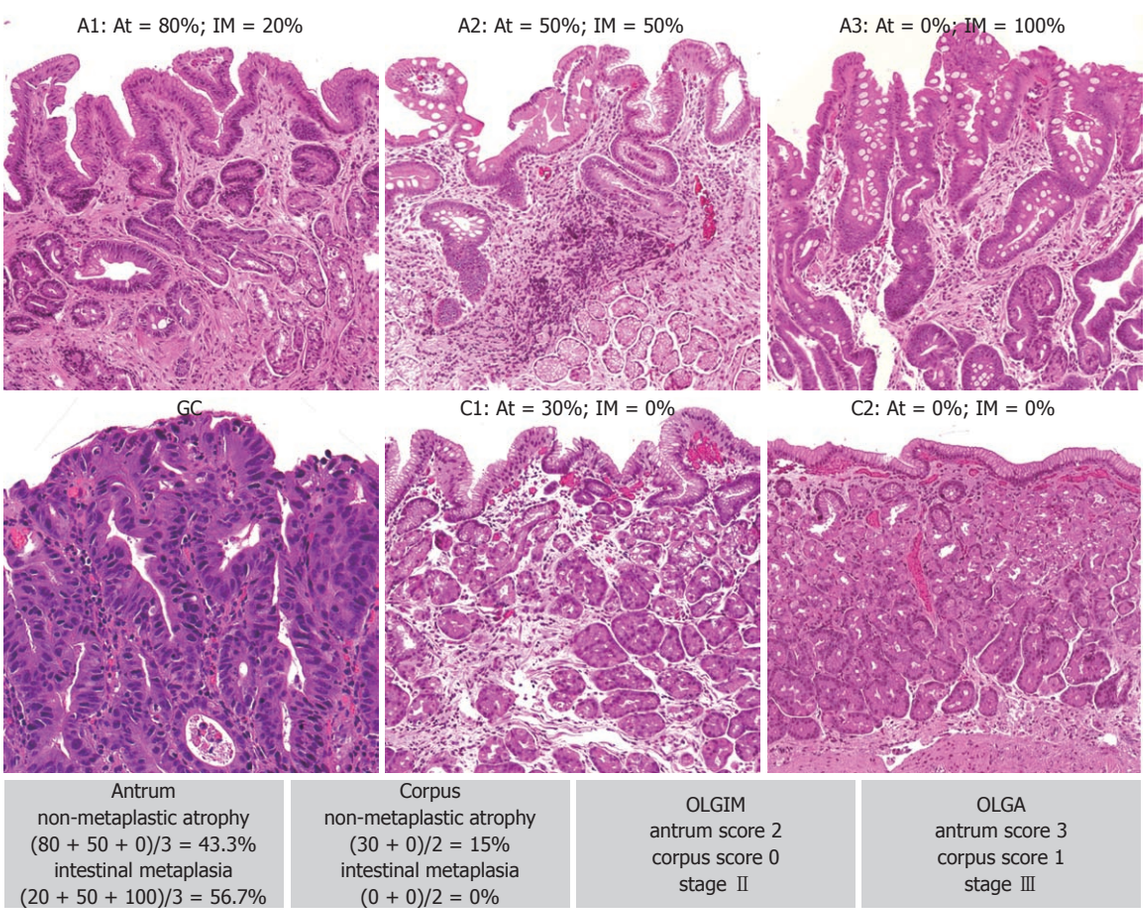

Figure 3 One of two gastric cancers developing in operative link for gastritis assessment stage Ⅲ, but the operative link for gastritis assessment stage Ⅱ, gastric mucosa. Representative images of 6 biopsy samples (3 from the mucosecreting/antral compartment, 1 from the lesion, and 2 from the oxyntic/corpus compartment) labeled according to site of origin (A: antral/angular; GC: gastric cancer; C: corpus), showing the percentages of atrophic/metaplastic lesions and the consequent operative link for gastritis assessment/operative link on intestinal metaplasia assessment stages.

DISCUSSION

Gastric cancer is still a health priority in Western Europe and it represents an epidemiological emergency in Eastern Europe, Central and Eastern Asia, and some South American regions[17-26].

Gastric mucosal atrophy is generally considered the "cancerization field" in which GC develops. Based on such a rationale, and incorporating the experience gained with the Sydney system[27], the OLGA staging system ranks the gastritis-associated cancer risk according to both the topography and the extent of gastric mucosa atrophy[2,4-6,26-28].

As regards topography, extensive biopsy sampling protocols (such as the one applied in the Houston experience) potentially increase the prognostic reliability of any staging system and they should be theoretically preferred. In line with the Sydney system[27], however, both OLGA and OLGIM systems require a (minimum) set of 5 biopsy samples for gastritis staging, which should strike a good compromise between the priority of obtaining a representative biopsy set and the operative limits of daily clinical practice[27].

The OLGIM proposal replaces the "global" atrophy score with a semiquantitative assessment of intestinal metaplasia (extent and site); according to its proposers, such a strategy should considerably increase the inter-observer agreement - an undeniable advantage[29,30].

In the present series of more than four thousand consecutive cases, 98% of stage Ⅲ-Ⅳ gastritis were consistently staged by applying either OLGA or OLGIM. The finding that patients' ages increase with higher stages further supports the clinico-biological plausibility of both systems.

It is worth noting that two intestinal-type GCs (both coexisting with OLGA stage Ⅲ gastritis) were found associated with OLGIM-Ⅱ gastritis (i.e., low-risk atrophic gastritis). In fact, by focusing on IM alone, OLGIM is less sensitive in identifying high-risk gastritis, and this may result in the down-staging of patients who should be offered follow-up[29,30]. Comparative studies involving non-GI (i.e., specialist) pathologists are needed to test which system (OLGA or OLGIM) provides more accurate results in relation to the time and effort spent on the histology assessment.

In his seminal work on gastric carcinogenesis, Pelayo Correa described mucosal atrophy as a cardinal step in the biological pathway that may eventually progress to gastric adenocarcinoma[31]. The current definition of gastric mucosal atrophy includes two different phenotypes: (1) loss (shrinkage or disappearance) of glands, which are replaced by fibrotic expansion of the lamina propria; and (2) metaplastic replacement of native glands by intestinalized and/or pseudopyloric glands (corpus antralization or SPEM). Focusing on IM alone excludes pseudopyloric metaplasia (i.e., SPEM) from the spectrum of atrophy, although it has recently been found increasingly important in gastric carcinogenesis (through transdifferentiation from mature chief cells following parietal cell loss)[32-34].

Lastly, considering IM alone carries the risk of us losing the correlation between gastric atrophy (as assessed by gastric serology, and PgI in particular) and its organic counterpart (resulting from the concurrence of the different phenotypes of gastric atrophy)[35-38].

In conclusion, gastritis staging effectively conveys an unequivocal message regarding the gastritis-associated cancer risk and may point to follow-up strategies tailored to a patient-specific clinico-pathological situation. This priority supports the inclusion of staging in gastritis histology reports and the demand for further efforts to improve the reproducibility of any staging criteria - bearing in mind that "easier" does not necessarily mean "better"!

COMMENTS

Background

Despite its declining incidence, gastric cancer is still the second cause of cancer-related death worldwide. Gastric atrophy is by far the main risk factor for non-hereditary, intestinal-type distal gastric cancer. Consistent evidence relates the extent and topography of atrophy to the gastric cancer risk.

Research frontiers

Building on current knowledge of the biology of gastritis, an international group of gastroenterologists and pathologists has proposed a system for reporting gastritis in terms of stage [the operative link for gastritis assessment (OLGA) staging system]. The OLGA staging system basically ranks gastric cancer risk according to the extent/topography of gastric atrophy. A recently proposed modification of the OLGA, called the OLGIM staging system, basically incorporates the OLGA frame, but considers intestinal metaplasia alone, instead of the global atrophy score.

Innovations and breakthroughs

In a large series of consecutive retrospective cases, the present study compares the reliability of the OLGA *vs* the operative link on intestinal metaplasia assessment (OLGIM) systems in ranking gastritis-associated cancer risk. Both staging systems stratify gastritis patients in different cancer risk classes, providing a clinico-biological rationale for a patient-tailored clinico-endoscopic follow-up. OLGIM staging, however, includes cases of gastritis at high risk of gastric neoplasia in its low-risk stage Ⅱ.

Applications

Gastritis staging conveys information on the clinico-pathological outcome of gastritis that is relevant to patient management, leading to an adequate patient stratification according to different cancer risks.

Terminology

OLGA is the acronym for "operative link for gastritis assessment", an innovative gastritis staging system proposed by an international group of gastroenterologists and pathologists that basically ranks the gastric cancer risk according to the extent and severity of gastric atrophy. OLGIM is the acronym for "operative link on intestinal metaplasia assessment", a modification of the OLGA staging system, which basically adopts the OLGA staging frame, but replaces the global atrophy score with an assessment of intestinal metaplasia alone.

Peer review

The authors compared the reliability of histological gastritis staging systems, OLGA and OLGIM, in ranking gastritis-associated cancer risk in a large series of consecutive cases. They concluded that both staging systems provide prognostically useful information on the gastritis-associated cancer risk even if OLGIM is less sensitive.

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BRIEF ARTICLE

Comparative study of laparoscopic *vs* open gastrectomy in gastric cancer management

Giuseppe S Sica, Edoardo Iaculli, Livia Biancone, Sara Di Carlo, Rosa Scaramuzzo, Cristina Fiorani, Paolo Gentileschi, Achille L Gaspari

Giuseppe S Sica, Edoardo Iaculli, Sara Di Carlo, Rosa Scaramuzzo, Cristina Fiorani, Paolo Gentileschi, Achille L Gaspari, Department of Surgical Sciences, "Tor Vergata" University Hospital, 00133 Rome, Italy
Livia Biancone, Department of Internal Medicine, Gastrointestinal Unit, "Tor Vergata" University Hospital, 00133 Rome, Italy
Author contributions: Sica GS and Iaculli E designed and wrote the paper; Iaculli E and Scaramuzzo R did the statistical analysis; Scaramuzzo R, Fiorani C, Di Carlo S and Gentileschi P performed database and analysis of the results; Biancone L and Gaspari AL reviewed the paper and edited it.
Correspondence to: Dr. Giuseppe S Sica, MD, PhD, Department of Surgical Sciences, "Tor Vergata" University Hospital, Viale Oxford 81, 00133 Rome, Italy. sica.giuseppe@fastwebnet.it
Telephone: +39-06-20902928 Fax: +39-06-20902926
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Abstract

AIM: To compare long-term results of gastric cancer patients undergoing laparoscopic and open gastrectomy in a single unit.

METHODS: From February 2000 to September 2004, all patients with adenocarcinoma of the stomach were assessed to entry in this longitudinal prospective non-randomized trial. Primary endpoint was cancer-related survival and secondary endpoints were overall survival, evaluation of surgical complications and mortality.

RESULTS: Fifty-eight patients were enrolled. Forty-seven patients were followed-up (range 11-103, median 38 mo). Four patients were lost at follow up. Twenty-two patients underwent a laparoscopic gastric surgery (LGS) and 25 had a standard open procedure (OGS). No statistical difference was found between the two groups in terms of 5 years cancer-related mortality rate (50% *vs* 52%, $P = 1$), and 5 years overall mortality rate (54.5% *vs* 56%, $P = 1$). Accordingly, cancer-related and overall survival probability by Kaplan-Meier method showed comparable results ($P = 0.81$ and $P = 0.83$, respectively). We found no differences in surgical complications in the 2 groups. There was no conversion to open surgery in this series.

CONCLUSION: LGS is as effective as OGS in the management of advanced gastric cancer. However LGS cannot be recommended routinely over OGS for the treatment of advanced gastric cancer.

Key words: Advanced gastric cancer; Laparoscopy; Laparoscopic cancer surgery; Long-term outcomes; Survival

Peer reviewer: Stephen M Kavic, MD, FACS, Assistant Professor of Surgery, Department of Surgery, University of Maryland School of Medicine, 22 South Greene Street, Room S4B09, Baltimore, MD 21201, United States

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INTRODUCTION

Radical surgical resection of the stomach and regional lymph nodes dissection is still the mainstream of the treatment of advanced gastric cancer (AGC). In 1994 Kitano *et al*[1] reported the first laparoscopic gastric resection and Billroth reconstruction in a patient with early gastric cancer (EGC). Laparoscopic gastric surgery (LGS) has been shown to improve short-term results and quality of life, compared to standard techniques[2,3,4] and has become an acceptable alternative approach in the management of

EGC especially in Japan and Korea[5,6,7]. The experience of laparoscopic gastric surgery in the western world is smaller because most of the gastric cancers are seen in an advanced stage and LGS is not considered an acceptable alternative to standard open surgery as yet. Little is known about long-term results and tumour recurrence after laparoscopic gastrectomy in AGC and most of the reported series are small[8].

However, the National Institute for Clinical Excellence in the United Kingdom has recently published an overview on interventional procedures regarding LGS[9]. LGS emerges as a feasible and oncologically secure procedure with several benefits.

Until large and well-conducted trials are undertaken also in the west and also including patients with AGC, case-control studies and preliminary data are important to guide the necessary acceptance to LGS.

LGS has been performed by our group since 1999. We herein report our 5 years results of a consecutive series of patients who have undergone LGS or standard open gastric surgery mostly for AGC over a four year period (February 2000-September 2004) and followed-up accordingly. The aim of this study was to evaluate long-term results of laparoscopy-assisted gastrectomy compared to open standard procedures in terms of overall and cancer-related survival probabilities.

MATERIALS AND METHODS

Between February 2000 and September 2004 all patients with preoperative diagnosis of non-metastatic gastric adenocarcinoma seen at our institution were evaluated to enter in this longitudinal prospective non-randomized trial. All patients underwent diagnostic work-up according to a standard protocol [endoscopy with biopsy, total-body computed tomography (CT) scan, endoscopic ultrasound (EUS) in selected patients]. Eligible patients were assigned either to laparoscopic or open procedure at the multidisciplinary cancer meeting, after the preoperative staging, solely on the basis of the availability of an upper gastrointestinal surgeon with experience in advanced laparoscopic surgery and accordingly to patients' preference. Demographics and preoperative clinical data analyzed included gender, age, body mass index (BMI), the American Association of Anaesthetists (ASA) score, pathological tumor-node-metastasis (pTNM) stage, tumour location, histological differentiation. Patients were stratified into 3 groups (proximal, distal, intermediate) according to tumour location.

The eligibility criteria were: histological diagnosis of gastric cancer at any stage ($\geqslant$ 1b), tumour location below the cardias and above the pylorus, pre-operative work-up indicating a surgical resection with an aim to perform R0 gastrectomy. Exclusion criteria were: age < 18 or > 75 years, BMI > 32, ASA score > 3, previous gastric surgery, presence of lynitis plastica, para-aortic lymph-nodes involvement, systemic metastases, contraindications to laparoscopy (severe cardiovascular disease, large abdominal wall or diaphragmatic hernias, *etc*). There was no selection bias during the enrollment process and inclusion criteria were the same for both groups.

Fifty-eight consecutive patients were enrolled in the study. Data from 47 patients who underwent open gastric surgery (OGS, $n = 25$) or LGS ($n = 22$) and then entered our oncologic follow-up (FU) protocol were included. Of this group of patients 91.5% had an AGC and underwent a D2 lymph nodes dissection. Four patients (8.5%) had a tumour in stage 1b and had a dissection of regional nodes (D1) + group β. The type of surgical resection and lymph nodes dissection were the same in both groups according to tumour location and disease stage. OGS required a midline xipho-umbilicus or a bilateral sub costal access. LGS was performed using a 3 ports plus liver retractor technique; at the end of the procedure the first port access was enlarged up to 5 cm to retrieve the specimen and to complete the already stapled esophago-jejunum side-to side anastomoses, if required. The same preoperative and postoperative care was provided to both groups. Adjuvant therapy, where appropriate, was similar for all patients. Patients were followed-up in the outpatient setting until cancer-related death or to the endpoint of the study, September 30, 2009. Follow-up schedule included history, clinical examination, blood tests and tumour markers every 6 mo. Upper gastrointestinal endoscopy was performed twice a year for the first 2 years then as clinically indicated. Total body CT scan was performed at 6 mo from surgery and then annually. Positron emission tomography (PET)-CT was indicated in selected cases.

Primary endpoints of the study were cancer related survival rate and disease-free interval. Secondary endpoints were overall survival rate and evaluation of number of harvested lymph nodes. Cancer related survival represents the number of patients alive at the end of the follow-up period (5 years). Disease free interval represents the period of time in which the patients were free of disease during the follow-up period.

All data were collected on a Microsoft® Excel spreadsheet and derived retrospectively from the database. Results were expressed as median and range of observed values. Statistical analyses were obtained with SPSS 17.0. Qualitative data were compared using Fisher's exact test or Pearson's χ^2 test; quantitative data showing a normal distribution were compared using a paired *t* test. Survival rates were assessed by Kaplan-Meier method and analyzed by the log-rank test. Regardless of the statistical analysis used, $P < 0.05$ was considered statistically significant.

RESULTS

Eighty-three patients with diagnosis of gastric cancer were referred to our unit from February 2000 to September 2004. Of these patients, 58 were eligible to enter the study. Eight patients were excluded thereafter because of distant nodal involvement, liver metastases or peritoneal carcinosis found intra-operatively. Three patients died within 90 d of surgery, one in the OGS group from complications related to surgery and one in each group due to pre-surgical co-morbidity. Ac-

Table 1 Clinical characteristics of patients

	LGS (n = 22)	OGS (n = 25)	P value
Median age, yr (range)	67 (38-75)	68 (54-75)	0.07
Gender (M/F)	13/9	13/12	0.4
BMI, kg/m^2 (range)	23 (18-25)	22 (18-26)	0.9
ASA score, median (range)	2 (0-3)	2 (0-3)	0.3
0	5 (23%)	2 (8%)	
1	3 (14%)	8 (32%)	
2	10 (45%)	10 (40%)	
3	4 (18%)	5 (20%)	
Tumor location (%)			0.9
Upper	5 (23%)	6 (24%)	
Middle	7 (32%)	7 (28%)	
Lower	10 (45%)	12 (48%)	
Lauren histology (%)			0.7
Intestinal	12 (54%)	12 (48%)	
Diffuse	10 (46%)	13 (52%)	
pTNM [1997] (%)			0.6
I b	2 (9%)	2 (8%)	
II	9 (41%)	13 (52%)	
IIIa/b	10 (45%)	7 (28%)	
IV	1 (5%)	3 (12%)	

LGS: Laparoscopic gastric surgery; OGS: Open gastric surgery; M/F: Male/Female; BMI: Body mass index; ASA: The American Association of Anaesthetists; pTNM: Pathological tumor-node-metastasis.

cording to this trial purpose, these cases were not included in the analysis of long-term results. Four patients were lost at follow-up (8.5%), two in the LGS group and 2 in the OGS group (P = 1). The median follow up was 38 mo (range 11-103) in the whole series, 39 mo in the LGS group (range 12-100) and 38 mo in the OGS group (range 11-103; P = 0.7).

Among the 47 patients left for the purpose of the study (26 males and 21 females; median age 68, range 38-75), 22 underwent LGS (46.8%) and 25 had a standard OGS (53.2%). According to tumour location and intraoperative findings, 12 patients had a total gastrectomy (5 LGS, 7 OGS) and 25 a sub-total gastrectomy (13 LGS, 13 OGS). No conversion from laparoscopic to open surgery occurred in the LGS group.

There were no significant differences between the two groups with respect to demographics and preoperative data considered, type of resection, type of lymphadenectomy and number of harvested lymph nodes. The characteristics of the study population and the type of surgical procedures are summarized in Tables 1 and 2 respectively.

A radical D2 lymph node dissection was performed in all 43 cases (91% in LGS and 92% in OGS) with a preoperative stage of T $\geqslant$ 2 or for any N+. Two patients in the LGS group (9%) and 2 in the OGS group (8%) had a preoperative T1\N0 diagnosis confirmed by EUS and underwent D1 + β lymphadenectomy according to our institution practice guidelines. The numbers of harvested lymph-nodes was 29 ± 7 in the LGS group and 30 ± 9 in the OGS group, thus showing no differences between the two techniques (P = 0.46). Three patients (1 in LGS, 4.5% and 2 in OGS, 8%) were found intra-operatively to have tumour invasion of the adjacent organs underwent an additional procedure, namely liver wedge resection, splenectomy and transverse colon resection.

Table 2 Operative findings

	LGS (n = 22)	OGS (n = 25)	P value
Type of gastric resection (%)			0.7
Subtotal	17 (77%)	18 (72%)	
Total	5 (23%)	7 (28%)	
Reconstruction (%)			0.6
Billroth 2	3 (14%)	2 (8%)	
Roux-en-Y	19 (86%)	23 (92%)	
Lymphadenectomy (%)			1
D1	2 (9%)	2 (8%)	
D2	20 (91%)	23 (92%)	
Lymph-nodes retrived	29 ± 7	30 ± 9	0.5

LGS: Laparoscopic gastric surgery; OGS: Open gastric surgery.

In the whole study group, 24 patients (51%) died of tumour recurrence. In the LGS group recurrence was observed in 11 patients (50%), the median disease free interval was 28 mo (range 12-60) and the median survival 39 mo (range 12-60). There was no port-site recurrence. In the OGS group recurrence was observed in 13 patients (52%) after a median of 26 mo (range 10-60) (P = 0.69). Median survival of the OGS group was 38 mo (range 11-60) (P = 0.73). There was no difference in the type of recurrence in the 2 study groups. Loco-regional recurrence (defined as tumor found by TC or TC-PET involving nodes or peritoneum in the region of the previous gastrectomy, including nodes around porta hepatis) or carcinosis occurred in 6 patients, liver metastases and jaundice in 12 patients and diffuse/systemic metastases in 6 patients

Cancer related mortality rate was comparable between the two groups (P = 1).

There was no difference in overall survival rate between the two groups (P = 0.9). Twelve patients (54.5%) in the LGS group and 14 patients (56%) in the OGS group died for any cause during the surveillance period.

Cancer related survival and overall survival (Figures 1 and 2, respectively) show similar Kaplan-Meier curves (P = 0.81 and 0.83, respectively) for OGS and LGS patients. Survival analyses are shown in Table 3.

DISCUSSION

Oncologically adequate surgical resection is the only therapeutic modality that offers curability in patients with gastric cancer.

According to our institution policy and current international guidelines the operation of choice for gastric cancer is a total or subtotal gastrectomy, depending on tumour location, with D2 dissection in advanced gastric cancer and with D1 ± β lymphadenectomy in non infiltrating tumours.

This paper reports a consecutive series of gastrectomies for cancer performed in a single unit over a 4-year period. The number of patients included in this report is small and come after a short learning curve experience but represents the first sensible group of patients who

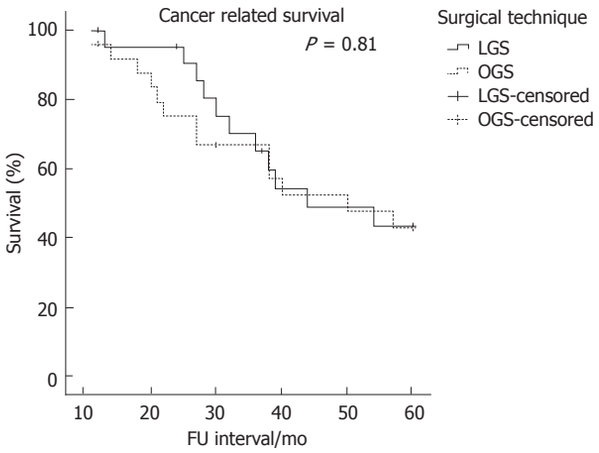

Figure 1 Kaplan-Meier estimates of cancer related survival. LGS: Laparoscopic gastric surgery; OGS: Open gastric surgery; FU: Follow up.

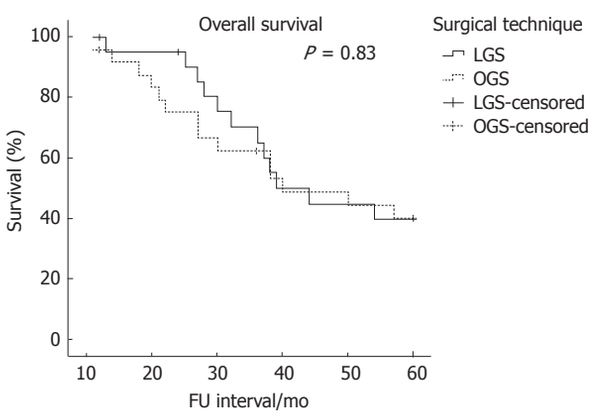

Figure 2 Kaplan-Meier estimates of overall survival. LGS: Laparoscopic gastric surgery; OGS: Open gastric surgery; FU: Follow up.

has completed follow-up so far. Almost half of the procedures were performed laparoscopically and the patients were not randomly assigned to one or the other procedure nor selected on the basis of pre-operative criteria such as tumour extension, BMI or other. However, to have a comparable number of patients, the 2 procedures were alternated whenever possible and this may well represent a sensible bias in the present series. Furthermore patients in the laparoscopic group were slightly younger than those operated with an open approach ($P = 0.07$); this is possibly related to an undesired selection bias based on patients' wishes.

Since the first report of laparoscopic gastric resection for cancer[1], various institutions around the world routinely perform LGS. However the vast majority of this experience comes from Asian medical institutions and is limited to the treatment of EGC. The standard lymphadenectomy for EGC is limited to the regional lymph nodes and even though LGS has been reported more difficult than OGS, from the 8th questionnaire survey of endoscopic surgery in Japan that recently examined 4799 cases of laparoscopic assisted distal gastrectomy, it appears clear that LGS is a safe procedure with an extremely low surgical mortality[10].

Table 3 Tumour recurrence and survival analyses

	LGS (n = 22)	OGS (n = 25)	P value
Overall 5 yr mortality rate	12 (54.5%)	14 (56%)	1
Cancer-related 5 yr mortality rate	11 (50%)	13 (52%)	1
FU (5 yr) accomplishment	20 (91%)	23 (92%)	1
Type of recurrence			0.9
Port-site M	0 (0%)		
Locoregional/carcinosis	2 (18%)	4 (31%)	
Hepatic M	6 (55%)	6 (46%)	
Distant M	3 (27%)	3 (23%)	
Median survival, mo (range)	39 (12-60)	38 (11-60)	0.7
Disease free interval, mo (range)	28 (12-60)	26 (10-60)	0.6

LGS: Laparoscopic gastric surgery; OGS: Open gastric surgery; FU: Follow up.

Table 4 Survival analyses by Kaplan-Meier method

	AGC + T1b (n = 47)	AGC (n = 43)
Cancer related survival probability analyses (P)	0.81	0.92
Overall survival probability analyses (P)	0.83	0.92

AGC: Advanced gastric cancer.

If the feasibility and safety of LGS in the treatment of EGC has been proven, it is also true that several reports have shown the efficacy of LGS in the cure of EGC with results comparable to those of an OGS series[11,12].

On the other hand, the role of LGS in the treatment of AGC is still controversial and many doubts remain about its safety and oncological efficacy. However it has to be said that many case series have been recently published showing adequate resection margins and lymph nodes retrieval for LGS in AGC whilst long term results still lack of comfortable numbers[13-16].

We hereby report our 5 years follow-up of a consecutive series of patients undergoing laparoscopic *vs* OGS, mostly with AGC. More precisely over 90% of the patients recruited for this study underwent surgery for AGC whilst four patients (2 from each group) were in stage 1b. The survival curves were re-analyzed after the exclusion of this subgroup of patients but no differences were found ($P = 0.9$) as shown in Table 4.

We demonstrate the technical feasibility of a radical lymph nodes dissection; a complete D2 dissection was always possible when appropriate, and the number of lymph-nodes dissected in all the cases of tumour > T1 complied with the Western (harvested N > 25) criteria of R0 resection for AGC. Also, no significant differences were found between the 2 groups (mean lymph nodes: LGS 29 *vs* OGS 30). Moreover the Kaplan-Meier survival curves show similar results when survival of the LGS group is compared to that of the OGS group (5 years cancer related survival 50% for LGS and 48% for OGS, $P = 0.81$).

Laparoscopic surgery may offer some advantages in

oncologic patients: less surgical stress, less blood loss and complications, better cellular immunity and cytokine release pattern[7,8,17,18].

Given the little data regarding gastric cancer and considering the characteristics of these patients (fragile, elderly, often malnourished and immune-compromised) and the specific biology of the tumour itself (subserosal cancer with transcoelomic metastatic capacity), at the moment we can only speculate that LGS could potentially offer a better outcome over OGS. This preliminary experience hasn't changed our clinical practice. However, currently we don't offer LGS to patients with known metastatic nodes to the lymph-nodes of the second level.

A large, multicenter and randomized trial should be designed to document long term benefit of LGS in the treatment of AGC according to tumour-stage (TNM) or preoperative evaluation such as age, co-morbidity, neo-adjuvant therapy.

In conclusion, LGS seems to be as effective as OGS in the treatment of advanced gastric cancer considering the long-term survival probabilities. However, given the lack of strong evidence on a large study population and the technical expertise required to complete a R0 laparoscopic gastrectomy, the authors cannot endorse LGS over OGS for the treatment of advanced gastric cancer outside clinical trials in specialized centres.

COMMENTS

Background

Gastric cancer is one of the leading causes of death for solid tumours worldwide. Surgical resection is the only hope of cure. Laparoscopic gastric resection is accepted as an alternative method of cure for early gastric cancer, especially in Asia. Its role in the treatment of advanced gastric cancer is a matter of debate.

Research frontiers

Laparoscopic surgery in cancers of the digestive tract (colon) has been demonstrated to bring some advantages, including a possible role in reducing recurrence. The authors looked at the role of laparoscopic gastric resection for advanced gastric cancer and showed that even this technically demanding procedure is safe and feasible in expert hands and that the long term results are equivalent to those of standard open surgery.

Innovations and breakthroughs

In this paper a case-control study was undertaken. Laparoscopic gastrectomy is compared to open surgery. This is one of the few papers with a long term follow-up of a consecutive series of patients with advanced gastric cancer undergoing laparoscopic surgical resection. It shows a good surgical result and reinforced the known advantages of gastrointestinal laparoscopic surgery. Case-control studies are important to guide surgical practice in cancer management.

Applications

This study could represent a step forward in minimally invasive surgery of the upper digestive tract. The long term results show that laparoscopic gastric resection is an oncologically adequate procedure that can be carried out with acceptable morbidity and mortality.

Peer review

This manuscript provides a single center comparison between laparoscopic and open gastrectomy, finding no significant difference in surgical resectability or oncologic outcomes. Overall, it is well written.

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BRIEF ARTICLE

"Liverscore" is predictive of both liver fibrosis and activity in chronic hepatitis C

Shoukat Ali Arain, Qamar Jamal, Amir Omair

Shoukat Ali Arain, Department of Pathology, Ziauddin University, 4/B, Shara-e-Ghalib, Block 6 Clifton, Karachi 75600, Pakistan
Shoukat Ali Arain, College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia
Qamar Jamal, Department of Pathology, Ziauddin University, 4/B, Shara-e-Ghalib, Block 6 Clifton, Karachi 75600, Pakistan
Amir Omair, Department of Medical Education, King Saud bin Abdulaziz University for Health Sciences, Riyadh 11426, Saudi Arabia
Author contributions: Arain SA and Jamal Q planned the study, carried out majority of the laboratory work and wrote manuscript; Omair A performed statistical analysis and was involved in final editing of the manuscript. All the authors have approved final draft.
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Correspondence to: Dr. Shoukat Ali Arain, Senior Lecturer in Pathology, College of Medicine, Alfaisal University, PO Box 50927, Riyadh 11533, Saudi Arabia. sa_arain@hotmail.com
Telephone: +966-1-2157677 Fax: +966-1-2157677
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Abstract

AIM: To formulate a noninvasive index predictive of severity of liver fibrosis and activity in chronic hepatitis C.

METHODS: This cross sectional study was conducted on polymerase chain reaction positive, treatment naïve patients. Fibrosis was staged on a five point scale from F0-F4 and activity was graded on a four point scale from A0-A3, according to the METAVIR system. Patients were divided into two overall severity groups, minimal disease (< F2 and < A2) and significant disease (≥ F2 or ≥ A2). Eleven markers were measured in blood. Statistically, the primary outcome variable was identification of minimal and significant overall disease. Indices were formulated using β regression values of different combinations of nine statistically significant factors. Diagnostic performance of these indices was assessed through receiver-operating characteristic curve analysis.

RESULTS: A total of 98 patients were included and of these 46 had an overall clinically significant disease. Our final six marker index, Liverscore for Hepatitis C, consisted of age, alanine transaminase, gamma-glutamyl transpeptidase, apolipoprotein A-1, alpha-2 macroglobulin and hyaluronic acid. The area under the curve was found to be 0.813. On a 0-1 scale, negative predictive value at a cutoff level of ≤ 0.40 was 83%, while positive predictive value at ≥ 0.80 remained 89%. Altogether, 61% of the patients had these discriminative scores.

CONCLUSION: This index is discriminative of minimal and significant overall liver disease in a majority of chronic hepatitis C patients and can help in clinical decision making.

Key words: Chronic hepatitis C; Staging and grading; Liver fibrosis and activity; Noninvasive assessment; Liverscore

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INTRODUCTION

Chronic hepatitis C (CHC) induces injury and inflammation of the liver, which appears to be responsible for

the associated fibrogenesis[1]. Morbidity and mortality in CHC is associated with the development of cirrhosis and its complications. The rate of fibrosis progression varies markedly from person to person and over time. The risk of developing cirrhosis varies from 10% to 20% over a period of 20 years[2]. Treatment of CHC is complex, costly, and associated with side effects that are difficult to accept in a population that is predominantly asymptomatic. Furthermore, about half of the patients with genotype 1, and slightly lesser than that in other genotypes, fail to respond to anti-viral therapy[3,4].

Treatment decisions are recommended to be individualized on the basis of severity of the liver disease, treatment response rates, co-morbid conditions and the readiness of the patient for treatment[5]. Therefore, assessment of the fibrosis stage and rapidity of progression of fibrosis (necro-inflammation) may help in determining the prognosis and the need of therapy in an individual patient. A prevalence peak of advanced fibrosis and cirrhosis in CHC patients is expected during this decade. Thus, increasing numbers of patients will require assessment. Furthermore, with the development of anti-fibrotic therapies, there will be a need for regular and more frequent monitoring[6].

In underdeveloped countries like Pakistan, with a high prevalence of disease and resource constrains[7,8], it seems unrealistic to offer treatment to all patients. There is a need to identify patient categories to rationalize the need for therapy. For patients showing minimal disease, treatment may be deferred with follow up for disease progression. Treatment may be offered to patients with progressive disease or else, safer, better tolerated, and more cost effective therapy will become available.

Liver biopsy is the current tool for the assessment of liver disease; nonetheless it is an invasive procedure and may be associated with complications. Moreover, the biopsy facility is not available in remote areas and is not always possible[9]. Alternative strategies are being actively evaluated, such as imaging and non-invasive biochemical monitoring of liver disease. Some of the biochemical markers, especially panels of multiple markers in the form of indices are promising, and may reduce the number of liver biopsies for assessment of liver disease[10]. The purpose of this study was to evaluate the predictive value of noninvasive biomarkers for the diagnosis of overall liver disease categories in CHC.

MATERIALS AND METHODS

This was a cross sectional study to determine the diagnostic accuracy of noninvasive biomarkers, conducted at Ziauddin University Hospital, Karachi, Pakistan from June 2006 to July 2010. The study was approved by Ethics Review Committee of the university. Treatment naïve polymerase chain reaction proven CHC patients, of 20 to 60 years of age, were included in the study. Patients with HBV co-infection and diabetes mellitus were excluded. Patients with history of other chronic inflammatory conditions, alcohol intake, and blood disorders requiring frequent blood transfusions were also excluded. A questionnaire was completed for every patient to document possible variables, such as demographic factors, and to rule out causes of exclusion. Written informed consent was obtained from each patient.

Liver biopsy

A percutaneous liver biopsy was performed with a 16-18 gauge modified Menghini aspiration needle (Surecu® TSK, Japan). Tissue was formalin fixed, paraffin-embedded, and processed for light microscopic examination. Along with standard hematoxylin and eosin staining, slides were also stained with connective tissue stains. Only liver biopsy specimens of more than 10 mm length and having not less than five portal tracts were included in the study[11]. Biopsy specimens with evident pathology, but without identification of the correct number of portal tracts, were also included. Histological features of the liver biopsy specimens were analyzed according to the METAVIR group scoring system[11,12], by one pathologist (Jamal Q), without any knowledge of the clinical or biochemical data. Every specimen was staged for fibrosis on a five-point scale; F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with rare septa; F3 = numerous septa without cirrhosis; and F4 = cirrhosis. There is an element of subjectivity in classifying patients in different stages, especially in biopsy specimens, because of the majority of hepatic lobules being partial. However, we considered any fibrosis beyond portal tracts as significant (F2). Fibrosis was considered F3 when 50% or more portal tracts showed fibrous septa extending beyond the portal tracts. Necroinflammatory lesions were graded on a four point scale on the basis of an algorithm based on the severity of focal lobular necro-inflammation and piecemeal necrosis; A0 = no histological activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity[11].

On the basis of fibrosis stage and necro-inflammatory grade patients were divided into two overall severity groups; minimal disease $\leqslant$ F2 and $<$ A2 and significant disease $\geqslant$ F2 or $\geqslant$ A2[13].

Biochemical markers

Chemical analysis was carried out for alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), total and direct bilirubin, apolipoprotein A-1 (Apo A-1), haptoglobin, alpha-2 macroglobulin (A2M), hyaluronic acid (HA), hydroxyproline (HYP) and proline.

The ALT assay was performed by an International Federation of Clinical Chemistry standardized ultraviolet enzymatic method, GGT was assayed by an enzymatic colorimetric method, the ALP assay was performed by a colorimetric method and total and direct bilirubin assays were performed according to the method described by Jendrassik and Grof. All enzymatic activities were measured at 37 ℃. All the above assays were performed using reagents from Roche Diagnostics®. A2M and haptoglobin assays were performed by an immuno-turbidimetric method using polyclonal rabbit anti-human antibodies (DakoCytomation Denmark Code. Nos. Q0102 &

Table 1 Baseline characteristics of all patients (*n* = 98)

Characteristic	Value
Age (yr)	36.0 ± 10.6
Male	52 (53)
Activity grade	
Absent (A0)	32 (33)
Mild (A1)	40 (41)
Moderate (A2)	20 (20)
Severe (A3)	6 (6)
Fibrosis stage	
No fibrosis (F0)	21 (20)
Portal fibrosis without septa (F1)	35 (36)
Portal fibrosis with rare septa (F2)	29 (30)
Numerous septa without cirrhosis (F3)	10 (10)
Cirrhosis (F4)	3 (3)

Results are shown as mean ± SD or *n* (%).

Table 2 Univariate analysis for significant and minimal disease

	Significant[1] (*n* = 46)	Minimal (*n* = 52)	*P* value
Age (yr)	39.6 ± 11.4	32.9 ± 8.7	0.002
Gender			0.15
Male	28 (61)	24 (46)	
Female	18 (39)	28 (56)	
Bilirubin total (mg/dL)	0.62 ± 0.34	0.49 ± 0.30	0.04
Bilirubin direct (mg/dL)	0.21 ± 0.17	0.15 ± 0.08	0.04
ALT (U/L)	53.2 ± 39.2	32.60 ± 15.90	0.002
ALP (U/L)	80.4 ± 26.9	81.70 ± 32.30	0.78
GGT (U/L)	51.8 ± 47.2	23.80 ± 16.90	< 0.001
Haptoglobin (g/L)	1.05 ± 0.45	1.05 ± 0.49	0.99
A2M (g/L)	2.56 ± 0.64	2.24 ± 0.55	0.009
Apo-A1 (mg/dL)	96.85 ± 23.70	114.2 ± 29.80	0.002
Hydroxyproline (μmol/L)	9.7 ± 7.0	13.5 ± 9.30	0.03
Proline (μmol/L)	123.0 ± 78.10	144.2 ± 106.4	0.27
Hyaluronic acid (ng/mL)	100 ± 150	23.90 ± 16.40	0.001

[1]Significant disease, A2, 3 or F2-4. Minimal disease, A0, 1 and F0, 1, Results are shown as mean ± SD or *n* (%). ALT: Alanine transaminase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; A2M: Alpha-2 macroglobulin; Apo-A1: Apolipoprotein A-1.

Q0330). The Apo-A1 assay was also performed immune-turbidimetrically, using reagents from Randox® UK (Cat. No. LP2989). HA was measured by an enzyme-linked binding protein microplate assay (Corgenix® Inc. United States). HYP and proline assays were carried out by high performance liquid chromatography (HPLC), using a method as described by Lange and Mályusz[14]. A HPLC unit LC-20AT was used with ultraviolet-visible spectroscopy photodiode array detector (SPD-M20A) and system controller, CBM-20A, all from Shimadzu Corporation Japan. A 6 mmID × 15 cm, C_{18} (Shim-pack CLC-ODS Japan) chromatography column was used.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (Chicago, IL). A χ^2 test was used to compare the qualitative and independent *t* test was used to compare the quantitative variables. A *P* value of less than 0.05 was considered statistically significant.

The primary outcome for statistical analysis was identification of the patients with minimal and significant disease. The factors identified in the univariate analysis were subjected to logistic regression. Keeping overall disease as binary variable, β regression coefficients of the factors were obtained to generate the indices. The diagnostic value of these indices was compared by calculating the area under receiver operating characteristic (AUROC) curve. The best suited 6 marker index, chosen for final analysis, was transformed into a standardized scale ranging from 0-1 through percent ranking as liverscore for hepatitis C.

Through ROC curve analysis, the diagnostic value of the Liverscore at various cutoff points was assessed by calculating sensitivity, specificity, and positive and negative predictive values, for overall disease.

RESULTS

Initially 104 CHC patients were enrolled. Six patients were excluded; the biopsy specimens of four patients had less than five portal tracts, one biopsy showed a granuloma, and assays for three biochemical markers could not be performed in one patient.

Baseline characteristics

Table 1 shows the baseline characteristics of 98 patients, 52 (53%) patients were male (Male: Female = 1.15:1). The mean age was 36.0 ± 10.6 years. On liver biopsy, 26 (27%) patients had an activity grade of A2-3 and 42 (43%) had F2-4 fibrosis stages. Except for 4 patients, all the patients with A2 or A3 activity on liver biopsy also had F2-4 fibrosis. The major determinant of overall significant disease category was thus found to be the stage of the disease. In aggregate, 46 (47%) patients were classified as having clinically significant overall disease (A2-3 or F2-4).

Univariate analysis

The demographic and biochemical variables were compared for their association with different overall disease categories (Table 2). The mean age of patients with significant disease was significantly higher (39.6 ± 11.4 year *vs* 32.9 ± 8.7 year, $P = 0.002$), while gender distribution was not different between significant and minimal disease groups ($P = 0.15$). The mean total and direct bilirubin levels were significantly higher in the significant disease group ($P = 0.04$ for both). The mean ALT level was significantly higher in significant disease group than the minimal disease group ($P = 0.002$), but the mean difference of ALP level was statistically insignificant between the two groups ($P = 0.78$). Mean GGT was significantly higher in the significant disease group ($P < 0.001$). Mean difference of haptoglobin was not statistically significant according to overall disease categories ($P = 0.99$). The mean of A2M was significantly higher as in the significant disease group ($P = 0.002$), but mean Apo-A1 was significantly lower in the significant disease group ($P = 0.002$). The mean hydroxyproline was also significantly lower in the significant disease group ($P = 0.03$), while proline was not statistically different in minimal and significant

Table 3 Scores of six marker indices with their coordinate liverscores

6 marker	Liverscore	6 marker	Liverscore	6 marker	Liverscore
-2.149	0.00	-0.883	0.34	0.270	0.68
-1.985	0.01	-0.869	0.35	0.277	0.69
-1.894	0.02	-0.855	0.36	0.364	0.70
-1.878	0.03	-0.853	0.37	0.383	0.71
-1.833	0.04	-0.851	0.38	0.400	0.72
-1.774	0.05	-0.819	0.39	0.482	0.73
-1.767	0.06	-0.805	0.40	0.518	0.74
-1.712	0.07	-0.778	0.41	0.549	0.75
-1.664	0.08	-0.777	0.42	0.551	0.76
-1.556	0.09	-0.751	0.43	0.926	0.77
-1.527	0.10	-0.740	0.44	0.980	0.78
-1.509	0.11	-0.712	0.45	0.999	0.79
-1.365	0.12	-0.636	0.46	1.238	0.80
-1.351	0.13	-0.593	0.47	1.316	0.81
-1.308	0.14	-0.579	0.48	1.373	0.82
-1.242	0.15	-0.572	0.49	1.384	0.83
-1.240	0.16	-0.510	0.50	1.554	0.84
-1.181	0.17	-0.499	0.51	1.816	0.85
-1.175	0.18	-0.448	0.52	2.244	0.86
-1.164	0.19	-0.362	0.53	2.377	0.87
-1.158	0.20	-0.218	0.54	2.607	0.88
-1.151	0.21	-0.158	0.55	2.781	0.89
-1.097	0.22	-0.135	0.56	2.835	0.90
-1.085	0.23	-0.063	0.57	4.061	0.91
-1.034	0.24	-0.054	0.58	4.745	0.92
-1.032	0.25	-0.047	0.59	5.617	0.93
-1.020	0.26	-0.024	0.60	5.631	0.94
-1.006	0.27	0.005	0.61	7.482	0.95
-0.979	0.28	0.028	0.62	7.968	0.96
-0.952	0.29	0.086	0.63	8.749	0.97
-0.948	0.30	0.147	0.64	11.602	0.98
-0.934	0.31	0.192	0.65	15.956	1.00
-0.890	0.32	0.213	0.67		

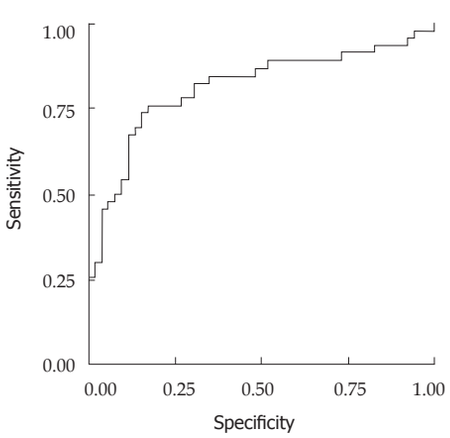

Figure 1 Receiver operating characteristiccurve showing the sensitivity and specificity of Liverscore for the prediction of overall disease categories on biopsy. Area under receiver operating characteristic was 0.813.

disease (P = 0.27). Mean hyaluronic acid was significantly higher in the significant disease group (P = 0.001).

Formulation and assessment of indices

In the univriate analysis, nine variables were identified as significantly associated with two groups of overall significant disease. These included age of the patient, bilirubin total and direct, ALT, GGT, A2M, Apo-A1, HYP and HA. As bilirubin total and direct were highly correlated (r = 0.85), we included bilirubin total only. Although gender was not significantly associated with histological categories, we included it in some indices to see if it improved their performance. Various combinations of the factors identified in the univariate analysis, were assessed by logistic regression. By keeping the overall disease categories as a binary variable, β regression coefficients of the factors were obtained to generate indices. The diagnostic value of these indices was assessed by an area under ROC (AUROC) curve. In the nine marker index, age, gender, bilirubin total, ALT, GGT, A2M, Apo-A1, HYP and HA were included. Bilirubin total was excluded in the eight marker index. Gender and HYP were excluded in the seven marker index. Gender, total bilirubin, and HYP were excluded in the six marker index. AUROC (±SE) was found to be 0.831 (0.05) for both the nine and eight marker indices and 0.813 (0.05) for both the seven and six marker indices. Although indices having HYP as a component i.e., the nine and eight marker indices performed slightly better, we excluded these indices because the difference was marginal and measurement of HYP is expensive and is not available in routine clinical laboratories. The AUROC was similar for seven and six marker indices.

Finally, we selected a six marker index formulated from beta regression values keeping overall disease category as a binary variable. The formula used to generate this index is given below (with ALT and GGT expressed as U/L, A2M as G/L, Apo A1 as mg/dL and HA as ng/mL).

Six marker index = -1.578 + 0.018 (age) + 0.023 (ALT) + 0.021 (GGT) + 0.152 (A2M) - 0.015 (Apo A1) + 0.014 (HA)

The obtained scores were converted to a standardized index ranging from 0-1 through percent ranking as Liverscore for Hepatitis C (Table 3).

The ROC curve showing the sensitivity and specificity for overall disease categories is shown in Figure 1, with an AUROC (± SE) of 0.813 (0.05). The specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) were calculated at different levels. Important cutoff points are shown in Table 4. Negative predictive value of Liverscore for Hepatitis C at a cutoff level of 0.30 was 84%. Six out of 31 patients who were below this cutoff were wrongly diagnosed (false nega-

Table 4 Diagnostic performance of liverscore at different cutoff points

Cut off	Sensitivity	Specificity	PPV	NPV
0.30	89	48	58	84
0.40	85	62	64	83
0.70	54	90	82	70
0.80	39	96	89	65

Positive predictive value and negative predictive value were calculated for a prevalence of 0.45, all the values are expressed as percentages. PPV: Positive predictive value; NPV: Negative predictive value.

tives). All patients had F2 fibrosis and minimal activity. At a cutoff point of 0.40, NPV remained at 83%. Seven out of 40 patients below this cutoff were false negatives, again all had F2 fibrosis. For the positive predictive value, to identify the presence of significant fibrosis, cutoff levels of $\geqslant 0.70$ and $\geqslant 0.80$ were compared, and had 82% and 89% positive predictive values, respectively. Out of 29 patients with a score of 0.70 and above, five were false positives, all with F1 fibrosis. At a cutoff point of 0.80, two patients out of 20 in this study were false positives. Again both had F1 fibrosis.

Thus, a liverscore for hepatitis C of 0.40 or below reliably excludes the presence of significant disease and a score of 0.80 and above confirms the presence of significant disease.

DISCUSSION

The rate of fibrosis progression in CHC patients varies markedly from person to person, and only a minority suffers from long term complications[2]. The current use of liver biopsy for the assessment of liver histology has many drawbacks. Noninvasive assessment of liver histology has been the focus of research for many years. Isolated markers of liver cell injury and fibrosis have not proved to be sufficiently reliable for clinical use[15]. Development of indices consisting of multiple markers is now being focused to distinguish between minimal and clinically significant fibrosis categories.

Our final index, liverscore for hepatitis C, consisted of six markers, ALT, GGT, A2M, Apo-A1, hyaluronic acid and age of the patient. AUROC with this index was found to be 0.813 for overall disease. This index was also evaluated for fibrosis stage and activity grade separately, with clinically acceptable diagnostic performance (data not shown). The negative predictive value of Liverscore for Hepatitis C at a cutoff level of $\leqslant 0.40$ was 83%. All the patients (7/40) diagnosed as false negatives had F2 fibrosis and minimal activity. For the positive predictive value, a cutoff level $\geqslant 0.80$ was found suitable, with a PPV of 89%. At this cutoff, two patients out of 20 were false positives, both had F1 fibrosis. The diagnostic performance of this index in terms of AUROC is comparable to other similar indices reported in the literature.

Forns' index[16] consists of age of the patient, GGT, cholesterol, and platelets. AUROC was 0.86 in the formulation and 0.81 in the validation group. Using the cut off score of < 4.2, presence of significant fibrosis could be excluded in 36% (125/351) patients, with a NPV of 96% in the formulation group. The majority of the patients in this cohort had genotype 1. This index includes cholesterol, which is metabolized differently in genotype 3; it has been suggested[17] that this index might not perform well in patients having genotype 3, the most common genotype affecting the Pakistani population[18].

AST to platelet ratio index (APRI) is a very simple and widely validated index that amplifies the opposing effects of liver fibrosis on AST and platelet counts[19]. The AUROC curve of APRI for prediction of significant fibrosis remained 0.80 in training and 0.88 in the validation set. In one study from Pakistan[20], it showed an AUROC of 0.82 for significant fibrosis. At a cutoff point of < 0.5, the authors could exclude the presence of significant fibrosis in 36% (43/120) patients, with an NPV of 78%. Our index performed slightly better than this at a cutoff point of $\leqslant 0.40$, with the exclusion of 41% patients and an NPV of 83%. Our index has a better NPV and these scores were present in 41% (40/98) patients. This has a clinical advantage of identifying patients that can safely be deferred for urgent treatment.

Patented Fibrotest® for fibrosis consists of age and gender of the patient, GGT, total bilirubin, haptoglobin, A2M and Apo A-1. In addition, the same authors have also reported Actitest® for necroinflammation, which includes ALT in addition to Fibrotest® biomarkers[13]. This is the most widely validated noninvasive marker and is in clinical use. The AUROC for the identification of liver fibrosis was 0.84 and 0.87 for the formulation and validation group, respectively. The PPV of this index was excellent (> 90% certainty of presence of F2, F3 or F4) for scores ranging from 0.60 to 1.00 (34% of all patients). This index could exclude the presence of significant fibrosis in 12% of patients, with a high negative predictive value (100% certainty of absence of F2, F3 or F4) for scores ranging from zero to 0.10. This high accuracy of Fibrotest was not uniform in different populations. A study carried out by Rossi et al., in the Australian population demonstrated, a PPV of 78% at a Fibrotest® score of > 0.6 and an NPV of 85% at < 0.1[21]. We included all the components of Fibrotest® in our initial analysis; however, haptoglobin and gender of the patients were not discriminative of minimal and clinically significant disease in our cohort of patients in univariate analysis. Thus, these were not included in the final index.

Hepascore consists of age and gender of the patient, bilirubin, A2M and HA. For significant fibrosis, it showed an AUROC of 0.85 and 0.82 in the training and validation groups, respectively. A score of $\geqslant 0.5$ was 92% specific and 67% sensitive in the training set and 89% specific and 63% sensitive in the validation group. At this cutoff it provided high PPVs of 87% and 88% in the training and validation set, respectively[22]. Authors have not reported negative predictive values for significant fibrosis. We have all the data required for the calculation of Hepascore and will evaluate the performance of this score in our patients.

All the above mentioned scores either predict fibrosis or activity. One advantage of our index is its prediction for overall disease, which includes both fibrosis stage and activity grade. Both these histopathological categories are important for prognosis and making treatment decisions[23]. Furthermore, the majority of the previous indices have been reported in populations infected predominantly with HCV genotype 1. Evidence points towards the possibility that HCV genotype 3 associated CHC is a metabolically different disease[24]. Our index

might perform better in genotype 3 patients, because it is formulated in a population predominantly infected with this genotype[18].

All the factors included in our index are available and easily programmable on automated instruments in routine clinical laboratories. Furthermore, factors included in the Liverscore for Hepatitis C have physiological rationale.

Gamma-glutamyl transpeptidase is synthesized by the liver cells, its synthesis increases with fibrosis. The mechanisms for this increase could be the stimulation of GGT synthesis by epidermal growth factor during fibrogenesis[25]. ALT is synthesized by hepatocytes and its release into serum is related to liver cell injury[26]. The synthesis of A2M increases during stellate cell activation in the course of fibrogenesis, and its serum concentration increases with fibrosis[27]. In liver fibrosis, Apo A-1 release from the hepatocytes is hampered by the collagen fibers decreasing its serum levels[28]. Hyaluronic acid is a nonsulfated glycosaminoglycan and is major component of extracellular matrix. Among the direct markers of liver fibrosis, HA has been most extensively studied in CHC. It increases in the liver during fibrogenesis and is released into the systemic circulation during remodeling. Recent indices consisting of HA in combination with indirect markers have shown promising results[22].

Some of the markers we evaluated were not helpful in differentiating minimal from significant disease. Total and direct bilirubin were significantly associated with different histological categories, but were highly correlated (r = 0.85). Direct bilirubin was therefore excluded. Total bilirubin was included in the seven marker index, but its exclusion did not affect the diagnostic value and was thus excluded from the final index. Serum levels of ALP are known to be raised in both alcoholic and non-alcoholic liver disease with advanced histological changes. In addition, ALP has shown a discriminative value for advanced fibrosis and cirrhosis previously[29]. It was also associated with mild and advanced fibrosis categories in our study, but not for overall disease, the primary outcome in our study, thus, we did not include ALP in our index. HYP and proline are amino acids present in collagen in large quantities. The HYP content of liver biopsies is found to increase with advancing stage of fibrosis[30]. The evidence that fibrosis is a dynamic two way process with fibrosis and its degradation occurring simultaneously, prompted us to include these amino acids as products of collagen degradation in our panel of biomarkers. We expected their high levels in serum because of the greater amount of collagen undergoing remodeling in advanced stages. We found no association of these amino acids of collagen degradation with severity of liver fibrosis or necroinflammatory activity. HYP, however, was statistically associated with overall disease category, but actually decreased. In the only study we could find, predictive value of these amino acids in sera of CHC patients was evaluated for advanced (F3 and 4) and mild (F0, 1 and 2) fibrosis[31]. Proline was not significantly different between the two groups, while HYP was found to be increased with advanced fibrosis, but showed a low (0.525) area under ROC curve. We found no study comparing these amino acids in minimal and significant fibrosis or overall disease. The evidence that their serum levels do not increase with increasing fibrosis might be explained by slower fibrosis degradation in advanced fibrosis. It has been shown that accumulation of fibrosis is the net effect of increased fibrogenesis and its decreased degradation[32].

One limitation of our study was that we could not validate our results in a different cohort of patients. This was not possible because of the smaller number of patients recruited in our study. We recommend an independent study for the validation of our index.

In conclusion, a liverscore for hepatitis C of 0.40 or below excludes the presence of significant disease. Thus, it can reliably exclude around 41% of CHC patients that do not require an urgent treatment. A score of 0.80 and above confirms the presence of significant disease. Using these cutoff values, the severity of the liver disease can reliably be predicted in around 61% of the CHC patients.

COMMENTS

Background

Chronic hepatitis C (CHC) is a leading cause of liver fibrosis and its complications, such as cirrhosis and hepatocellular carcinoma. On the other hand, the disease progresses slowly and may even remain non progressive in many patients. Thus, assessment of severity of liver fibrosis and activity guides prognosis and treatment decisions. Liver biopsy, the current gold standard for monitoring liver histology, is associated with complications and is not possible in all patients.

Research frontiers

Noninvasive assessment of liver histology has been a focus of research for many years. The majority of these noninvasive indices have been developed in western populations with predominantly hepatitis C virus (HCV) genotype 1. The research hotspot is to develop a noninvasive index in our population with a predominantly HCV genotype 3.

Innovations and breakthroughs

The noninvasive index score of 0.40 or below (on a scale of 0-1) excludes the presence of significant liver disease and a score of 0.80 and above confirms the presence of significant disease reliably. With these cutoff points, this index was discriminative for minimal and significant overall disease in 61% of our patients.

Applications

The development of a convenient monitoring tool will allow physicians to make evidence based clinical decisions and closely monitor disease progression.

Terminology

Activity grade is a measure of necroinflammation in liver tissue. Higher grades are related to rapid disease progression. Fibrosis stage is a measure of the amount of fibrosis in the liver. Overall disease is regarded as minimal when both grade and stage are < 2 and is significant when either of the grade or stage is ⩾ 2. Significant disease is an indication for institution of antiviral treatment when liver histology is known.

Peer review

This cross sectional study was conducted on polymerase chain reaction positive, treatment naïve patients to formulate a noninvasive index predictive of severity of liver fibrosis and activity in CHC. This study represents an attempt to accomplish such a goal by determining the diagnostic accuracy of a group of noninvasive biomarkers in the assessment of liver fibrosis.

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BRIEF ARTICLE

Comparison of presentation and impact on quality of life of gastroesophageal reflux disease between young and old adults in a Chinese population

Shou-Wu Lee, Chia-Ming Chang, Chi-Sen Chang, Ai-Wen Kao, Ming-Chih Chou

Shou-Wu Lee, Chi-Sen Chang, Ming-Chih Chou, Institute of Medicine, Chung Shan Medical University, Taichung 40242, Taiwan, China
Shou-Wu Lee, Chi-Sen Chang, Division of Gastroenterology, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung 40242, Taiwan, China
Shou-Wu Lee, Chi-Sen Chang, Department of Medicine, Chung Shan Medical University, Taichung 40242, Taiwan, China
Chia-Ming Chang, Ai-Wen Kao, Institute of Gerontology, National Cheng Kang University, Tainan 70101, Taiwan, China
Chia-Ming Chang, Division of Geriatrics, Department of Internal Medicine, National Cheng Kang University Hospital, Tainan 70101, Taiwan, China
Ai-Wen Kao, Division of Gastroenterology, Department of Internal Medicine, National Cheng Kang University Hospital, Tainan 70101, Taiwan, China
Author contributions: Lee SW and Chou MC contributed equally to this work; Lee SW, Chang CM and Chang CS designed the research; Lee SW and Chang CS performed the research; Kao AW and Chou MC contributed new reagents/analytic tools; Lee SW and Chang CM analyzed the data; and Lee SW, Chang CM and Chang CS wrote the paper.
Correspondence to: Dr. Ming-Chih Chou, Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan 110, Section 1, Chien Kuo North Road, Taichung 40242, Taiwan, China. ericest429@yahoo.com.tw
Telephone: +886-4-23592525-3310 Fax: +886-4-23741331
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Abstract

AIM: To compare the presentation and impact on quality of life of gastroesophageal reflux disease (GERD) in old and young age groups.

METHODS: Data from adult patients with GERD diagnosed by endoscopic and symptomic characteristics were collected between January and November 2009. Exclusion criteria included combined peptic ulcers, malignancy, prior surgery, antacid medication for more than 2 mo, and pregnancy. Enrolled patients were assigned to the elderly group if they were 65 years or older, or the younger group if they were under 65 years. They had completed the GERD impact scale, the Chinese GERD questionnaire, and the SF-36 questionnaire. Data from other cases without endoscopic findings or symptoms were collected and these subjects comprised the control group in our study.

RESULTS: There were 111 patients with GERD and 44 normal cases: 78 (70.3%) and 33 patients (29.7%) were in the younger and elderly groups, respectively. There were more female patients (60.3%) in the younger group, and more males (72.7%) in the elderly group. The younger cases had more severe and frequent typical symptoms than the elderly patients. Significantly more impairment of daily activities was noted in the younger patients compared with the elderly group, except for physical functioning.

CONCLUSION: Elderly patients with GERD were predominantly male with rare presentation of typical symptoms, and had less impaired quality of life compared with younger patients in a Chinese population.

Key words: Gastroesophageal reflux disease; Quality of life; Age factors

Peer reviewer: Bernabe Matias Quesada, MD, Department of Surgery, Hospital Cosme Argerich, Talcahuano 944 9A, Buenos Aires 1013, Argentina

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic disease which has a considerable, impact on the everyday lives of affected individuals, and interferes with physical activity, impairs social functioning, disturbs sleep and reduces productivity at work[1]. The prevalence of the disease increases with age[2,3], and advanced age has been identified as a significant risk factor for relapse of esophagitis[4,5]. Furthermore, elderly patients present less frequently with the typical symptoms of heartburn, acid regurgitation, and abdominal or chest pain, which are quite different from those of young subjects[6,7]. Several previous studies assessed clinical symptoms and quality of life of GERD patients using the GERD impact scale[8], Chinese GERD questionnaire (GERDQ)[9], and the Short Form 36 (SF-36) questionnaire[10,11]. However, to date, no studies have been conducted to compare the quality of life of young and old patients with GERD. The aim of this study was to compare the presentation and impact on quality of life of GERD in younger and elderly patients in a Chinese population.

MATERIALS AND METHODS

Data from consecutive patients with GERD in our hospital were collected between January 2009 and November 2009. All patients underwent an open-access transoral upper gastrointestinal endoscopy, and those who were diagnosed with erosive esophagitis, or had typical symptoms, were considered for inclusion in the study. Exclusion criteria were as follows: (1) GERD combined with other structural gastrointestinal disorders, such as peptic ulcer disease, esophageal or gastric malignancy; (2) prior gastric surgery; (3) use of chronic antacid medication, such as proton pump inhibitors or H2-receptor antagonists, for more than 2 mo prior to enrollment; and (4) pregnancy.

The enrolled patients were assigned to the younger group if they were under the age of 65 years; patients 65 years and over were assigned to the elderly group. All cases were asked to complete the GERD impact scale, the Chinese GERDQ, and the SF-36 questionnaire (Chinese version), and results were compared between the two groups. Data from other cases without endoscopic findings of erosive esophagitis or symptoms related with GERD were collected and these subjects served as the control group in our study. All study participants completed the SF-36 questionnaire, a widely used health survey.

Data are expressed as mean ± SD deviation for each of the measured parameters. A P value below 0.05 was considered statistically significant. Statistical comparisons were made using Pearson's χ^2 test, or Fisher's exact test, to compare the effects of gender and endoscopic or clinical reflux esophagitis severity, and the independent Student t test was used to analyze scores of each scale and questionnaire.

Table 1 Characteristics and endoscopic symptoms in younger and elderly cases with gastroesophageal reflux disease

Variable	Younger group (n = 78, 70.3%)		Elderly group (n = 33, 29.7%)		
	n (%)	mean ± SD	n (%)	mean ± SD	P value
Mean age (yr)		37.73 ± 10.17		72.94 ± 9.58	
Symptom duration (yr)		2.82 ± 3.92		2.50 ± 2.99	0.384[1]
Gender					0.001[2]
M	31 (39.7)		24 (72.7)		
F	47 (60.3)		9 (27.3)		
Endoscopic reflux esophagitis severity					
NERD	15 (19.2)		0		0.001[3]
L.A. Grade A	50 (64.1)		16 (48.5)		
L.A. Grade B	8 (10.3)		8 (34.2)		
L.A. Grade C	5 (6.4)		6 (18.2)		
L.A. Grade D	0		3 (9.1)		

[1]Pearson's χ^2 test, [2]Independent t test, [3]Fisher's exact test. F: Females; M: Males; L.A.: Los Angeles classification; SD: standard derivation; NERD: Non erosive reflux disease.

RESULTS

Among all enrolled consecutive patients between January and November 2009, there were 111 with GERD and 44 normal cases. Patients' characteristics and endoscopic reflux esophagitis severity are shown in Table 1. Among the patients with GERD, there were 78 patients (70.3%) in the younger group and 33 cases (29.7%) in the elderly group. A similar duration of symptoms was noted between the younger cases (mean 2.82 years) and the elderly cases (mean 2.50 years). Comparing the gender of each group, there were significantly more female patients (60.3%) in the younger group, and more male patients (72.7%) in the elderly group. The elderly patients had greater endoscopic disease severity than the younger patients, based on the Los Angeles classification.

The presentation of the younger and elderly patients with GERD in our study are summarized in Table 2, which show the results of the GERD impact scale. As shown in Table 2, the young cases had a higher prevalence of typical symptoms than the elderly patients, including burning (48.7% *vs* 15.2%, P = 0.005), pain in chest (64.3% *vs* 33.3%, P = 0.001), regurgitation of food (79.5% *vs* 54.6%, P = 0.058), hoarseness (73.1% *vs* 39.4%, P = 0.010) and chronic conditions related to heartburn (44.8% *vs* 33.3%, P = 0.708).

The quality of life results in the two groups measured by the GERD impact scale are shown in Table 3, and the SF-36 questionnaire results are shown in Table 4 and Figure 1. As shown in Table 3, significantly impaired activities of daily living, including sleep disturbance (52.6% *vs* 18.2%, P = 0.004), feeding and drinking problems (64.1% *vs* 12.1%, P = 0.001), and impact on social activites (43.6%

Table 2 Symptom presentation measured by gastroesophageal reflux disease impact scale in younger and elderly patients

GERD impact scale	Young group (*n* = 78, 70.3%)	Elderly group (*n* = 33, 29.7%)	
	n (%)	*n* (%)	*P* value
Burning			0.005
None of the time	30 (38.50)	22 (66.70)	
A little of the time	34 (43.60)	1 (3.00)	
Some of the time	9 (11.50)	9 (27.30)	
All of the time	6 (6.40)	1 (3.00)	
Pain in chest			0.001
None of the time	40 (51.30)	28 (84.80)	
A little of the time	28 (35.90)	4 (12.20)	
Some of the time	9 (11.50)	0	
All of the time	1 (1.30)	1 (3.00)	
Food coming into mouth			0.058
None of the time	16 (20.50)	15 (49.40)	
A little of the time	48 (61.50)	13 (39.40)	
Some of the time	7 (9.00)	2 (6.10)	
All of the time	7 (9.00)	3 (9.10)	
Hoarseness			0.01
None of the time	21 (55.20)	20 (60.60)	
A little of the time	37 (26.90)	8 (24.20)	
Some of the time	15 (11.50)	4 (12.20)	
All of the time	5 (6.40)	1 (3.00)	
Chronic heartburn			0.708
None of the time	43 (55.20)	22 (66.70)	
A little of the time	21 (26.90)	6 (18.10)	
Some of the time	9 (11.50)	3 (9.10)	
All of the time	5 (6.40)	2 (6.10)	

All were analyzed by Fisher's exact test. GERD: Gastroesophageal reflux disease.

Table 3 Quality of life measured by quality of life of gastroesophageal reflux disease impact scale in younger and elderly cases

GERD impact scale	Young group (*n* = 78, 70.3%)	Elderly group (*n* = 33, 29.7%)	
	n (%)	*n* (%)	*P* value
Sleep disturbance			0.004
None of the time	37 (47.40)	27 (81.80)	
A little of the time	26 (33.30)	4 (12.10)	
Some of the time	8 (10.30)	0	
All of the time	7 (9.00)	2 (6.10)	
Food and drink problems			0.001
None of the time	28 (35.90)	29 (78.90)	
A little of the time	31 (39.70)	3 (9.10)	
Some of the time	6 (7.7)	0	
All of the time	13 (16.70)	1 (3.00)	
Social interference			0.005
None of the time	44 (56.40)	28 (84.80)	
A little of the time	27 (34.60)	3 (9.10)	
Some of the time	6 (7.70)	0	
All of the time	1 (1.30)	2 (6.10)	

All were analyzed by Fisher's exact test. GERD: Gastroesophageal reflux disease.

vs 15.2%, $P = 0.005$), were noted in the younger patients.

As shown in Figure 1, comparing the quality of life between cases with and without GERD, patients with GERD had lower scores in all items of the SF-36 questionnaire, except physical functioning. With regard to age,

Table 4 Scores of SF36 questionnaire in younger and elderly cases with gastroesophageal reflux disease

SF-36	Younger group (*n* = 78, 70.3%)	Elderly group (*n* = 33, 29.7%)	
	mean ± SD	mean ± SD	*P* value
Total SF 36 score	64.58 ± 16.84	70.73 ± 18.50	0.106
Total physical health scores	64.54 ± 15.96	60.03 ± 19.06	0.24
Total metal health scores	54.77 ± 16.18	66.18 ± 15.10	0.011
Physical functioning	90.26 ± 15.52	88.36 ± 21.00	0.342
Role-physical	72.76 ± 41.91	71.21 ± 39.09	0.853
Bodily pain	62.70 ± 18.35	69.18 ± 17.99	0.089
General perception	46.47 ± 18.06	58.09 ± 19.60	0.005
Vitality	51.22 ± 18.36	61.63 ± 19.46	0.013
Social functioning	72.18 ± 18.70	76.76 ± 19.73	0.263
Role-emotional	66.24 ± 45.76	76.82 ± 36.78	0.203
Mental health	54.77 ± 16.18	66.18 ± 15.10	0.001

All were analyzed by independent *t* test.

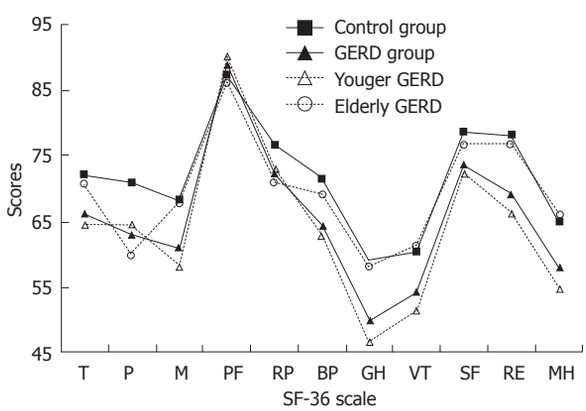

Figure 1 Scores of SF36 questionnaire among the control cases, and younger and elderly cases with gastroesophageal reflux disease. T: Total SF36 scores; P: Total physical health scores; M: Total metal health scores; RP: Role-physical; RF: Physical functioning; BP: Bodily pain; GH: General perception; VT: Vitality; MH: Mental health; SF: Social functioning; RE: Role-emotional; GERD: Gastroesophageal reflux disease.

as shown in Table 3, younger patients had higher scores than elderly patients in the items of physical functioning (mean 90.26 *vs* 86.39, $P = 0.342$), role-physical limitations (mean 72.76 *vs* 71.21, $P = 0.853$), and total physcial health status (mean 64.54 *vs* 60.03, $P = 0.240$). In contrast, younger patients had lower scores than elderly patients for the following items: bodily pain (mean 62.70 *vs* 69.18, $P = 0.089$), general perception of health (mean 46.47 *vs* 58.09, $P = 0.005$), vitality (mean 51.22 *vs* 61.36, $P = 0.013$), social functioning (mean 72.18 *vs* 76.76, $P = 0.263$), emotional limitations (mean 66.24 *vs* 76.82, $P = 0.203$), mental health status (mean 54.77 *vs* 66.18, $P = 0.001$), total mental health status (mean 58.15 *vs* 67.76, $P = 0.011$), and total health status (mean 64.58 *vs* 70.73, $P = 0.106$).

DISCUSSION

GERD is a chronic disease that tends to relapse and develop complications[12], and it is reported to be more severe and to have a higher incidence of severe complica-

tions in older *vs* younger patients[4,13,14]. Moreover, clinical features of GERD in old patients are quite different from those of younger adult subjects, with the elderly presenting less frequently with the typical symptoms of heartburn, acid regurgitation, and pain[15]. Our study findings showed similar results in that most typical symptoms of GERD occurred in the younger patients, but occurred more rarely in the older subjects. The only exception in our study was that acid regurgitation was more frequent in older patients than in young ones, but this difference was non-significant. Hence, the typical esophageal symptoms of GERD, such as heartburn, are unreliable markers when diagnosing this disease in elderly patients[16].

Interestingly, the symptom of hoarseness, which is often considered an extraesophageal symptom of GERD[17], was also more frequently noted in the younger patients. Our results demonstrated that the younger patients with GERD not only had more typical esophageal symptoms but also more extraesophageal symptoms than in the elderly group. Furthermore, a previous study reported roughly one-third of elderly patients may have none of the symptoms usually associated with GERD[6]. It has been suggested that old patients may display symptoms in a different manner compared with young patients.

Patients with GERD may present with a broad range of troublesome symptoms that can damage the quality of their daily lives[18,19]. The negative effects of GERD are dependent on the frequency and severity of symptoms rather than the presence of esophagitis. Studies conducted in Sweden's general population, which assessed the impact of the severity and frequency of GERD symptoms showed that even symptoms rated as mild are associated with a clinically meaningful reduction in wellbeing[20]. A German study determined that patients with symptoms of GERD had substantially impaired physical and psychosocial aspects of wellbeing compared with the general population, and felt restricted as a result of food and drink problems, disturbed sleep, and impaired vitality and emotional wellbeing[18]. A Chinese study reported that the largest decrements in quality of life scores among all subscales in subjects with GERD symptoms were related to bodily pain and role limitations[21]. Another study found that patients with GERD reported more impairment due to pain but less impairment with regard to physical and role functioning[11]. Our study yielded similar results, namely, that patients with GERD reported impaired quality of life in all dimensions, except physical functioning, compared with quality of life in the normal population.

The quality of life findings revealed slightly lower physical scores in elderly patients with GERD. It is possible that the increased disability or frailty associated with aging may account for the lower physical functioning. In addition, compared with elderly patients, significantly impaired mental health and vitality in the younger GERD patients was found in our study. That is, severity and frequency of symptoms of GERD were associated with impaired quality of life. This finding is consistent with the results of a previous study which showed both severity and perceived discomfort of symptoms were valid markers of the degree of quality of life impairment[22,23].

There were some limitations in our study. Firstly, some personal matters and other co-morbidity of diseases, which tend to influence quality of life, such as psychiatric factors[24], central obesity[25], chronic heart failure, and chronic obstructive pulmonary disease or asthma[26] were not considered. These might have led to scoring bias in the questionnaire. Secondly, overlap of GERD with inflmmatory bowel syndrome and functional dyspepsia should be considered. The overlaps were common and might worsen scores of questionnaries in our study according to a previous report[27]. Thirdly, our study design was hospital-based. Further research should include representative samples from the general population to confirm these results.

In conclusion, in the present study, elderly and younger adult patients with GERD in a Chinese population had different characteristics. Elderly GERD patients were predominantly male, rarely presented with typical GERD symptoms and their quality of life was less impaired compared with younger GERD patients.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a chronic disease which has an impact on the everyday lives of affected individuals. The prevalence of the disease increases with age, but elderly patients present less frequently with typical symptoms of heartburn and acid regurgitation, which are quite different from symptoms of young subjects.

Research frontiers

Several previous studies assessed clinical symptoms and quality of life of GERD patients using the questionnaire. However, to date, no studies have been conducted to compare the quality of life of young and old patients with GERD. The aim of this study was to compare the presentation and impact of quality of life in younger and elderly GERD patients in a Chinese population.

Innovations and breakthroughs

The study found more female patients in the younger group, and more males in the elderly group. The younger cases had more severe and frequent typical symptoms than the elderly patients. Significantly impaired daily activities were noted in the younger patients compared with the elderly group, except for physical functioning.

Applications

The diagnostic features of GERD should be determined in the elderly, since these patients have rare presentations of typical symptoms, and less impaired quality of life compared with younger cases in a Chinese population.

Peer review

It's an interesting and important manuscript in which the vluable index based on patient has been shown.

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BRIEF ARTICLE

Training gastroenterology fellows to perform gastric polypectomy using a novel *ex vivo* model

Ming-Jen Chen, Ching-Chung Lin, Chia-Yuan Liu, Chih-Jen Chen, Chen-Wang Chang, Ching-Wei Chang, Chien-Wei Lee, Shou-Chuan Shih, Horng-Yuan Wang

Ming-Jen Chen, Ching-Chung Lin, Chia-Yuan Liu, Chih-Jen Chen, Chen-Wang Chang, Ching-Wei Chang, Chien-Wei Lee, Shou-Chuan Shih, Horng-Yuan Wang, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10449, Taiwan, China
Ming-Jen Chen, Ching-Chung Lin, Chia-Yuan Liu, Chih-Jen Chen, Chen-Wang Chang, Ching-Wei Chang, Chien-Wei Lee, Shou-Chuan Shih, Horng-Yuan Wang, Mackay Medicine, Nursing and Management College, Taipei 11260, Taiwan, China
Ming-Jen Chen, Ching-Chung Lin, Chia-Yuan Liu, Chih-Jen Chen, Clinical Skills Training Center, Department of Medical Education, Mackay Memorial Hospital, Taipei 10449, Taiwan, China
Ming-Jen Chen, Shou-Chuan Shih, Horng-Yuan Wang, Mackay Medical College, Taipei 10449, Taiwan, China
Author contributions: Chen MJ designed the experiments and wrote this article; Lin CC, Liu CY and Chen CJ participated in experimental design, data analysis and discussion; Wang CW and Chang CW conducted the literature review; Lee CW coordinated the *ex vivo* experimental design; Shih SC supported this work and performed critical reading of the manuscript; Wang HY supported this work and supervised the final editing. All authors read and approved the final manuscript.
Correspondence to: Dr. Horng-Yuan Wang, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, No. 92, Sec. 2, Chungshan North Road, Taipei 10449, Taiwan, China. hywang@ms2.mmh.org.tw
Telephone: +886-2-25433535-2260 Fax: +886-2-25433642
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Abstract

AIM: To evaluate the effect of hands-on training of gastroenterology fellows in gastric polypectomy using an *ex vivo* simulator.

METHODS: Eight gastroenterology fellows at Mackay Memorial Hospital, Taipei were evaluated in gastric polypectomy techniques using a pig stomach with artificial polyps created by a rubber band ligation device. The performance of four second year (year-2) fellows who had undergone one year of clinical training was compared with that of four first year (year-1) fellows both before and after a 4-h workshop using the *ex vivo* simulator. The workshop allowed for hands-on training in the removal of multiple artificial polyps and the placement of hemoclips at the excision site. Evaluation included observation of technical skills, procedure time, and the fellows' confidence scale.

RESULTS: One week after the workshop, the year-1 fellows were re-evaluated and had significantly improved mean performance scores (from 17.9 ± 1.8 to 22.5 ± 0.7), confidence scale (from 4.5 ± 1.0 to 7.8 ± 0.5) and procedure time (from 615.0 ± 57.4 s to 357.5 ± 85.0 s) compared with their baseline performance. After 4 h of training using the *ex vivo* simulator, the skills of the year-1 fellows were statistically similar to those of the year-2 fellows.

CONCLUSION: Use of this *ex vivo* simulator significantly improved the endoscopic gastric polypectomy skills of gastroenterology fellows who had not had previous clinical training in gastric polypectomy.

Key words: *Ex vivo*; Animal tissue model; Hands-on training; Pig stomach; Polypectomy

Peer reviewer: Javier San Martín, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay

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INTRODUCTION

Interventional endoscopy continues to advance, requiring that endoscopists obtain practical training in skills needed for therapeutic intervention. This has traditionally been accomplished by having trainees perform endoscopy on patients under close supervision. However, the use of animal models is a safe way to practice the techniques used in therapeutic endoscopy[1]. Since the mid-1990s, an *ex vivo* porcine-tissue simulator has been widely used in many hands-on training programs. It has been shown to facilitate skill in a variety of endoscopic procedures, such as hemostasis for arterial or variceal bleeding[2,3]. It is also useful in training trainers for endoscopy programs[4].

Endoscopic polypectomy is one such skill that must be acquired, including removal of gastric polyps incidentally discovered during endoscopic evaluations. Hyperplastic polyps are by far the most common histologic type of gastric polyp, occurring most commonly in the antrum[5]. When such polyps are larger than 1 cm, the risk of neoplastic transformation increases. Therefore, endoscopic polypectomy may be necessary both for accurate diagnosis and as definitive treatment[6,7]. Larger polyps, however, may be more difficult for inexperienced endoscopists to manage, as are those in more challenging locations. Fellows in gastroenterology training programs need to become familiar with the practical skills required, both in removing the polyp and closing the resulting mucosal defect[8].

We developed a method to simulate artificial polyps in an *ex vivo* pig-stomach model for use in our gastroenterology fellowship training program. As far as we are aware, this novel approach has not previously been described. We designed this study to evaluate this model in training gastroenterology fellows.

MATERIALS AND METHODS

The study group comprised eight gastroenterology fellows at Mackay Memorial Hospital, Taipei, Taiwan, four in the second year (year-2) and four in the first year (year-1) of subspecialty training. Year-2 fellows had already learned to perform endoscopic gastric polypectomy in patients under the supervision of an experienced supervisor certified by the Digestive Endoscopic Society of Taiwan. Year-1 fellows were certified in diagnostic endoscopy after one year of training but had no prior experience performing gastric polypectomy, although they had assisted other endoscopists in placing hemoclips. None of the eight fellows had ever used an *ex vivo* pig-stomach simulator prior to this study. One instructor conducted workshop training, and two reviewers evaluated the fellows' skill in performing polypectomy. The instructor and reviewers each had more than 10 years' experience in interventional endoscopy and were certified as instructors by the Digestive Endoscopic Society of Taiwan.

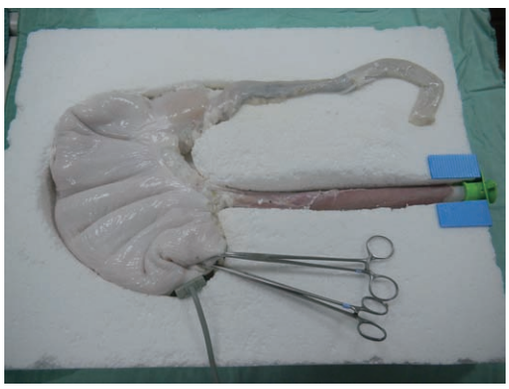

Figure 1 ***Ex vivo*** **porcine organ package including esophagus, stomach and duodenum in a handmade simulator shell.**

The *ex vivo* pig-stomach simulator was a modified version of the compactEASIE model[2,3]. Fresh pig stomachs, including short segments of the lower esophagus and duodenum, were purchased from a slaughterhouse early in the morning of the day they were to be used and were stored in cool saline until preparation. For the simulator, the stomach was placed in a hand-made container composed of layered polystyrene boards cut so as to accommodate the esophagus, stomach, and duodenum (Figure 1). It was irrigated copiously with tap water until clean. Defects in the viscera were closed using appropriately sized Kelly forceps. A flexible overtube (Sumitoma Corp, Tokyo, Japan) with an air-tight valve was inserted into the short segment of the lower esophagus and a plastic band placed to seal the space between the overtube and esophagus. The distal end of the small bowel was closed with Kelly forceps for a good air-tight effect. An electronic-conduction pad was placed between the stomach and the polystyrene board, and an electrosurgical generator (VIO 200D; ERBE Corp., Tuebingen, Germany) with standard settings was used.

The instructor created simulated gastric polyps by using a pneumatically-activated esophageal variceal ligation device (MD-48709; Sumitoma Corp, Tokyo, Japan). For the skills assessment portion of the study, a polyp was created in the cardia in essentially the same location for each fellow being evaluated (Figure 2A). The endoscope (GIF Q230; Olympus Optical Co. Ltd, Tokyo, Japan) and other equipment (polypectomy snare: SD-8P-1; Olympus Optical Co. Ltd, Tokyo, Japan; hemoclip applicator: HX-100 LR; Olympus Optical Co. Ltd, Tokyo, Japan) were retired from clinical use and all used exclusively in animals at the time of the study. The Institutional Review Board at Mackay Memorial Hospital approved this training project.

Polypectomy techniques

Fellows were assessed for their ability to set up the equipment, to remove the entire polyp safely and to close the mucosal defect so as to prevent hemorrhage or perfora-

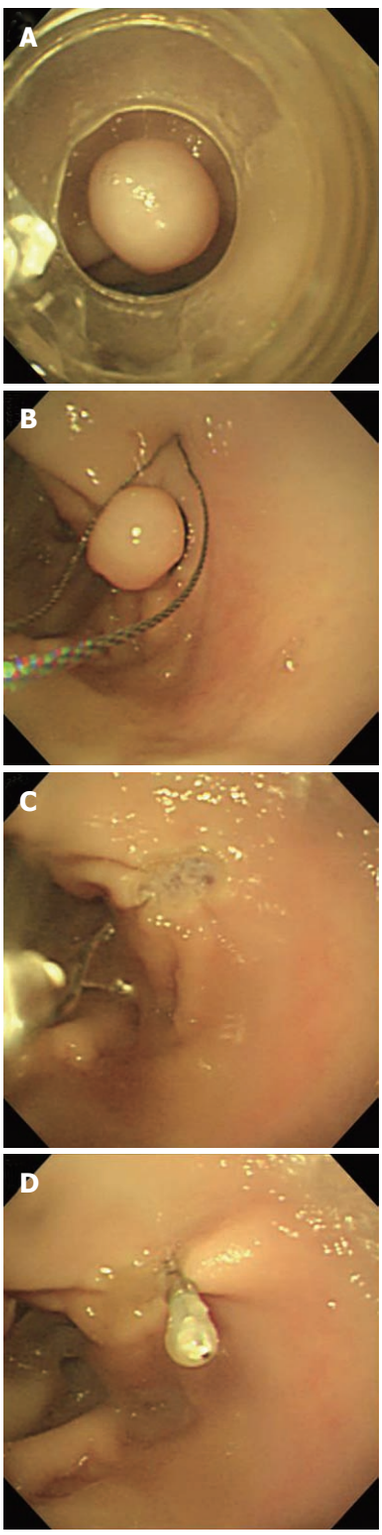

Figure 2 Use of the simulator for evaluation. A: One approximate 8-mm gastric polyp artificially created with a rubber band ligation device in the porcine stomach cardia; B: Snare being advanced to encircle the polyp; C: Successful removal of the entire lesion; D: Application of a hemoclip.

tion. They were required to advance the snare to encircle the target tissue and then transect the polyp with an electrosurgical cutting device attached to the snare. They then had to ensure closure of the mucosal defect by placing hemoclips, which required understanding of how to load the clips, and also direct an assistant in proper placement (Figure 2B-D).

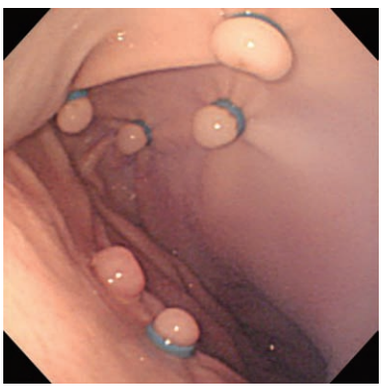

Figure 3 Use of the simulator for training: several artificial polyps placed in different position in the porcine stomach. Use of a multiband ligation device reduced the time required to load rubber bands, allowing efficient creation of multiple artificial polyps.

Workshop training

Following a baseline skills assessment, the four year-1 fellows attended a 4-h workshop led by one instructor, with an assistant to handle the instruments. There was an initial 1-h lecture about gastric polypectomy, followed by the instructor demonstrating the procedure for 1 h, including proper techniques, equipment settings, and communication with the assistant. The final 2 h involved hands-on practice by the fellows (30 min each). For this session, multiple gastric polyps were artificially created ahead of time in various locations within the porcine stomach using a preloaded multiband esophageal variceal ligation device (Speedband Superview Super 7; Microvasive, MA, United States). This allowed each fellow to practice removing a number of simulated polyps for training (Figure 3). While one fellow was practicing, the other three acted as observers and benefitted by hearing the instructions, feedback, and correction of technical errors given to the others.

Outcome measures

Skills assessments were all conducted by two reviewers who had not conducted the training session, and again with an assistant for the actual procedure. All eight fellows were assessed at baseline. A second evaluation was conducted for year-1 fellows a week after they had attended the workshop. Before each evaluation, fellows indicated on a 10-point visual analogue scale their own degree of confidence in performing the procedure. The reviewers met before each evaluation session to standardize the evaluation criteria. Each independently completed a standard assessment checklist for each fellow. The time to complete the procedure was recorded. The standard rating score was based on a 5-point score (1-5 from poor to excellent) for each of 5 items: (1) setting up and testing the equipment; (2) issuing correct instructions to the

Table 1 Assessments at baseline (all fellows) and of year-1 fellows after training on the *ex vivo* simulator

	Year-2 initial assessment	Year-1 initial assessment	Year-1 final assessment	*P* value[1]	*P* value[2]	*P* value[3]
Setting up equipment	3.6 ± 0.5	3.3 ± 0.5	5.0 ± 0.0	0.160	< 0.001	0.003
Communication with the assistant	4.5 ± 0.6	3.6 ± 0.3	4.6 ± 0.3	0.180	0.35	0.007
Proper localization and snaring	4.4 ± 0.5	4.0 ± 0.8	4.4 ± 0.5	0.229	0.50	0.107
Removal of polyp and avoidance of tissue injury	4.1 ± 0.3	4.5 ± 0.3	4.6 ± 0.3	0.104	0.15	0.091
Successful application of hemoclips	4.4 ± 0.5	2.6 ± 0.5	3.6 ± 0.9	0.001	0.088	0.081

[1]Year-2 fellow assessment *vs* year-1 fellow initial assessment; unpaired *t* test; [2]year-2 fellow assessment *vs* year-1 fellow final assessment; unpaired *t* test; [3]year-1 fellow initial assessment *vs* year-1 fellow final assessment; paired *t* test.

assistant; (3) proper localization of the polyp; (4) removal of the polyp with minimal tissue injury; and (5) successful application of the hemoclips. The scores for each item were summed for the final performance score.

Statistical analysis

The primary aim was to assess changes in performance score, procedure time, and confidence among the year-1 fellows before and after workshop training. This was done by using paired *t* tests. The results for the year-1 fellows at both baseline and again after training were also compared with those of the year-2 fellows, using unpaired *t* tests. Date was analyzed using SPSS 11.0 (SPSS Inc, Chicago, Ill). Results were considered to be statistically significant if the *P* value was < 0.05.

RESULTS

Baseline fellow characteristics and skills

Year-1 and -2 fellows were of comparable age and academic background. Year-2 fellows had done about twice as many diagnostic endoscopies as the year-1 fellows. At baseline assessment, year-2 fellows had a significantly higher mean performance score (21.1 ± 0.9 *vs* 17.9 ± 1.8), confidence scale (7.8 ± 0.5 *vs* 4.5 ±1.0), and shorter procedure time (377.5 ± 156.3 s *vs* 615.0 ± 57.4 s) than year-1 fellows.

Assessment of learning progress in year-1 fellows

One week after the workshop using the *ex vivo* simulator, the year-1 fellows were re-evaluated and had significantly improved mean performance scores (from 17.9 ± 1.8 to 22.5 ± 0.7), confidence scale (from 4.5 ± 1.0 to 7.8 ± 0.5) and procedure time (from 615.0 ± 57.4 s to 357.5 ± 85.0 s) compared with their baseline performance. The significantly improved scores were attributed to doing a better job of setting up the equipment and in communicating with the assistant (Table 1). There were no significant differences between the final assessments for the year-1 fellows and those of the year-2 fellows.

DISCUSSION

Our study demonstrated the feasibility of using the *ex vivo* pig stomach with artificially created polyps to improve the practical skills of gastroenterology fellows learning the techniques of gastric polypectomy. In theory, this should minimize risks to patients, as the fellows can practice the necessary maneuvers on the *ex vivo* model.

The most important advantage of the method we devised is the ease with which artificial polyps can be created. A previously described method is to lift the mucosa with surgical forceps and ligate the base with a suture[9]. However, using the multiband esophageal variceal ligator to raise the tissue and band allows rapid creation of multiple polyps in different locations within the stomach and allows the fellows to practice on a variety of lesions such as they might encounter clinically. Reducing the time required to prepare the model makes it more user-friendly. The entire setup is relatively inexpensive and easily portable.

Practicing with the *ex vivo* simulator has several other advantages over traditional clinical training in which an instructor supervises the trainee who performs the procedure in an actual patient. Clinical endoscopy may be a prolonged and difficult procedure in some patients. If the patient is unstable, the instructor may have to take over the procedure. Even if able to carry out the entire procedure themselves, fellows may feel less free to ask questions or receive feedback in the clinical setting. Also, unlike polyps of the colon, gastric polyps are relatively uncommon, with an incidence of less than 2%[10,11]. This means that in the course of subspecialty training, a fellow is unlikely to have many opportunities to perform gastric polypectomy under supervision. The four year-2 fellows we assessed had performed only a mean of 5 gastric polypectomies, with only one having the chance to do two such procedures in the second year of training.

A satisfactory outcome for gastric polypectomy requires expertise in multiple skills, including preparation of the electrosurgical cutting device and accessories, in addition to the endoscopic techniques such as proper snaring, stretching the polyp away from the intact mucosa, safe cutting and finally application of hemoclips to close the mucosal defect. Knowing that so many skills are required puts additional pressure on trainees, especially the first few times they perform the procedure. When they have to do so in a real patient, lack of confidence may increase the risk of technical errors and unpredictable outcomes[12]. Our year-1 fellows had considerable improvement in the confidence scale from a mean score of 4.5 ± 1.0 before training to 7.8 ± 0.5 after using the simulator in the work-

shop. They were able to practice the procedure a number of times without fear of injuring a patient. This contention is supported by the fact that their performance scores did appear to improve after the training.

As noted above, the most obvious improvement after the workshop was in setting up the equipment and communicating with the assistant. The educational experience in this workshop transcends a "simulation experience" because it also involves lectures, student-student interaction, and interaction with a faculty member which are all in a non-simulated environment. Of course this exposure would be in addition to a formal teaching setting with mentoring, and this by no means will or can replace that. While the mean score for hemoclip application improved from baseline (2.6 ± 0.5 to 3.6 ± 0.9), it did not reach 4 points. This experience is similar to that noted by Hochberger *et al*[1] when they first designed the compactEASIE model. This particular technique involves more intricate maneuvers and several steps, increasing the opportunities to make mistakes. It thus appears the application of hemoclips may require more training sessions for the fellows to achieve competency, which is all the more reason for ensuring that a good simulator is available. Familiarity with loading of the device, delivery, and deployment may enhance safe and successful application[13].

The expense of conducting a hands-on workshop may be a limiting factor for some training programs. Our 4-h workshop cost about US $350 overall using our hand-made simulator, endoscopes and accessories retired from clinical practice and pig stomachs. Because this workshop was a project of our Clinical Skills Training Center, the instructor, reviewers and assistants were all volunteers, and no facility fee was needed. The Olympus GIF Q230 endoscopes, polypectomy snare, and hemoclip device were retired from our clinical service and cost almost nothing to use. The hemoclips and rubber band ligation cost US $300. The price of one hand-made simulator was US $35 and pig stomachs were US $15 each. We therefore believe this hand-made *ex-vivo* porcine-stomach simulator could be used by most institutions without requiring a huge capital outlay.

Our study had a number of limitations. Firstly, this was an in-house project; we didn't recruit fellows from other sites therefore had a very small sample, precluding rigorous statistical analysis. The performance evaluations by the two reviewers were unblinded. Because we didn't invite outside experts as reviewers, there may have been observational bias. Evaluation with a video recording of the entire procedure is an alternative blinded method, but this approach can't adequately assess the equipment setup process or communication with the assistant. Secondary, we presume it's a very realistic simulator to represent the task of gastric polypectomy no matter the size of polyp, techniques or procedures time and it is easily transferable to clinical care. Whether those results will translate into better clinical performance of polypectomy as the year-1 fellows continue on into the second year of training remains to be seen. A number of factors may possibly result in incomplete transfer of simulator-acquired skills to the real world[14], including differences between the training environment and the clinic, anxiety and stress of learners[15]. Conducting such a study on a larger scale with an appropriate sample size and assessment of the clinical data is therefore still necessary.

Our pilot study demonstrates the feasibility of using the *ex vivo* pig stomach with artificially created polyps to improve the practical skills of gastroenterology fellows learning the techniques of gastric polypectomy. The workshop allowed the year-1 fellows to improve their skills nearly to the level of those acquired by year-2 fellows who were trained clinically. In theory, this should minimize risks to patients, as the fellows can practice the necessary maneuvers on the *ex vivo* model. We believe this is a simple and cost-effective way to train gastroenterology fellows in endoscopic polypectomy and regard this program as preceding training in more complicated polypectomy once competent in standard removal of small and pediculated lesions.

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COMMENTS

Background

Since the mid-1990s, an ex vivo porcine-tissue simulator had been widely used in many hands-on training programs. It has been shown to facilitate skill in a variety of endoscopic procedures.

Research frontiers

Endoscopic polypectomy is one such skill that must be acquired, including removal of gastric polyps incidentally discovered during endoscopic evaluations. The authors developed a method to simulate artificial polyps in an *ex vivo* pig-stomach model for use in their gastroenterology fellowship training program.

Innovations and breakthroughs

The most important advantage of the method the authors devised is the ease with which artificial polyps can be created. Using the multiband esophageal variceal ligator to raise the tissue and band it allows rapid creation of multiple polyps in different locations within the stomach.

Applications

Reducing the time required to prepare the model makes it more user-friendly. The entire setup is relatively inexpensive and easily portable.

Peer review

The authors should be congratulated in their efforts on this manuscript. Though the number of fellows is small, the differences and improvements were profound. The educational experience herein transcends a "simulation experience" because it also involves lectures, student-student interaction, and interaction with a faculty member - all in a non-simulated environment. The topic is timely. It would be a benefit if this improvement could be tied to a clinical parameter to remove doubt that all that occurred was improving performance in a simulation experience.

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BRIEF ARTICLE

Primary intestinal non-Hodgkin's lymphoma: A clinicopathologic analysis of 81 patients

Guo-Bao Wang, Guo-Liang Xu, Guang-Yu Luo, Hong-Bo Shan, Yin Li, Xiao-Yan Gao, Jian-Jun Li, Rong Zhang

Guo-Bao Wang, Guo-Liang Xu, Guang-Yu Luo, Hong-Bo Shan, Yin Li, Xiao-Yan Gao, Jian-Jun Li, Rong Zhang, Department of Laser Treatment and Endoscopy, Sun Yat-Sen University Cancer Center, Guangzhou 510060, Guangdong Province, China

Guo-Bao Wang, Guo-Liang Xu, State Key Laboratory of Oncology in South China, Guangzhou 510060, Guangdong Province, China

Author contributions: Wang GB designed the study and wrote the manuscript; Xu GL performed statistical analysis; Luo GY and Shan HB analyzed data and edited the manuscript; Li Y, Gao XY and Li JJ acquired and inputted data; Zhang R provided analytical tools and writing assistance.

Correspondence to: Guo-Liang Xu, Professor, Department of Laser Treatment and Endoscopy, Sun Yat-Sen University Cancer Center, 651 Dongfeng Road, Guangzhou 510060, Guangdong Province, China. manuscript_doris@yahoo.cn

Telephone: +86-13751889486 Fax: +86-020-87343272

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Abstract

AIM: To analyze the clinicopathologic features and the prognosis of primary intestinal lymphoma.

METHODS: Patients were included in the study based on standard diagnostic criteria for primary gastrointestinal lymphoma, and were treated at Sun Yat-sen University Cancer Centre between 1993 and 2008.

RESULTS: The study comprised 81 adults. The most common site was the ileocaecal region. Twenty-two point two percent patients had low-grade B-cell lymphoma. Fifty-one point nine percent patients had high-grade B-cell lymphoma and 25.9% patients had T-cell lymphoma. Most patients had localized disease. There were more patients and more early stage diseases in the latter period, and the origin sites changed. The majority of patients received the combined treatment, and about 20% patients only received nonsurgical therapy. The wverall survival and event-free survival rates after 5 years were 71.6% and 60.9% respectively. The multivariate analysis revealed that small intestine and ileocaecal region localization, B-cell phenotype, and normal lactate dehydrogenase were independent prognostic factors for better patient survival. Surgery based treatment did not improve the survival rate.

CONCLUSION: Refined stratification of the patients according to the prognostic variables may allow individualized treatment. Conservative treatment may be an optimal therapeutic modality for selected patients.

Key words: Gastrointestinal lymphoma; Non-Hodgkin's lymphoma; Gastrointestinal oncology; Prognostic factors

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INTRODUCTION

In the recent years, the incidence of non-Hodgkin's lymphoma (NHL) has increased worldwide, especially for primary extranodal lymphoma (ENL). Primary gastrointestinal lymphoma (PGIL) is the commonest ENL, which accounts for 30%-45% of ENL. Gastric lymphoma is the most frequent (55%-70%), followed by intestinal lymphoma (20%-35%), and colorectal lymphoma (5%-10%)[1].

Due to a lack of characteristic symptoms and a low

incidence rate, primary intestinal lymphoma (PIL) is easily misdiagnosed until serious complications occur, such as perforation and ileus. Furthermore, the optimal treatment for PIL remains controversial, and the prognosis is unsatisfactory. Although during the past two decades, the diagnosis and treatment of PGIL has changed tremendously, results from studies of gastric lymphoma are controversial regarding the benefit of surgical resection[2-4]. However, a large-scale prospective investigation of PIL is difficult due to its low incidence and complicated histologic subtypes.

Numerous retrospective studies report the PGIL in European countries[5-7], but there were no large-sample studies from Asia in this decade besides Japan[8]. Our study consisted of 81 Chinese patients with PIL who were diagnosed and treated at Sun Yat-sen University Cancer Centre between 1993 and 2008. It was a single center study, which retrospectively analyzed the clinical features, anatomic and histologic distribution, time trends and the prognosis of the PIL in Chinese patients.

MATERIALS AND METHODS

Patients

This study consisted of 81 adult Chinese patients with PIL who were diagnosed and treated at Sun Yat-sen University Cancer Centre between 1993 and 2008. Patients were included in the study based on standard diagnostic criteria for PGIL; patients had to present gastrointestinal (GI) symptoms or predominant lesions in the GI tract[9]. The patients who presented with second malignancies, had missing confirmation of histologic subtype, or had no follow-up information were excluded from the study.

The diagnostic workup included: details of the patients' history and physical examination with inspection of Waldeyer's ring, blood cell count, serum chemistry, chest radiographs, abdominal ultrasound, computed tomography (CT) scan of the chest and abdomen, bone marrow aspiration or biopsy, and endoscopy examination with multiple biopsy. Endoscopic ultrasound and positron emission tomography (PET)/CT were carried out when available.

The histologic specimens were obtained by endoscopic biopsy or surgery. These specimens were stained routinely with hematoxylin and eosin. Immunohistochemical staining for CD3 and CD20 was performed on all 81 specimens. In some selected cases, additional staining and polymerase chain reaction-based amplifications of genes for immunoglobulin heavy chain or T-cell receptor gamma chain were done. All slides were reviewed separately by two pathologists and a common consensus was reached in all cases. Histologic classification was done according to WHO criteria[10].

Patients were then staged according to the Ann Arbor system with modifications[11].

Treatment and response

The treatment modalities included surgical resection, conservative treatment (chemotherapy and radiotherapy), and the combined treatment. The main first line chemotherapy were four to six cycles of cyclophosphamide (CHOP) 750 mg/m^2, doxorubicin 50 mg/m^2, and vincristine 1.4 mg/m^2 (maximum, 2mg) each on day 1, plus prednisone 100 mg on days 1 to 5) or RCHOP (rituximab 375 mg/m^2 on day 0, plus CHOP). The regimen of cyclophosphamide, vincristine, doxorubicin, high-dose methotrexate (CODOX-M), alternating with ifosfamide, etoposide and high-dose cytarabine (IVAC), were used for patients with Burkitt's lymphoma[12].

International Workshop Criteria was used for response evaluation. Therapeutic response after the initial treatment was designated as complete remission (CR), partial remission, stable disease, or progression of disease.

Follow up and statistical analysis

Overall survival (OS) was defined as the time from diagnosis to the date of death for any reason or to the last follow-up. Event-free survival (EFS) was measured from the date of diagnosis to the date that event occurred. Events included disease progression, relapse and patient death for any reason.

The statistical differences were evaluated using the Student's *t*-test, Mann-Whitney *U* test (rank sum test) and the χ^2 test. Survival curves were calculated according to the Kaplan-Meier method, and the value was compared using the log rank test. All variables that influenced the prognosis ($P < 0.10$) were put into a multivariate analysis using the Cox proportional hazards model. Differences were considered significant if the *P*-value was < 0.05. All statistical analyses were performed using the Statistical Software Package for the Social Sciences version 17.0 (SPSS 17.0).

RESULTS

Patient clinical features

From January 1993 to December 2008, 81 patients with PIL were recruited for the study. The patients had a mean age of 46 years (range, 18-75 years) and comprised of 58 men and 23 women.

The clinical symptoms of PIL were unspecific. Pain was the main diagnostic symptom in most cases (75.3%), followed by hematochezia (25.9%) and diarrhea (16.9%). Palpable mass, constipation and loss of appetite were less common symptoms. Perforation was scarce (7.4%). "B" symptoms occurred in 42.0% patients.

Most patients had localized disease. Sixty-six (81.5%) patients had Stage Ⅰ~Ⅱ disease and 15 (18.5%) had Stage Ⅲ~Ⅳ disease. Most patients (75.3%) received the combined treatment, while about one fifth of patients only received nonsurgical therapy. The clinical features are shown in Table 1.

Sites of origin

The origins of PIL were confirmed by surgery, endoscopy, or CT. They were subdivided as follows: (1) duodenum; (2) jejunum; (3) ileum; (4) ileocaecal region; (5)

Table 1 Clinicopathologic features *n* (%)

Characteristics	Number of patients (*n* = 81)
Gender	
Male	58 (71.6)
Female	23 (28.4)
Histology	
Low-grade B-cell	18 (22.2)
High-grade B-cell	42 (51.9)
T cell	21 (25.9)
Stage	
Ⅰ~Ⅱ	66 (81.5)
Ⅲ~Ⅳ	15 (18.5)
Treatment	
Surgery alone	3 (3.7)
Nonsurgical	16 (19.8)
Both	61 (75.3)
No	1 (1.2)
Treatment response	
Complete remission	56 (69.1)
Partial remission	7 (8.6)
Stable disease	3 (3.7)
Progression	15 (18.5)

Table 2 Sites of origin *n* (%)

Sites of origin	Number of patients (*n* = 81)
Duodenum	2 (2.5)
Jejunum	4 (4.9)
Ileum	5 (6.2)
Ileocaecal region	31 (38.3)
Colon	20 (24.7)
Rectum	8 (9.9)
Multiple sites	11 (13.6)

Table 3 Immunohistological phenotypes *n* (%)

Histologic type	Patients		Total (*n* = 81)
	Male (*n* = 58)	Female (*n* = 23)	
B cell	42 (72.4)	18 (78.3)	60 (74.1)
MALT	6 (10.3)	9 (39.1)	15 (18.5)
Follicular lymphoma	3 (5.2)	0 (0.0)	3 (3.7)
Mantle cell lymphoma	3 (5.2)	0 (0.0)	3 (3.7)
DLBCL	27 (46.6)	8 (34.8)	35 (43.2)
Burkitt-like	2 (3.4)	1 (4.3)	3 (3.7)
Burkitt lymphoma	1 (1.7)	0 (0.0)	1 (1.2)
T cell	16 (27.6)	5 (21.7)	21 (25.9)
ETCL	6 (10.3)	1 (4.3)	7 (8.6)
Anaplastic	3 (5.2)	0 (0.0)	3 (3.7)
NK/T	0 (0.0)	1 (4.3)	1 (1.2)
PTCL, NOS	4 (6.9)	1 (4.3)	5 (6.2)
Others	3 (5.2)	2 (8.7)	5 (6.2)

MALT: Marginal zone lymphoma; DLBCL: Diffuse large B-cell lymphoma; ETCL: Enteropathy-type T cell lymphoma; PTCL, NOS: Peripheral T-cell lymphoma, not otherwise specified.

colon; (6) rectum; and (7) multiple sites. The ileocaecal region was defined as involvement of terminal ileum, cecum, appendix and or lower part of ascending colon. The most common site of PIL was the ileocaecal region (38.3%), followed by the colon (24.7%) and 13.6% small intestine (Table 2).

Histologic subtypes

PIL are heterogeneous diseases. In the current study, B cell NHL and T-cell NHL were both classified into five common subtypes. The B cell subtypes, arranged by order, were: diffuse large B-cell lymphoma (DLBCL) 35 (43.2%), marginal zone B-cell lymphoma 15 (18.5%), Burkitt and Burkitt-like lymphoma 4 (4.9%), follicular lymphoma 3 (3.7%), and mantle cell lymphoma 3 (3.7%). The ranks of the T cell subtypes were: enteropathy-type T cell lymphoma 7 (8.6%), peripheral T-cell lymphoma, not otherwise specified 5 (6.2%), uncertain subtype 5 (6.2%), anaplastic 3 (3.7%), and NK/T 1 (1.2%). All types of intestinal lymphoma showed broadly similar patterns in both sexes, apart from marginal zone lymphoma (MALT) which was predominant in women (9 in 23, 39.1%) (Table 3).

Time trends of intestinal lymphoma

The study was divided into two 8 year periods due to the use of Rituximab after 2000. Twenty-seven (33.3%) patients belonged to Period A, and 54 (66.7%) to Period B. Over these two periods, the average age of patients didn' t change (43.9 years *vs* 47.2 years, $P = 0.454$). The sites of origin were different ($P = 0.0469$), whereas the histology differences were not significant ($P = 0.4975$). More patients were in the early stage in period B ($P < 0.0001$), however the treatment and response did not change significantly ($P = 0.686$ *vs* $P = 0.6842$, respectively).

Treatment and prognosis

The follow-up after the diagnosis ranged from 18 to 183 mo (mean, 72 mo). The OS and EFS rates after 5 years were 71.6% and 60.9% respectively. Prognostic factors on univariate analysis were shown in Table 4.

Female patients showed a better OS, but EFS did not different between two groups. The sites of origin were prognostic (Figure 1). EFS in different sites were significantly changed ($P = 0.025$), and OS in the small intestine and ileocaecal region were significantly longer compared with rectum and multiple sites ($P = 0.016$). Histologic subtype was prognostic for EFS and OS ($P = 0.002$ and $P < 0.001$, respectively). B cell phenotype had a better prognosis than T cell phenotype (Figure 2). Patients who had perforation showed a poorer EFS and OS than those did not perforate. In the normal lactate dehydrogenase (LDH) group, EFS and OS were significantly better compared with the elevated LDH group ($P = 0.010$ *vs* $P = 0.034$, respectively) (Figure 3).

We could not detect any significant influence of age, stage, bulky disease, B symptom, treatment or time trend in EFS and OS of the PIL.

Cox multivariate analysis revealed that small intestine and ileocaecal region localization, B-cell phenotype, and normal LDH were independent prognostic factors for better OS and EFS (Table 5).

We also did prognostic analyses for the subgroups of B-cell lymphomas. Kaplan-Meier curves showed the sur-

Table 4 Prognostic factors on univariate analysis[1]

Characteristics	*n* = 81	5-year EFS	*P* value	5-year OS	*P* value
Age					
59 or younger	70	56.1	0.939	68.9	0.852
60 or older	11	56.8		68.4	
Gender					
Male	58	56.4	0.843	62.3	0.058
Female	23	56.2		85.6	
Sites of origin					
Small intestine	11	63.6	0.025	80.8	0.219
Ileocaecal region	31	71.0		79.6	
Colon	20	54.5		65.2	
Rectum	8	37.5		50.0	
Multiple sites	11	27.3		46.8	
Histology					
Low-grade B-cell	18	66.3	0.002	87.5	< 0.001
High-grade B-cell	42	71.3		79.2	
T cell	21	23.8		34.8	
Stage					
Ⅰ~Ⅱ	66	58.8	0.387	69.0	0.727
Ⅲ~Ⅳ	15	45.7		71.4	
Bulky disease					
< 8 cm	54	60.6	0.189	70.1	0.837
⩾ 8 cm	27	48.1		67.7	
Perforation					
Yes	6	28.6	0.074	25.0	0.024
No	75	58.9		72.5	
B symptom					
Yes	34	55.1	0.768	59.5	0.172
No	47	57.1		75.0	
LDH					
Elevated	25	34.2	0.010	52.2	0.034
Normal	56	65.9		76.9	
Treatment					
Surgery-based	64	54.3	0.668	70.9	0.371
Nonsurgical	16	61.9		60.9	
Radical surgery					
Yes	54	57.7	0.218	70.3	0.599
No	10	40.0		77.8	
Period					
A	27	51.9	0.594	66.7	0.808
B	54	58.8		71.3	

[1]Assessed by the log-rank test. EFS: Event-free survival; OS: Overall survival; LDH: Lactate dehydrogenase.

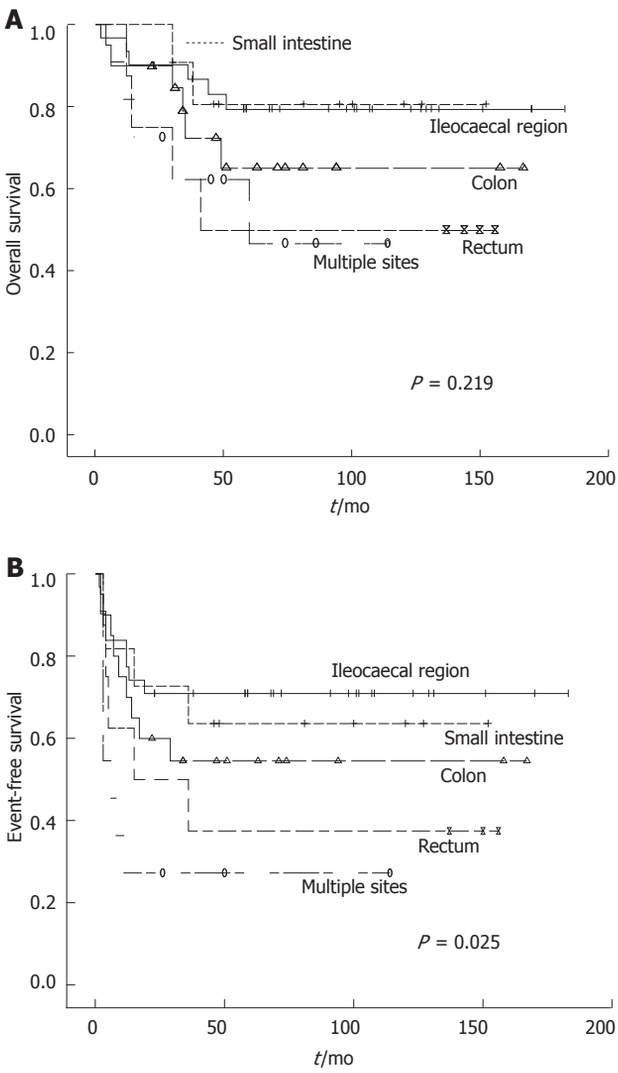

Figure 1 The survival curves stratified by the five groups according to anatomic site of origin. A: Overall survival among five groups (P = 0.219). Small intestine and ileocecal region *vs* rectum and multiple sites (P = 0.016); B: Event-free survival among five groups (P = 0.025).

vival of follicular lymphoma was better than MALT and DLBCL, and Burkitt lymphoma was the worst. However, the *P* values were not significant.

Cox multivariate analysis revealed that normal LDH were protective factors for better EFS (P = 0.007), and the gender and sites of origin were independent prognostic factors (P = 0.037 and P = 0.05, respectively). More specifically, females had better OS, and small intestine and ileocaecal region had better prognosis than rectum and multiple sites. We could not detect any significant influence of pathology, age, stage, bulky disease or treatment in B cell PIL (Table 6).

Numbers of T-cell lymphoma were too small for subanalyses. The small number of patients made it difficult to get an accurate analysis.

DISCUSSION

There are many publications about the epidemiology and clinical features of PIL. Only a few of the publications recruited more than 80 PIL patients[1,6,8], however, all of them were published before 2003. Except for a report on PGIL in Chinese patients which recruited 184 intestinal patients in 1995 from Hong Kong[13], there was no relative large-sample report from mainland China. Due to the differences in living habits and environment effects between Hong Kong and mainland China, it was necessary to analyze the epidemiology and prognosis of PIL in mainland China.

PIL is a male predominant disease, and male:female ratio was 2.5:1. In our series, we found that the age of the patients (median age = 46 and mean age = 46) were younger than the other reports[5,6,8,13-15].

The ileocaecal region was the most common site, with a frequency of 38.3%. Unlike the other reports[5,8,13,16], the colorectal involvement was more frequent. The rates of primary sites varied considerably in the other publications, especially noticeable for the rates of the ileocaecal region. The data ranged from 9.5% to 38.3% in our re-

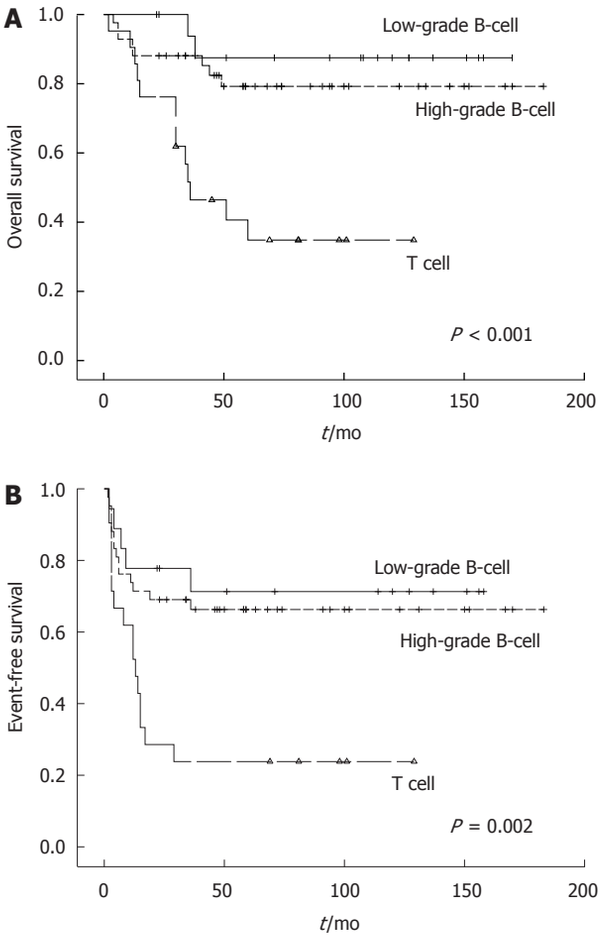

Figure 2 The survival curves stratified by the three histologic subgroups. A: Overall survival among the three groups ($P < 0.001$); B: Event-free survival among the three groups ($P = 0.002$).

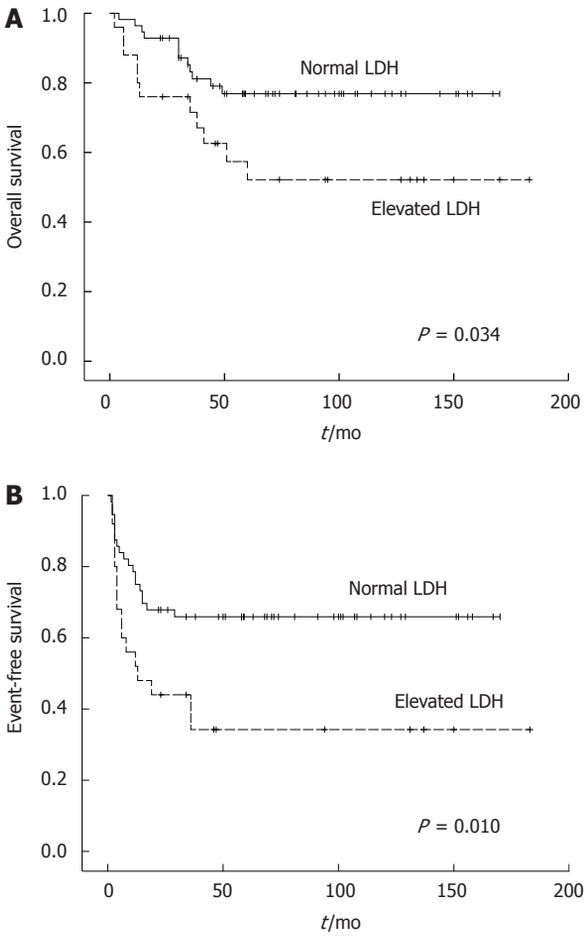

Figure 3 The survival curves stratified by the two groups according to lactate dehydrogenase levels. A: Overall survival between the two groups ($P = 0.034$); B: Event-free survival between the two groups ($P = 0.010$). LDH: Lactate dehydrogenase.

port[16]. We considered that the main reason for the difference was probably the precise definition of the ileocaecal region, which was missing in most reports. We believe it is important to distinguish it as a separate site, because our data show a significantly better prognosis for this region.

High-grade B cell lymphoma was the most common subtype in all the patients. This was followed by T cell lymphoma, which is apparently more than in Europe[5,6]. In our opinion, the reason for this is due to the higher ratio of T cell lymphoma in the NHL in China than European countries. Two publications reported T cell lymphoma only comprised 5% and 20.6% of the primary intestinal lymphoma in Chinese patients[17,18]. The difference between the reported series is difficult to interpret. Geographic variations in the prevalence of viral or bacterial infection, celiac disease, diet or other environmental factors may be the cause of the difference. However, the most likely reason may be due to these other studies having too small a sample size.

Our data confirmed the rising trend of PIL over the past 16 years in China, which was similar to reports from the United States[19] and Europe[6]. Furthermore, the number of patients with early stages or multiple sites has also increased. Advances in diagnostic procedures have led to an improvement in the accuracy in the diagnosis of lymphoma. However, an actual increase in the incidence of intestinal lymphoma should also be considered. Increasing exposure or susceptibility to risk factors, such as *H. pylori* infection, excessive protein or fat in the diet, and environmental pollution also may have contributed to this increase[6,20].

Most of the patients received combined treatment including surgery and chemotherapy with or without radiotherapy, 69.1% patients reached CR. The OS and EFS rates after 5 years were 71.6% and 60.9%, respectively. This was similar to the other reports[5,8].

Multivariate analysis revealed that small intestine and ileocaecal region localization, B-cell phenotype, and normal LDH were independent prognostic factors for better OS and EFS. Female gender[21] and no perforation indicated better OS in univariate analysis, but not significantly in multivariate analysis.

We also did prognostic analyses for the subgroups of B-cell lymphomas. The pathology subtypes of the B-cell lymphomas were not significant prognostic factors. Perhaps the small number of subtypes made it difficult to get an accurate analysis.

Stage and age were prognostic factors in many reports[2,22-25], and the size of the mass were also mentioned,

Table 5 Prognostic factors assessed by multivariate analysis[1]

Variable	Overall survival		Event-free survival	
	Exp(B)	*P* value	Exp (B)	*P* value
Pathology (B cell *vs* T cell)	4.566	0.002	3.833	0.001
LDH (normal 240 U/L)	1.002	0.013	1.003	0.002
Sites of origin	1.370	0.052	1.442	0.010
Gender	2.980	0.089	NE	
Perforation	0.835	0.778	0.625	0.438

[1]Assessed by Cox multivariate analysis. LDH: Lactate dehydrogenase.

Table 6 Prognostic factors for the subgroups of B-cell lymphomas[1]

Variable	Overall survival		Event-free survival	
	Exp(B)	*P* value	Exp (B)	*P* value
Pathology (subtypes of B cell lymphoma)	1.426	0.174	1.141	0.500
LDH (normal 240 U/L)	3.671	0.127	4.663	0.007
Gender	16.328	0.037	0.804	0.716
Sites of origin	1.604	0.050	1.306	0.151

[1]Assessed by Cox multivariate analysis. LDH: Lactate dehydrogenase.

but we found no find significant effect. Other factors reported to have contributed to survival included surgical resection and a good performance status[3].

We found surgery based treatment and radical surgery did not improve the survival rate. This fact raised the question of whether surgery is really necessary. Usually, the diagnostic difficulties, as well as the high rate of initial complications, led to primary resection of PIL in most patients. However, the efficacy of this procedure has not been evaluated so far. Furthermore, we observed complete remissions of PIL in patients receiving only conservative therapy[8,26,27].

It is clear that there was no benefit from lymph node clearance in lymphoma patients, which makes a case against extended surgical procedures. Surgery is still controversial as first-line therapy in patients with early stages of gastric lymphoma. Our findings indicate that patients with a clear diagnosis, better prognostic factors, and without initial complications should consider the conservative approach so that they may have a better quality of life. However, emergency operation is required when treatment related complications occur, such as perforation, ileus or bleeding.

PIL are heterogeneous diseases. In our point of view, the different prognostic factors and controversial treatment may be caused by the different constitution of these sub-types, ignoring racial and environment factors. Thus, further randomized prospective studies with a large number of patients are essential for this to be established.

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COMMENTS

Background

In recent years, the incidence of primary extranodal lymphoma has increased, and gastrointestinal lymphomas are the most frequent subtype. However, the optimal treatment for primary intestinal lymphoma (PIL) remains controversial.

Research frontiers

Numerous retrospective studies reported primary gastrointestinal lymphoma in Europe, but there were no large-sample studies from China. The optimal treatment for PIL remains controversial, and the prognostic data for patients with PIL remain to be elucidated.

Innovations and breakthroughs

This study analyzed the clinical feature, anatomic and histologic distribution, time trends, treatment responses and the prognosis of primary intestinal lymphoma in Chinese patients. The authors found surgery based treatment and radical surgery did not improve the survival rate. The fact raised the question of whether surgery is really necessary. The findings indicate that patients with a clear diagnosis, better prognostic factors, and without initial complications should consider the conservative approach, so that they may have a better quality of life.

Applications

Refined stratification of patients with PIL, according to the prognostic variables, may allow individualized treatment. Conservative treatment may be an optimal therapeutic modality for selected patients.

Terminology

PIL: Primary intestinal lymphoma; marginal zone lymphoma, mantle cell lymphoma, diffuse large B-cell lymphoma, enteropathy-type T cell lymphoma and peripheral T-cell lymphoma, not otherwise specified *et al* are histologic types of non-Hodgkin's lymphoma.

Peer review

This is an interesting manuscript on primary gastrointestinal-lymphoma in China. The authors analyzed the clinicopathologic features of PIL with special reference to time trends. They determined the prognostic factors for intestinal lymphoma and evaluated the influence of therapeutic modalities on the prognosis. The results showed that conservative treatment might be an optimal therapeutic modality for selected patients with PIL.

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CASE REPORT

S-1 induced secondary acute erythroid leukemia with a chromosome inv(12)(p13;q13)

Kensuke Matsumoto, Akira Kitanaka, Makiko Uemura, Fusako Waki, Tetsuya Fukumoto, Hiroaki Ohnishi, Yoshitsugu Kubota, Toshihiko Ishida

Kensuke Matsumoto, Makiko Uemura, Fusako Waki, Tetsuya Fukumoto, Hiroaki Ohnishi, Toshihiko Ishida, First Department of Internal Medicine, Faculty of Medicine, Kagawa Univesity, 1750-1 Miki-cho, Kita-gun, Kagawa prefecture 761-0793, Japan
Akira Kitanaka, Hematology, Faculty of Medicine, Kagawa Univesity, 1750-1, Miki-cho, Kita-gun, Kagawa prefecture 761-0793, Japan
Yoshitsugu Kubota, Department of Transfusion Medicine, Kagawa University,1750-1 Miki-cho, Kita-gun, Kagawa prefecture 761-0793, Japan
Author contributions: Matsumoto K, Kitanaka A contributed equally to this work; Uemura M, Waki F, Fukumoto T, Ohnishi H, Kubota Y, Ishida T designed research; Matsumoto K and Kitanaka A wrote the paper.
Correspondence to: Kensuke Matsumoto, MD, PhD, First Department of Internal Medicine, Faculty of Medicine, Kagawa Univesity, 1750-1, Miki-cho, Kita-gun, Kagawa prefecture, Japan. matsumot@med.kagawa-u.ac.jp
Telephone: +81-87-8912145 Fax: +81-87-8912147
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Abstract

Adjuvant chemotherapy by S-1 following gastrectomy is considered standard treatment in Japan. Analysis of follow-up data have proved the efficacy of S-1 administration, and that hematological adverse events were relatively rare. Pyrimidine anti-metabolites, including S-1, have shown relatively lower risks for secondary hematological malignancies in comparison to alkylating agents and topoisomerase-Ⅱ inhibitors. We here report a case of therapy-related leukemia after S-1 administration. A patient who had received S-1as the sole adjuvant chemotherapy was diagnosed with acute erythroid leukemia. To the best of our knowledge, our patient represents the first report of S-1 induced acute leukemia.

Key words: S-1; Secondary leukemia; Acute erythroid Leukemia; Gastric cancer

Peer reviewers: Limas Kupcinskas, Professor, Department of Gastroenterology, Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT44307, Lithuania

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INTRODUCTION

S-1 (tegafur + gimeracil + osteracil) oral administration for long periods has been widely used in East Asia as an adjuvant chemotherapy and for a advanced gastric cancer with little caution regarding the development of secondary malignancy[1]. The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC), a randomized study comparing S-1 adjuvant therapy with surgery only proved the efficacy of S-1adjuvant therapy. In the ACTS-GC study, hematological adverse events of grade 3 or 4 were relatively rare[2].

Therapy-related leukemia (TRL) may be separated into two types. The first type which usually develops 3-6 years after chemotherapy with alkylating agents, is usually preceded by a preleukemic phase. It is associated with specific unbalanced cytogenetic aberrations mostly involving chromosome 5 or 7, with an acute myeloid leukemia (AML) and invariably carries a poor prognosis. The second type is found in patients treated with topoisomerase Ⅱ inhibitors, and lacks a preleukemic phase. Rather, it often develops after a short latency, and presents with

cytogenetic rearrangements specific to *de novo* AML, such as t(8;21), inv(16), t(15;17) or often with a balanced translocation between 11q23 and other chromosomes, primarily t(6;11), t(9;11) and t(11;19)[3-5].

Although alkylating agents and topoisomerase Ⅱ inhibitors are well known as drugs that are related to the development of therapy-related leukemia, pyrimidine antimetabolites, including S-1, have been thought to be rarely associated with the development of leukemia[6]. In fact, therapy related acute leukemia by the sole administration of pyrimidine antimetabolites is very rare. Therapy-related leukemia induced by S-1 has not yet been reported. A recent study, however, revealed that pyrimidine antimetabolites could cause damage to DNA[7].

CASE REPORT

We report a 67-year-old male who developed acute erythroid leukemia after adjuvant chemotherapy using S-1 following distal gastrectomy (D2 resection) for primary gastric cancer (T2N1M0 stage Ⅱ, poorly differentiated adenocarcinoma non-solid type). Peripheral blood analysis showed no abnormalities before chemotherapy.

Ten courses of chemotherapy S-1 (120 mg/d) were orally administered between April 2008 and July 2009. He had not received any other chemotherapeutic agents. In August 2009, peripheral blood analysis demonstrated mild anemia and leukocytopenia. He was referred to our department for further examination. Peripheral blood analysis showed anemia (Hb9.0 g/dL), leukocytopenia (1840/μL) and thrombocytopenia (101 000/mL). Bone marrow aspiration revealed hypercellular marrow with 57.1% of the erythroblasts showing megaloblastic morphologic changes. Blast comprised 38.0% of non-erythroid cells.

Bone marrow pictures revealed morphologically dysplastic nuclei and cytoplasm in all three hematopoietic cell lineages. The patient was diagnosed with acute erythroid leukemia, according to the WHO classification. Immunophenotypical analysis by flowcytometry demonstrated that the leukemic cells were CD 4- CD13+, CD33dim, CD34+, CD56+, CD117+, MPO-/+, TdT- and HLA-DR+. Chromosomal analysis showed 45, XY, del(5q), inv(12)(p13;q13), -17, -17, add (22)(q13), +marl[7]/47, sl, +10, +11, +22, add (22)[4]48, sdl1, +8[2]/46, XY[3].

The patient was initially treated with idarubicin and Ara-C, but failed to achieve complete remission and was subsequently administered an alternative induction chemotherapy regimen [G-CSF + Fludarabine + Ara-C + Mitoxantrone (FLAGM)]. Treatment with salvage chemotherapy failed to induce remission. She died after 3 mo from diagnosis from sepsis and liver failure.

DISCUSSION

The introduction of adjuvant chemotherapy after successful surgical or radiotherapeutic eradication of cancers has been considered to improve relapse-free survival. However, treatment-related malignancy has emerged as a serious complication. The accumulation of genetic aberrations induced by anti-cancer agents in hematopoietic stem cells ultimately leads to myelodysplastic syndrome (MDS)/AML[8]. On the other hand, oral administration of pyrimidine anti-metabolites for long periods has been widely used in Japan as an adjuvant chemotherapy with little caution regarding the development of secondary malignancy.

Abe *et al*[9] reported a case of tegafur-induced AML who developed AML 8 years after starting tegafur (Table 1). This patient showed del(5) chromosomal change, as in our case. Other patients Table 1 were also administrated a large amount of pyrimidine anti-metabolites; in all of them it took at least 24 mo to develop AML. Our patient developed AML after a cumulative S-1 dose of 33.6g only 13 mo after starting S-1.

Acute erythroid leukemia accounts for less than 5% of all leukemia cases. The incidence for leukemia among 65-to-69-year-old males in Japan is $18.9/10^5$ per year[12]. We estimate that the possibility of the coincidence of two malignancies would not be very high given this data. However, the patient's leukocytes might have had an abnormal gene that did not present phenotypically before, and S-1 administration might have caused a further gene mutation that caused the leukemia.

S-1 is a combination preparation consisting of tegafur, gimeracil [5-chloro-2,4-dihydroxypyridine (CDHP)] and oteracil potassium (Oxo) in a molar ratio of 1:0.4:1. CDHP reversibly inhibits the function of dihydropyrimidine dehydrogenase (DPD), which mediates the rate-limiting process of 5-‰Fluorouracil (5-FU) elimination, thereby increasing the plasma concentration of 5-FU. UFT is another combination preparation consisting of tegafur and uracil in a molar ratio of 1:4. Although uracil, like CDHP, inhibits DPD, its inhibitory potency is far weaker than that of CDHP[13]. The content of tegafur in UFT is also 3-to 5-fold higher than that in S-1. Therefore, a short time duration and a small cumulative dose of S-1 could still induce secondary leukemia as in our case. Two cases of S-1 -induced chronic myeloid leukemia (CML) have been reported. The cumulative doses of S-1 were only 41.5 g and 92.5 g, respectively[14]. The present case should serve as a cogent warning that even a relatively short period of S-1 intake may result in the development of lethal leukemia.

Therapy-related leukemias are often refractory to conventional treatment and are associated with poor survival, with a few exceptions such as acute promyelocytic leukemia. As recently reviewed by Pedersen-Bjergaard *et al*[15], survival after post -transplant t-MDS/AML is estimated to be 6 mo.

Manola *et al*[16] reported that t(12;12)(p13;q13) constitutes a disruption of the *ETV6*(*TEL*) gene. The 12p13 region is genetically unstable and fragile, with subsequent translocations and insertions into other chromosomes[17]. It has been reported that multiple chromosome breaks in this region are likely to have been induced through

Table 1 Therapy-related leukemia cases induced by (adjuvant therapy constituted) mainly of pyrimidine anti-metabolites

No.	Age/sex	Primary tumor	Type of treatment for (primary) tumor	Duration from prior therapy (mo)	FAB	Karyotype	Survival duration (mo)	Ref.
1	81/M	Colon	c.r+ 1086 g tegafur/uracil	24	M4	47XY, +8, t(11;17)(q23;q25)	14	[10]
2	54/M	Colon	c.r+ 315 g of UFT +210 mg of MMC	40	M6	44, XY, dic(5;17)(q13;p11), -7, add (15)(q24) 44, as above, -dic(5;17), +mar1	3 >	[11]
3	67/M	Colon rectum	c.r+ 645 g of tegafur, 560 mg of Ara-C, 56 mg of MMC	96	M2	45XY, -5, -6, 7q-, -8, -20, +3mar	6	[9]
4	67/M	Colon	c.r+ 252g of UFT, 80mg of MMC	108	M2	47XY, +1, der(1;7)(q10,p10), -7, +8	10	[9]

c.r: Curative resection; M: Male; UFT: Tegafur/uracil; MMC: Mitomycin C; Ara-C: Cytarabine; FAB: French-American-British classification.

chemo/radiotherapy or mutagens, and are associated with a subgroup of patients with extremely bad prognoses. Although the 12p13 region is considered genetically unstable, t(12;12)(p13;q12~q13) is a rare cytogenetic abnormality. Only one case of therapy-related acute leukemia with t(12;12)(p13;q13) has been reported[16].

Our case suggests that therapy-related leukemia may develop after exposure to pyrimidine anti-metabolites. Thus, S-1 may induce TRL even when used for shorter durations and at lower cumulative doses than other pyrimidine anti-metabolites. Adjuvant chemotherapy by S-1 is a standard therapy for locally advanced gastric cancer in Japan, and is often used in China and Singapore as well. Therefore, more caution should be taken against the possibility of t-MDS/AML caused by S-1 in these countries.

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CASE REPORT

Autoimmune pancreatitis characterized by predominant CD8+ T lymphocyte infiltration

She-Yu Li, Xiang-Yang Huang, Yong-Tao Chen, Yi Liu, Sha Zhao

She-Yu Li, Department of Endocrinology and Metabolism and Department of Rheumatology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Xiang-Yang Huang, Yong-Tao Chen, Yi Liu, Department of Rheumatology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Sha Zhao, Department of Pathology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
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Author contributions: Li SY collected the clinical data of the case and wrote the manuscript; Huang XY collected the clinical data, performed the histological investigations, and reviewed the manuscript; Liu Y reviewed the manuscript; Chen YT, collected the clinical data of the case; Zhao S reviewed the pathology of the specimen; Li SY and Huang XY contributed equally to this work.
Correspondence to: Xiang-Yang Huang, MD, Associate Professor of Medicine, Department of Rheumatology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. yangyang1029@yahoo.com.cn
Telephone: +86-28-85422393 Fax: +86-28-85423459
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Abstract

Autoimmune pancreatitis (AIP) is a rare form of pancreatitis characterized by prominent lymphocyte infiltration and pancreatic fibrosis resulting in organ dysfunction. The pathogenesis and pathology of AIP remain unknown. A 64-year-old Chinese man presented with symptoms and signs of bile duct obstruction diffuse enlargement of the head of pancreas, elevated IgG levels, and negative autoimmune antibody responses. A pylorus-preserving pancreatoduodenectomy was performed and a pancreatic tumor was suspected. However, periductal lymphoplasmacytic infiltration and fibrosis were found in the head of pancreas and nearby organs instead of tumor cells. Four months after surgery, the patient was readmitted because of reoccurrence of severe jaundice and sustained abdominal distension. Prednisone 30 mg/d was administered orally as an AIP was suspected. One and a half months later, the symptoms of the patient disappeared, and globulin, aminotransferase and bilirubin levels decreased significantly. Over a 9-mo follow-up period, the dose of prednisone was gradually decreased to 10 mg/d and the patient remained in good condition. We further demonstrated dominant CD3+/CD8+ populations, CD20+ cells and a few CD4+ cells in the pancreatic parenchyma, duodenum and gallbladder wall by immunohistochemical assay. This AIP case presented with significant CD8+ T lymphocyte infiltration in the pancreas and extra-pancreatic lesions, indicating that this cell population may be more important in mediating AIP pathogenesis than previously known and that AIP might be a poorly defined autoimmune disease with heterogeneous pathogenesis.

Key words: Autoimmune pancreatitis; Pancreas; Prednisone; CD8+ T and CD4+ T lymphocytes; CD20; Inflammatory cell; Infiltration

Peer reviewers: Basil Ammori, MD, Department of Surgery, Salford Royal Hospital, Stott Lane, Salford, Greater Manchester, M6 8HD, United Kingdom; Dr. Thiruvengadam Muniraj, MBBS, MD, PhD, MRCP (UK), University of Pittsburgh Medical Center, 100 Chatham Park Drive, Apt 511, Pittsburgh, PA 15220, United States

Li SY, Huang XY, Chen YT, Liu Y, Zhao S. Autoimmune pancreatitis characterized by predominant CD8+ T lymphocyte infiltration. *World J Gastroenterol* 2011; 17(41): 4635-4639 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i41/4635.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i41.4635

INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare form of pancre-

atitis characterized by prominent lymphocyte infiltration and pancreatic fibrosis resulting in organ dysfunction[1]. This disease has been mainly reported in Japan and Europe with an estimated incidence of 4%-6% among chronic pancreatitis patients[2,3]. In China, only a few AIP cases have been reported even though approximately 5% of chronic pancreatitis cases resulted from autoimmune-associated responses[4]. To date, the pathogenesis and pathology of AIP remain undefined. Previously, CD4+ T lymphocytes were thought to be critical (relative to CD8+ T lymphocytes) in mediating AIP pathogenesis[5-7]. However, the case of AIP described herein demonstrated a significant CD8+ infiltration and an absence of CD4+ T lymphocytes in the pancreas and surrounding organs, including the duodenum.

CASE REPORT

A 64-year-old Chinese man was admitted to the West China Hospital on May 8, 2004 with a history of 1-mo abdominal distension and 3-d jaundice. He was examined with blood biochemical tests, including direct bilirubin (DB, 114.71 μmol/L), indirect bilirubin (IB, 44.9 μmol/L), alanine amino transferase (ALT, 70 IU/L), aspartate amino transferase (AST, 51 IU/L), globulin (45.8 g/L) and fasting plasma glucose (FPG, 6.1 mmol/L) (Figure 1). Computed tomography (CT) showed a 3 cm × 3 cm mass at the head of the pancreas suspected to be a pancreatic tumor. Therefore, a pylorus-preserving pancreatoduodenectomy was performed on May 11 and a 3 cm × 3 cm mass was identified at the head of the sclerified pancreas, with cholestatic changes in the liver and gallbladder hydrop with dilation of the common bile duct (1.5-2 cm; the normal range is 0.3-0.8 cm). Pathological examination showed chronic inflammation with fibrosis in the pancreatic and common bile ducts as well as moderate to severe epithelial dysplasia (Figure 2). Adenoepithelial hyperplasia but not tumor cells were found in the pancreatic parenchyma. The patient was discharged 2 wk after surgery when jaundice symptoms abated.

Four months later on September 13, 2004, the patient was readmitted to the West China Hospital due to a recurrence of severe jaundice and sustained abdominal distension. No obvious abnormal findings were identified following physical examination other than jaundice in the skin and sclera. Blood biochemical results (Figure 1) demonstrated the levels of DB at 91.5 μmol/L, IB at 37.7 μmol/L, ALT at 41 IU/L, AST at 59 IU/L, globulin at 60.4 g/L, FPG at 4.3 mmol/L, immunoglobulin gamma (IgG) at 47.2 g/L, IgA > 3000 mg/L and C-reactive protein at 14.3 mg/L. Detections of anti-nuclear antibodies, anti-double strand DNA antibodies (anti-dsDNA), anti-Smith antibodies, anti-Sjogren syndrome A antibodies, anti-Sjogren syndrome B antibodies, anti-Jo-1 antibodies, anti-mitochondrial M2 antibodies, anti-liver-kidney microsome antibodies, anti-liver cytosol antibody type 1 and anti-soluble liver antigen antibodies were all negative. Magnetic resonance cholangiopancreatography

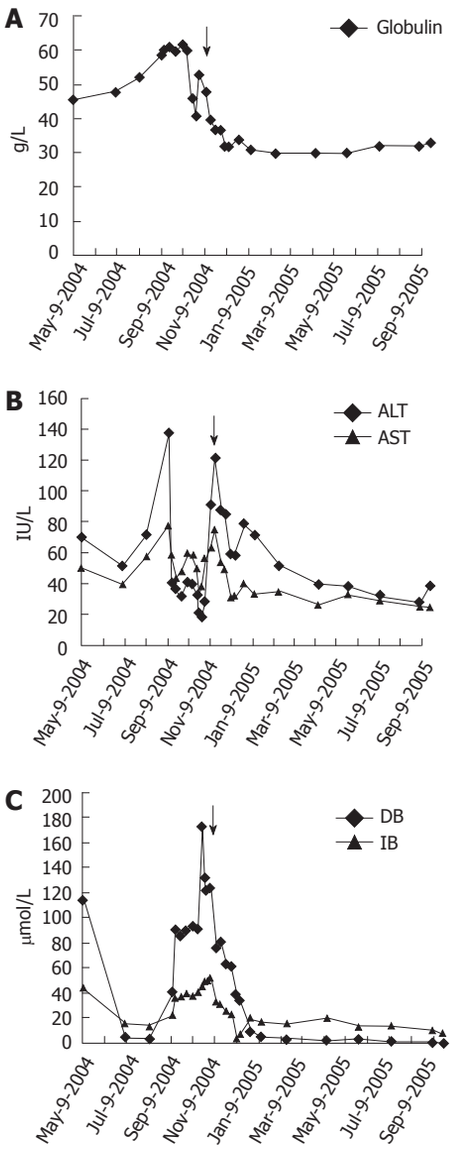

Figure 1 Blood biochemical analyses. Pre- and post-prednisone treatment levels of (A) immunoglobulin, (B) aminotransferase and (C) bilirubin. The black arrow indicates the start of prednisone treatment. ALT: Alanine amino transferase; AST: Aspartate amino transferase; DB: Direct bilirubin; IB: Indirect bilirubin.

showed a dilated intrahepatic biliary duct and a thickened and asperous wall of the common bile duct.

Based on the hypergammaglobulinemia and elevated IgG levels observed, this patient was suspected to have pancreatitis of autoimmune origin. Prednisone 30 mg/d was administered orally beginning on November 2, 2005. One and a half months later, the patient was discharged from the hospital after the jaundice and abdominal distension disappeared and the globulin (Figure 1A), aminotransferase (Figure 1B) and bilirubin (Figure 1C) levels decreased significantly. Over a 9-mo follow-up period, the dose of prednisone was gradually decreased to 10 mg/d and the patient remained in good condition without the presentation of either jaundice or abdominal distention and his globulin (Figure 1A), aminotransferase (Figure 1B) and bilirubin (Figure 1C) levels were within the nor-

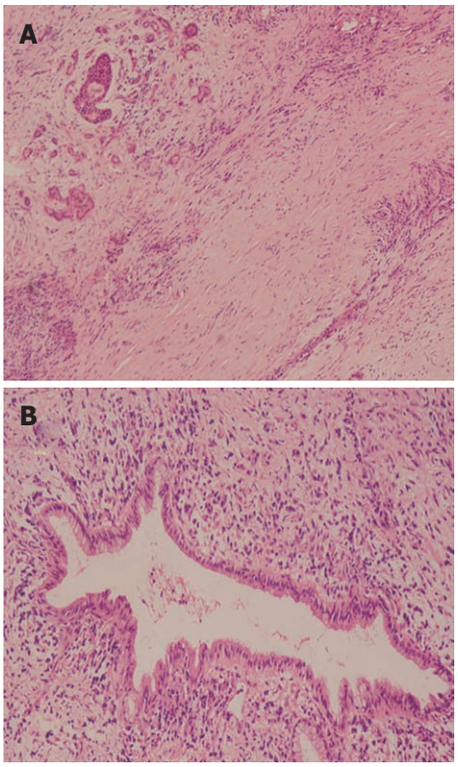

Figure 2 Characterization of pathological changes. The head of the pancreas was examined following Hematoxylin and eosin staining, demonstrating (A) pancreatic ductal atrophy and lymphocytic infiltration with fibrotic proliferation and (B) periductal infiltration of lymphocytes.

mal range.

To further characterize the pathogenesis of AIP in this case, histopathologic and immunohistochemical analyses were carried out to determine the CD3, CD4, CD8 and CD20 positive profiles in paraffin sections taken from the head of the pancreas, duodenum and gallbladder. Hematoxylin and eosin staining of the head of pancreas showed ductal atrophy and periductal lymphocytic infiltration with fibrotic proliferation (Figure 2), and high levels of CD3+ cells were identified in the periductal region of the head of the pancreas (Figure 3A), duodenal villi (Figure 3B) and the gallbladder wall (Figure 3C). Interestingly, CD4+ cells were present at low levels in the specimens from the head of the pancreas (Figure 3D), duodenum (Figure 3E) and the gallbladder wall (Figure 3F). Parallel to the CD3 expression levels, CD8+ cells were detected at high levels in the periductal region of the head of the pancreas (Figure 3G), duodenal villi (Figure 3H) and the gallbladder wall (Figure 3I). The CD20 was expressed at low levels in the periductal region of the head of the pancreas (Figure 3J) and focally expressed in the duodenal villi (Figure 3K). No CD20 expression was detected in the gallbladder wall (Figure 3L).

DISCUSSION

AIP, also described as chronic pancreatitis of autoimmune origin, is clinically similar in presentation to pancreatic carcinoma. AIP patients may undergo pancreatoduodenectomy as a consequence of treatment. It is reported that 2.5%-11% of the patients diagnosed with pancreatic malignancy actually had a benign pancreatic disorder confirmed by pathological examination after surgery[7] and 9.9% of the patients underwent pancreatoduodenectomy following a diagnosis of pancreatitis, 38% of them were reported to be caused by autoimmune responses[8,9].

AIP is characterized by an irregular narrowing of the main pancreatic duct, massive lymphoplasmacytic inflammation of the pancreatic parenchyma, hypergammaglobulinemia and a fair response to glucocorticoid treatment[10]. The case presented in this report was mainly characterized by symptoms and signs of bile duct obstruction, diffuse enlargement of the head of pancreas, elevated IgG levels, negative autoimmune antibody responses and periductal lymphoplasmacytic infiltration and fibrosis. After a strict treatment regimen of prednisone, the patient recovered quickly and his globulin, aminotransferase, and bilirubin levels returned to normal for more than half a year. In our case, AIP was diagnosed based on the criteria established by the Japan Pancreas Society[2,11].

AIP presentation has recently been divided into either subtype type 1 or 2[12-14]. In Asia, type 1 AIP presents at a higher frequency and is also referred to as lymphoplasmacytic sclerosing pancreatitis or AIP without granulocyte epithelial lesions (GELs). Type 2 AIP is referred to as idiopathic duct-centric pancreatitis or AIP with GELs. The case presented here with periductal lymphoplasmacytic infiltration (without granulocytes), fibrotic proliferation and pancreatic ductal atrophy is consistent with the histopathological features of type 1 AIP[13,15,16].

It was previously reported that infiltrating cells in AIP cases predominantly consisted of CD4+ T lymphocytes with few detectable CD8+ T lymphocytes and B lymphocytes[2,7]. T helper imbalance (Th1 *vs* Th2) is believed to be associated with the initiation of AIP[17] and elevated Th1 responses were reported in both pancreatic and extra-pancreatic lesions in AIP patients[18,19]. CD4+CD25+ regulatory T lymphocytes have also been demonstrated to contribute significantly to the pathology of AIP-associated lesions compared to lesions resulting from other pancreatic disorders[6,19]. In animal AIP disease models, administration of amylase-sensitized CD4+ T lymphocytes elicited autoimmune pancreatitis, suggesting that CD4+ T lymphocytes may be the most important components in mediating disease pathogenesis[20]. This observation is supported by the cases presenting with a reduced number of infiltrating CD4+ T lymphocytes in AIP lesions.

In contrast, our case presented with infiltrating CD8+ T (primarily cytotoxic T lymphocytes) instead of CD4+ T lymphocytes in both pancreatic and extra-pancreatic lesions. Since CD4+ T helper lymphocyte is believe to be indispensable in mediating various types of immune responses, we hypothesize that CD4+ T lymphocytes may have originally infiltrated these tissues and then recruited more CD8+ T lymphocytes functioning as effector cells of AIP pathogenesis. These data demonstrated that the presence of CD8+ T lymphocytes in AIP lesions may be

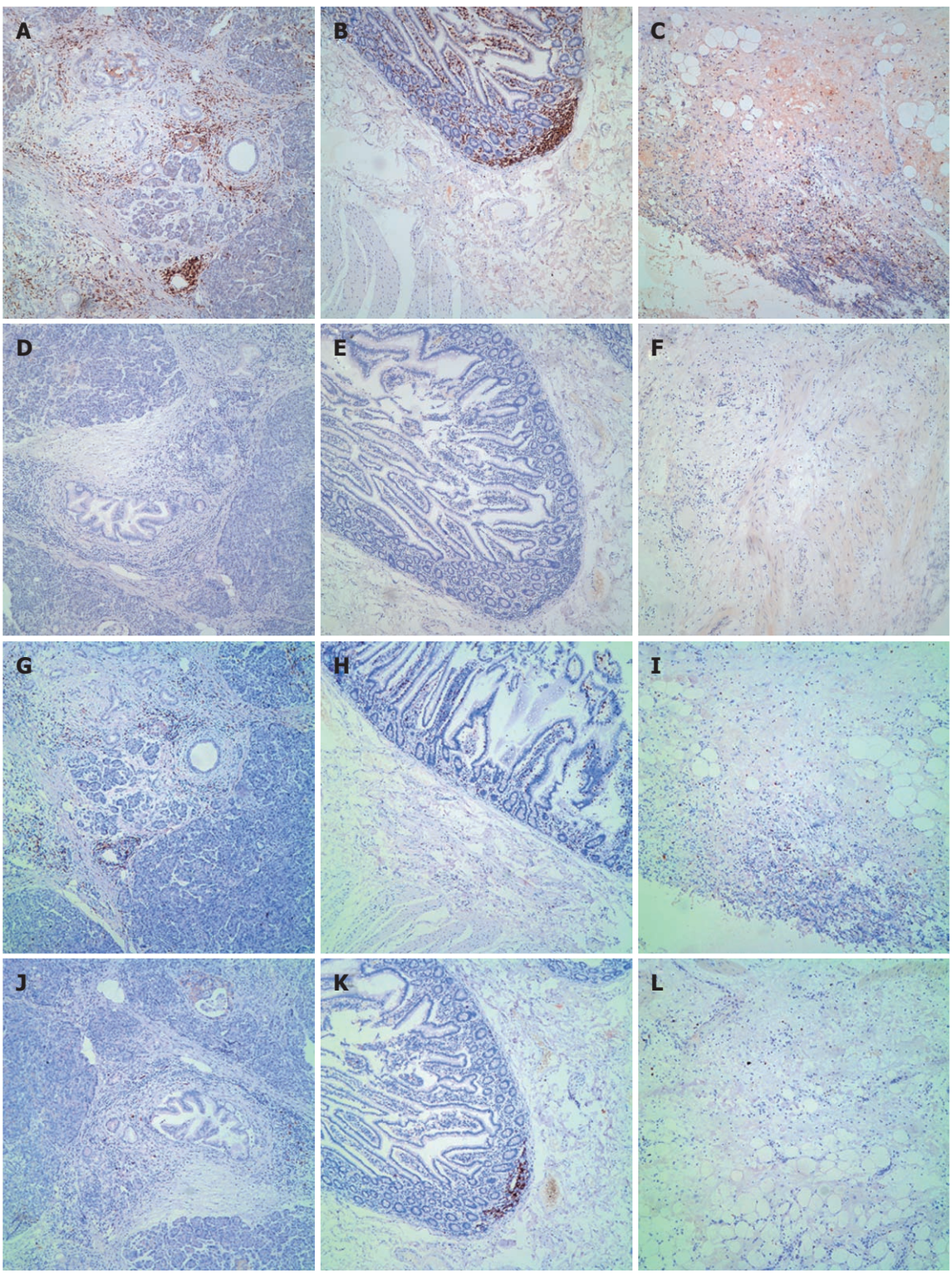

Figure 3 Immunohistochemical characterization. The head of the pancreas, duodenum and the wall of the gallbladder were examined for the presence of CD3+, CD4+, CD8+ and CD20+ cells; magnification × 100. A: CD3+ lymphocyte infiltration in the periductal region of the head of the pancreas; B: CD3+ lymphocyte infiltration and their focal concentration in duodenal villi; C: CD3+ lymphocyte infiltration in the wall of the gallbladder; D: CD4+ lymphocytes infiltration in the head of the pancreas; E: CD4+ lymphocyte infiltration in the duodenum; F: CD4+ lymphocyte infiltration in the gallbladder; G: CD8+ lymphocyte infiltration in the periductal region of the head of the pancreas; H: CD8+ lymphocyte infiltration in the duodenal villi; I: CD8+ lymphocyte infiltration in the wall of the gallbladder; J: CD20+ lymphocyte infiltration in the periductal region of the head of the pancreas; K: Focal CD20+ lymphocyte infiltration in duodenal villi; L: CD20+ lymphocyte infiltration in the wall of the gallbladder.

more important than previously thought and that elicitation of AIP may have heterogeneous origins. A limitation in these observations is that they have only been reported in a single case, more investigations about the role of

CD8+ T lymphocytes are required to further understand the pathogenesis of AIP.

In summary, we identified a significant number of CD8+ T lymphocytes infiltrating in both pancreatic and extra-pancreatic AIP lesions, instead of CD4+ T lymphocytes as commonly expected. It suggests that AIP might possess heterogeneous autoimmune origins.

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Pancreatic cancer risk variant *ABO rs505922* in patients with cholangiocarcinoma

Marcin Krawczyk, Florentina Mihalache, Aksana Höblinger, Monica Acalovschi, Frank Lammert, Vincent Zimmer

Marcin Krawczyk, Florentina Mihalache, Frank Lammert, Vincent Zimmer, Department of Medicine Ⅱ, Saarland University Medical Center, 66421 Homburg, Germany
Florentina Mihalache, Monica Acalovschi, Department of Medicine Ⅲ, University Iuliu Hatieganu, 400162 Cluj-Napoca, Romania
Aksana Höblinger, Department of Internal Medicine Ⅰ, University Hospital Bonn, 53127 Bonn, Germany
Author contributions: Krawczyk M and Mihalache F contributed equally to this work; Mihalache F performed genotyping; Mihalache F, Höblinger A, Acalovschi M and Zimmer V included patients and controls in the study; Krawczyk M, Lammert F and Zimmer V analyzed the data; Zimmer V and FLammert F designed and supervised the project; Krawczyk M, Zimmer V and Lammert F wrote and edited the paper.
Correspondence to: Dr. Marcin Krawczyk, Department of Medicine Ⅱ, Saarland University Medical Center, Kirrberger Str. 1, 66421 Homburg, Germany. marcin.krawczyk@uks.eu
Telephone: +49-6841-1623242 Fax: +49-6841-1623570
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Abstract

The aim of this study was to investigate an association between the development of cholangiocarcinoma (CCA) and the *ABO* variant *rs505922* (known to increase pancreatic cancer risk) in a large cohort of European individuals with CCA. In total, 180 individuals with CCA and 350 CCA-free controls were included. The *ABO* variant *rs505922* was genotyped using a polymerase chain reaction-based assay. Association between this single nucleotide polymorphism (SNP) and CCA was tested in contingency tables. Neither allele distributions nor association tests and regression analysis provided evidence for an increased risk of CCA among carriers of the *ABO* variant (all $P > 0.05$). Nevertheless, we documented a deviation from Hardy-Weinberg equilibrium in the entire CCA cohort ($P = 0.028$) and for patients with intrahepatic ($P = 0.037$) but not extrahepatic tumor localization ($P > 0.05$). The association tests did not provide evidence for a prominent role of the investigated SNP in the genetic risk of CCA. However, Hardy-Weinberg disequilibrium in the entire cohort and the intrahepatic CCA subgroup warrants future studies investigating a potential CCA risk modulation by individual blood groups.

Key words: *ABO*; Biliary tract cancer; Blood groups; Genetic risk; Single nucleotide polymorphism

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TO THE EDITOR

We were very interested to read the recent report by Greer *et al*[1], which further substantiates the association between an individual's blood group and the risk of pancreatic cancer. In line with previous data, Greer *et al*[2] demonstrate that individuals with blood group O have a lower risk of pancreatic cancer relative to blood groups A or B. These serological data are consistent with results from a large genome-wide association study comprising 2457 patients with pancreatic cancer, whereby the common variant *rs505922* (C > T) in the *ABO* locus was identified as a genetic risk factor for this malignancy. Interestingly, the [TT] genotype, which proved to be protective against pancreatic malignancy, is in complete linkage disequilibrium

Table 1 Allele and genotype distribution and association tests

ABO rs505922 allele/genotype	Counts of alleles/genotypes	
	Controls (2N = 700)	Cases (2N = 360)
C	246 (0.35)	124 (0.34)
T	454 (0.65)	236 (0.66)
CC	50 (0.14)	28 (0.16)
CT	146 (0.42)	68 (0.38)
TT	154 (0.44)	84 (0.46)
Association test	χ^2	*P*
Allele frequency difference test	0.05	0.82
Armitrage's trend test	0.05	0.83
OR statistics	OR	95% CI
[T]↔[C]	1.03	0.79-1.35
[TT]↔[CC]	0.94	0.57-1.66
[TT]↔[CT + CC]	1.11	0.78-1.60

Patients with cholangiocarcinoma are defined as cases, the [C] allele represents the assumed cholangiocarcinoma risk allele. CI: Confidence interval; OR: Odds ratio.

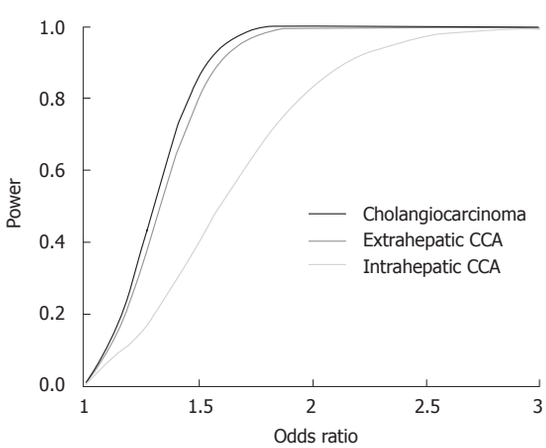

Figure 1 Statistical power as a function of the effect size (odds ratio) in the cholangiocarcinoma cohort (black) and in extra- and intrahepatic cholangiocarcinoma subgroups (dark grey and grey, respectively) (α set at 0.05). CCA: Cholangiocarcinoma.

(r^2 = 1.0) with blood group O. Conversely, the [C] allele is present in individuals with blood groups A, B or AB.

Cholangiocarcinoma (CCA) albeit uncommon, represents the second most prevalent primary liver cancer, and is globally increasing in incidence[3]. As with pancreatic cancer, CCA is usually diagnosed in the late stages with locally advanced or metastatic disease, and is therefore characterized by poor prognosis. Hence, the identification of genetic variants contributing to CCA development is warranted, to further elucidate the pathobiological mechanisms modulating disease risk, and to assist with the development of novel screening strategies for detecting patients at risk of biliary malignancy. Many low-risk variants have been postulated to confer an increased risk for cancers, including CCA[4]. Indeed, our previous study demonstrated the genetic risk of CCA to be modulated by heterozygosity for the *alpha1-antitrypsin* Z allele[5].

In the current study, we therefore specifically assessed the potential role of blood groups in CCA risk using a single nucleotide polymorphism (SNP)-based approach in a large European CCA cohort consisting of 180 individuals with CCA and 350 CCA-free controls. The details of this cohort are described in our previous study[5]. With regards to our methodology, the intronic variant *rs505922* was genotyped with a 99% success rate, using a polymerase chain reaction-based assay with 5'-nuclease and fluorescence detection (Taqman, Applied Biosystems, Foster City, CA, United States). The consistency of our genotyping results with the Hardy-Weinberg equilibrium (HEW) was verified by exact tests (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). An association between the *ABO* variant and biliary cancer was tested in contingency tables (genotypes, Armitrage's trend test; alleles, χ^2 test) and by regression analysis using SPSS software (version 18.0).

Table 1 summarises the genotyping results. The frequency of [TT] individuals (known to carry blood group O) is consistent with the distributions reported in European populations (http://www.bloodbook.com/world-abo.html). As shown in Table 1, allele distributions did not differ significantly between cases and controls (P > 0.05). The association tests [common odds ratio (OR) = 1.01, P = 0.83)] and regression analysis (OR for the [TT] variant = 1.11, P = 0.56) did not provide evidence for the involvement of the *ABO* variant in CCA. Similarly, subsequent exploratory data analysis stratifying cases according to gender and intra- *vs* extrahepatic tumour localisation yielded no significant association between *rs505922* and CCA (all P > 0.05). Interestingly, we detected a departure from HWE in patients with CCA (P = 0.028) and in the subgroup of cases with intrahepatic CCA (P = 0.037), but not in cases with extrahepatic disease (P > 0.05). Although the SNP was not associated with CCA in the above statistical tests, the presence of Hardy-Weinberg disequilibrium might however, be indicative of a possible association, since the assumption of no selection implies that the gene is not associated with the disease[6]. Confirmed consistency with HWE in the much larger control cohort and 100% consistent results in re-genotyped cancer individuals (n = 20) argue against genotyping errors as a reason for departure from HWE in the cancer group. However, the exploratory data analysis in the current study may be underpowered due to the limited sample size of the intrahepatic CCA subgroup (n = 40) (Figure 1). Of note, recent data from an Italian hospital-based cancer registry report a non-significant underrepresentation of serological blood group 0 in a chimeric cancer subgroup referred to as "*liver and intrahepatic bile ducts*" (36% *vs* 46 %; P = 0.015; n = 78)[7].

In conclusion, the blood group polymorphism investigated in this study does not appear to alter the general risk of developing CCA. Furthermore, due to the relatively small number of patients with intrahepatic CCA, departure from HWE should be interpreted with caution. Nevertheless, further dedicated studies exploring the possible functional role of *ABO* blood types in cholangiocarcinogenesis in selected groups of patients (i.e., with

intrahepatic CCA) may provide further insight into the pathobiological mechanisms that enhance the risk of this malignancy.

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2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

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International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

World Journal of Gastroenterology

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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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]

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.

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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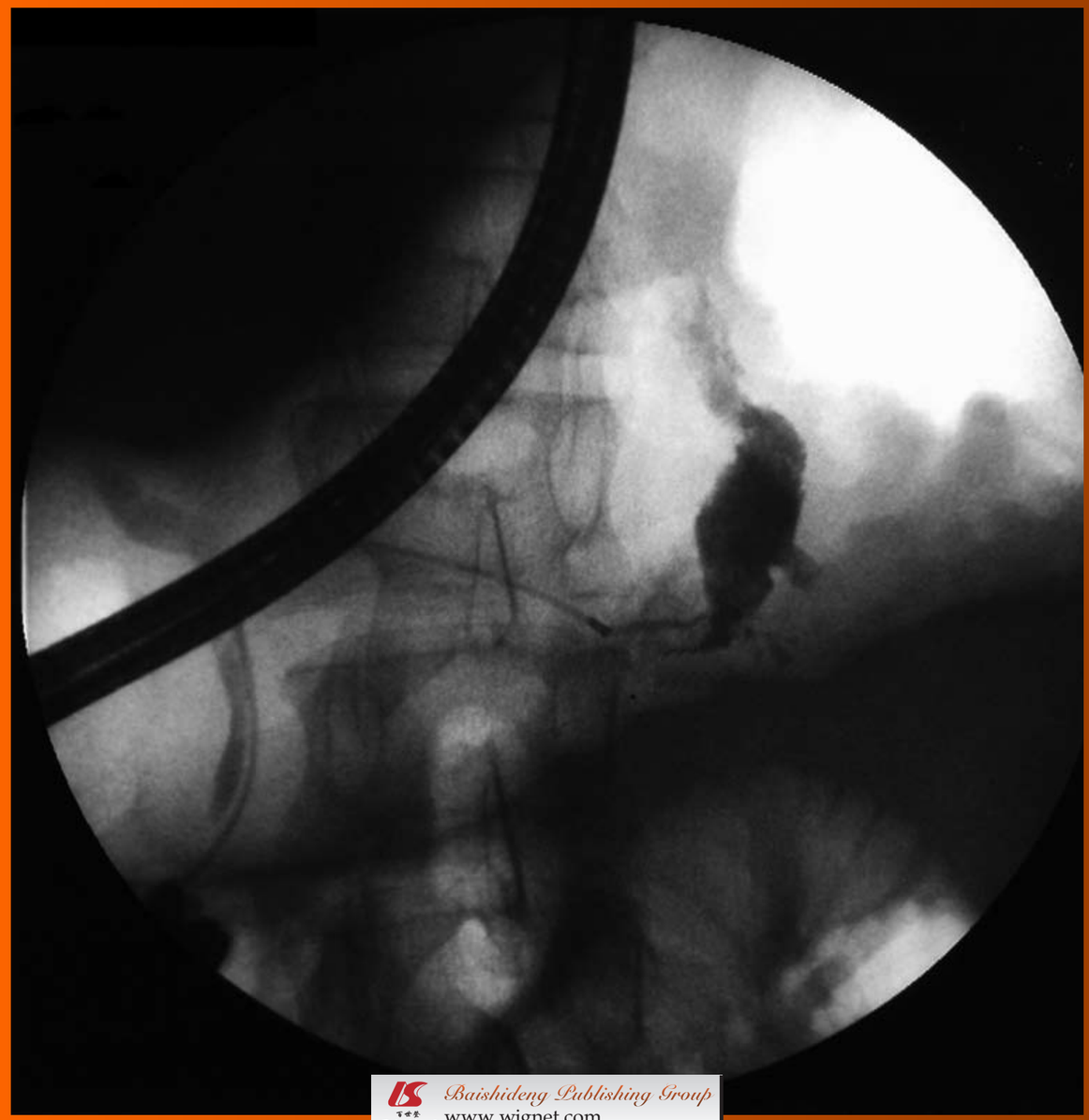

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EDITORIAL

Rifaximin in the treatment of inflammatory bowel disease

Mario Guslandi

Mario Guslandi, Gastroenterology Unit, S Raffaele University Hospital, 20132 Milan, Italy
Author contributions: Guslandi M contributed solely to this editorial.
Correspondence to: Mario Guslandi, Professor, Gastroenterology Unit, S Raffaele University Hospital, Via Olgettina 60, 20132 Milan, Italy. guslandi.mario@hsr.it
Telephone: +39-02-26432744 Fax: +39-02-26433491
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Abstract

The gut microbiota plays a role in promoting and maintaining inflammation in inflammatory bowel diseases (IBD), hence the rationale for the use of antibiotics in the treatment of those disorders. Antibiotics, however, may induce untoward effects, especially during long-term therapy. Rifaximin α polymer is an antibacterial agent that is virtually unabsorbed after oral administration and is devoid of systemic side effects. Rifaximin has provided promising results in inducing remission of Crohn's disease (up to 69% in open studies and significantly higher rates than placebo in double blind trials) and ulcerative colitis (76% in open studies and significantly higher rates than placebo in controlled studies) and might also have a role in maintaining remission of ulcerative colitis and pouchitis. The potential therapeutic activity of rifaximin in IBD deserves to be further investigated and confirmed in larger, controlled studies. The optimal dosage still needs to be better defined.

Key words: Antibiotics; Gut microbiota; Inflammatory bowel disease; Rifaximin

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INTRODUCTION

The etiology of inflammatory bowel diseases (IBD) still remains obscure. Genetic, immunological, environmental, and psychological factors can all play a role in the pathophysiology of both ulcerative colitis and Crohn's disease.

The gut microbiota is now recognized as a further important factor involved in promoting and/or maintaining the inflammatory process typical for IBD[1-3].

The concentration of intestinal bacteria in IBD patients is higher than normal, gradually increasing with the severity of the disease[1]. A breakdown in the qualitative balance between protective and harmful bacteria (dysbiosis) has also been proposed as a potential mechanism[2].

Indeed, in Crohn's disease, an increased presence of *Campylobacter concisus*[4] and *E. Coli*[5], as well as a substantial decrease in the amount of the anti-inflammatory commensal *Faecalibacterium prausnitzii*[5,6], has been reported.

On the other hand, it has been suggested that *Fusobacterium varium* can promote the development of ulcerative colitis[7].

The above data constitute a good rationale for the use of antibiotics in IBD[8].

Various meta-analyses have demonstrated that antibiotics such as metronidazole, ciprofloxacin, clofazimine and antibiotic combinations can be successfully employed in the treatment of Crohn's disease[9-11], ulcerative colitis[12] and pouchitis[13].

However, prolonged administration of antibiotics is accompanied by systemic adverse effects.

Rifaximin α-polymer, a rifampicin derivative, is a

locally acting antibacterial agent that is virtually unabsorbed after oral administration, is mostly excreted as unchanged drug in the stools in the course of intestinal disorders, and is thus devoid of systemic side effects.

It exhibits a broad-spectrum activity against enteric bacteria and a lack of clinically relevant acquired resistance[14-16]. Currently approved in the United States for the treatment of travellers' diarrhea, rifaximin is being used in a variety of gastrointestinal disorders, such as small intestine bacterial overgrowth, colonic diverticular disease, *Clostridium difficile* infection, as well as in the treatment of portal systemic encephalopathy[16,17].

In particular, rifaximin has been shown to modulate the colonic microbiota of patients with Crohn's disease by increasing the concentration of *Bifidobacteria* and *Faecalibacterium prausnitzii*[18].

In addition, experimental studies have shown that the drug can reduce the development of trinitrobenzene sulfonic acid-induced colitis and accelerate healing by preventing bacterial translocation[19], as well as exerting anti-inflammatory activities by increasing the expression of pregnane-X-receptor and by antagonizing the effects of tumor necrosis factor -α on intestinal epithelial cells[20,21].

The possible therapeutic role of rifaximin in the treatment of IBD has been repeatedly investigated in recent years.

RIFAXIMIN AND CROHN'S DISEASE

Further to an open-label study where rifaximin 200 mg tid administered for 16 wk to 29 patients with active Crohn's disease reduced Crohn's disease activity index (CDAI) score by more than 40% and induced clinical remission in 59% of cases[22], and a recent retrospective analysis of the charts of 68 patients receiving adjunctive therapy with rifaximin (mean dose 600 mg/d for 16 wk) showing remission in up to 70% of cases[23], two controlled studies were carried out.

A multicenter, double-blind, placebo controlled trial including 83 patients with mild-to-moderate Crohn's disease[24] found that monotherapy with rifaximin 800 mg bid for 12 wk was superior to placebo in promoting clinical remission (CDAI < 150), which was observed in 52% of cases compared with 33% in the placebo group. The difference in remission rates, however, was statistically significant ($P = 0.032$) only between the subgroups of patients with baseline values of C reactive protein above the upper normal limit.

A recent, international, multicenter, randomised study enrolling 402 patients from 55 centers in Europe and Israel demonstrated that an extended intestinal release formulation of rifaximin 400 mg in daily doses of 400-1200 mg bid for 12 wk was significantly superior to placebo in inducing remission (as defined as a CDAI < 150).

The best results were observed at the dose of 800 mg bid (remission rate 62.2% *vs* 42.6% in the placebo group: $P = 0.005$) and the effects were maintained during a subsequent 12-wk follow-up without treatment[25].

RIFAXIMIN AND ULCERATIVE COLITIS

In an open-label study, 30 patients with a mild-to-moderate flare-up of ulcerative colitis during maintenance treatment with mesalazine, and in whom steroid treatment was not advisable because of a history of poor tolerability, rifaximin 400 mg bid was added for four weeks[26]. Clinical remission was obtained in 76.6% of cases.

On the other hand, a group of 28 patients refractory to steroid therapy received an adjunct therapy with either rifaximin 400 mg bid or placebo for 10 d, in a double blind fashion. In the rifaximin group clinical improvement was observed in 64.3% of patients, who showed a significant reduction in stool frequency ($P < 0.02$), rectal bleeding ($P < 0.05$) and sigmoidoscopic score ($P < 0.05$) compared with placebo[27].

A small pilot experience on six mesalazine-intolerant patients with ulcerative colitis, who were in remission after a course of oral steroids, employed a combination of rifaximin 400 mg + the probiotic agent *Saccharomyces boulardii* 500 mg as a maintenance treatment for three months. At the end of the treatment period, all patients were still in clinical remission, which suggests that this therapeutic combination can be useful in preventing early relapses of ulcerative colitis[28].

Rifaximin, in doses ranging from 200 to 1800 mg/d, was also assessed as a maintenance therapy in 51 patients who had undergone restorative proctocolectomy and ileal pouch-anal anastomosis for ulcerative colitis, affected by antibiotic-dependent pouchitis[29]. At 3 mo, remission was maintained in 65% of patients. 79% of these patients were still in remission at 6 mo, 58% at 12 mo and 6% at 24 mo.

A combination of rifaximin 1000 mg bid and ciprofloxacin 500 mg bid for 15 d had been previously found capable of promoting either improvement (55.5%) or remission (33.3%) in eighteen patients with chronic active pouchitis[30].

CONCLUSION

The role of the gut microbiota in the development and maintenance of inflammation in IBD provides the rationale for the use of antibiotics in the medical treatment of both Crohn's disease and ulcerative colitis. Systemic antibiotics, such as ciprofloxacin and/or metronidazole, are commonly employed with good results, but possible side effects limit their use, especially for prolonged periods.

On the other hand, rifaximin α polymer, thanks to its negligible intestinal absorption, represents a safer and more attractive alternative. Both in open and in controlled studies, rifaximin, either in monotherapy or as an adjunctive treatment, was found to provide satisfactory results when administered for up to 12 wk (Table 1).

In a preliminary experience in children with IBD, rifaximin induced encouraging results and proved to be well tolerated[31].

Additional controlled study are warranted to fur-

Table 1 Rifaximin in inflammatory bowel disease

Ref.	Year	Patient (*n*)	Study type	Dose (mg)	Duration (wk)	Concomitant medication	Outcome
Crohn's disease							
Shafran *et al*[22]	2005	29	Open	200 tid	16	Various	Remission up to 59%
Shafran *et al*[23]	2010	68	Open	200 tid	16	Steroids (46%)	Remission rate up to 65%
Prantera *et al*[24]	2006	83	Db RCT	800 bid	12	None	Remission rate > placebo
Prantera *et al*[25]	2010	402	Db RCT	400-1200 bid	12	Various (no steroids)	Remission rate > placebo (also at 12 wk follow-up)
Ulcerative colitis							
Guslandi *et al*[26]	2006	30	Open	400 bid	4	Mesalazine	Remission rate 76%
Gionchetti *et al*[27]	1999	28	Db	400 bid	10 d	Steroids	Clinical improvement > placebo
Guslandi *et al*[28]	2010	6	Open, pilot	400 od	12	*S.boulardii*	Remission maintained in 100%

Db: Double blind; RCT: Randomized controlled trial; >: Significantly higher.

ther confirm and expand the currently available data on the possible role of rifaximin in the treatment of IBD patients and to better define the optimal dosage of the drug in this clinical setting.

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EDITORIAL

Prevention and management of non-steroidal anti-inflammatory drugs-induced small intestinal injury

Sung Chul Park, Hoon Jai Chun, Chang Don Kang, Donggeun Sul

Sung Chul Park, Chang Don Kang, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kangwon National University School of Medicine, Chuncheon 200-722, South Korea
Hoon Jai Chun, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University Anam Hospital, Korea University College of Medicine, Seoul 136-705, South Korea
Donggeun Sul, Graduate School of Medicine, Korea University, Seoul 136-705, South Korea
Author contributions: Park SC and Chun HJ wrote the article; Kang CD and Sul D assisted in drafting and revising the paper.
Correspondence to: Hoon Jai Chun, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University Anam Hospital, Korea University College of Medicine, 126-1, Anam-dong 5 ga, Seongbuk-gu, Seoul 136-705, South Korea. drchunhj@chol.com
Telephone: +82-2-9206555 Fax: +82-2-9531943
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Abstract

Non-steroidal anti-inflammatory drug (NSAID)-induced small bowel injury is a topic that deserves attention since the advent of capsule endoscopy and balloon enteroscopy. NSAID enteropathy is common and is mostly asymptomatic. However, massive bleeding, stricture, or perforation may occur. The pathogenesis of small intestine injury by NSAIDs is complex and different from that of the upper gastrointestinal tract. No drug has yet been developed that can completely prevent or treat NSAID enteropathy. Therefore, a long-term randomized study in chronic NSAID users is needed.

Key words: Non-steroidal anti-inflammatory drugs; Small intestinal injury; Prevention; Treatment

Peer reviewer: Dr. Francesco Manguso, MD, PhD, UOC di Gastroenterologia, AORN A. Cardarelli, Via A. Cardarelli 9, Napoli 80122, Italy

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most commonly used pharmaceuticals worldwide. They are used for prevention and treatment of inflammatory diseases, arthritis, collagen diseases, pain, fever, and ischemic cerebrovascular disorders because of their anti-inflammatory, analgesic, antipyretic, and anti-platelet functions. In recent years, it has also been reported that they are effective for the prevention of colorectal cancer[1].

NSAIDs function by inhibiting cyclooxygenase (COX), the enzyme responsible for synthesis of prostaglandin. However, there are side effects with the use of NSAID-based therapy. The most common side effects are disorders of the digestive tract mucosa[2]. In addition to upper gastrointestinal complications, such as gastric and duodenal ulcers, complications in the small intestine and colon can occur, which cause bleeding, perforation, stricture, and chronic problems, such as iron deficiency anemia and protein loss[3].

The adverse effects of NSAIDs on the gastrointestinal tract are often unrelated to abdominal symptoms. In patients with suspected gastrointestinal bleeding, but who are not found to have bleeding lesions on gastroscopy and colonoscopy, NSAID-induced small intestine ulcerative lesions should be suspected[4]. The use of

NSAIDs has recently increased; therefore, increased awareness of the gastrointestinal side effects is needed. However, effective prevention and treatment of the side effects of NSAIDs in the small intestine have not yet been determined. In this manuscript, we review the studies conducted to date on the prevention and treatment of small intestine damage caused by NSAIDs.

EPIDEMIOLOGY

Until recently, gastrointestinal injury by NSAIDs was studied mainly in upper gastrointestinal organs, such as the stomach and duodenum, but there have been few studies on the small intestine. Among chronic NSAID users, up to 25% suffer from upper gastrointestinal ulcers, while bleeding or perforation occurs in 2%-4%[5].

Upper gastrointestinal complications in the stomach or duodenum are relatively easy to examine by endoscopy and upper gastrointestinal series, whereas it is more difficult to observe complications of the small intestine and determine the prevalence of injuries to this organ[6]. However, according to autopsy results published by Allison *et al*[2] in 1992, small intestinal ulcers were found in 0.6% of patients who did not take NSAIDs, whereas they were found in 8.4% of individuals taking NSAIDs. In more than 70% of arthritis patients receiving NSAID therapy for more than three months, intestinal inflammation accompanied by bleeding and protein loss was induced; even after the therapy ended, and these symptoms could persist longer than 16 mo[7]. Iron deficiency anemia due to blood loss in the small intestine was found in 41% of rheumatoid arthritis patients taking NSAIDs[1].

According to a recent study, gross damage was observed in 68% of volunteers who were administered 75 mg of diclofenac for 2 wk[8]. Another report found that macroscopic injury occurred in 80% of patients who took low doses of aspirin for 2 wk[9]. NSAID-related damage mainly occurred in the distal small bowel and colon, most commonly in the ileocecal region[10].

PATHOGENESIS

Administering NSAID increases intestinal permeability within 12 h and inflammation in the small intestine within 10 d[10]. The mechanism underlying small intestine injury by NSAIDs, unlike complications of the upper gastrointestinal tract, has not been elucidated because of the presence of intestinal bacteria in the small intestine and other complicating factors. The results of studies on the mechanism of injury by NSAIDs are still not sufficient, but can be summarized as combined systemic and local effects.

Currently, this has been described as a "three hit hypothesis"[3]. First, the phospholipids in cell surface membrane are damaged by direct injury by the NSAID, and damage to mitochondria within the cells subsequently occurs. Damage to mitochondria cause a reduction of energy generation within the cells (uncoupling of oxidative phosphorylation), release of intracellular calcium, and generation of free radicals. This leads to a decrease of integration between the cells and increased permeability of the small intestine. Through the increased intestinal permeability, various materials such as bile acids, food, intestinal bacteria, and proteolytic enzymes damage the weakened intestinal barrier and secondary inflammation occurs by the activation of neutrophils[3].

In experimental studies, Gram-negative bacteria invade the mucous membrane and activate Toll-like receptors, which are the receptors for Gram-negative bacterial lipopolysaccharide (LPS). It was reported that Toll-like receptors stimulate the inflammatory response and play an important role in small intestine damage[11,12].

If intestinal bacteria secrete endotoxins, intestinal bacterial translocation can occur. That is, LPS originating from endotoxins can spread to other places in addition to the intestine. LPS increases the expression of inducible nitric oxide synthase (iNOS) and iNOS leads to the production of peroxynitrite, the cytotoxic moiety from nitric oxide (NO) and superoxide. Ampicillin and metronidazole inactivate LPS and reduce iNOS expression[13].

Enterohepatic circulation plays an important role in gut injury. If NSAIDs do not enter the enterohepatic circulation, they will not damage the small intestine. For example, sundilac or aspirin, which do not enter the enterohepatic circulation, are less toxic to the small intestine[14]. However, if the intestine is continuously exposed to the drugs in the bloodstream *via* the enterohepatic circulation, damage may occur[14].

NSAIDs are conjugated to acryl glucuronides in the liver and excreted through the canalicular membrance of hepatocytes into bile[15]. Electrophilic NSAID-acyl glucuronides contact the brush border proteins of the enterocyte, causing the uptake of the NSAID into the cell. Acyl glucuronide also plays a role in the transport of NSAIDs to the target site-the distal part of jejunum/ileum. However, the role of acyl glucuronides in NSAID enteropathy is not yet clear.

There are two types of COX: COX-1 and COX-2. Prostaglandins derived from COX-1 are considered to be important for maintaining intestinal mucosa homeostasis. Previously, it was found that COX-1 had "housekeeping" characteristics, and inhibition of this factor reduced blood circulation in the mucosa and increased intestinal permeability, thereby causing injury to the gastrointestinal tract. Inhibition of COX-2 is not associated with gastrointestinal damage[16]. However, in a recently study using an animal model, intestinal mucosa damage occurred when both COX-1 and COX-2 were inhibited[17]. This finding suggests that COX-2 acts as immunomodulator and is involved in the healing process of inflammation. Thus, there could be an immunological mechanism whereby the inhibition of COX-2 causes gastrointestinal damage[3].

Heme oxygenase-1 (HO-1) is the rate-limiting en-

zyme in heme catabolism, and the upregulation of HO-1 produces anti-inflammatory or anti-oxidative effects. HO-1 is thought to be involved in the inhibition of small intestinal damage associated with NSAID. Pretreatment with an HO-1 inhibitor, SnPP (tin-protoporphyrin Ⅸ), exacerbates damage to the small intestine by indomethacin. Lansoprazole ameliorates small intestine ulcers induced by indomethacin through the upregulation of HO-1[18].

DIAGNOSIS

In the past, the documentation of NSAID-induced enteropathy was based on the measurement of small intestine permeability and an analysis of indicators of inflammation, such as fecal calprotectin. In recent years, intestinal mucosa have been able to be viewed directly by capsule endoscopy and enteroscopy[19,20].

For diagnosis, there should be a history of NSAID use, no history of antimicrobial agent use, and no bacterial growth in the stool or tissue cultures. There should be no vasculitis or granuloma in tissue specimens, and, after stopping an NSAID, clinical symptoms and the lesions found by endoscopy should disappear.

Intestinal permeability test

The intestinal permeability test, which examines damage to the intestinal barrier, is primarily used to measure the amount of an orally administered test reagent that is discharged in the urine[21]. Within 12 h after NSAID therapy, increased intestinal permeability can be observed. The material that is used for an intestinal permeability test is rarely absorbed into the normal intestinal barrier, but its absorption increases in damaged intestinal barrier, after which it is transported into the bloodstream and excreted in urine. Most of the material used for this test is excreted in urine within a certain time and is not metabolized *in vivo*[1]. The probes used in intestinal permeability tests include polyethylene glycol, cellobiose, sugars (such as lactulose and mannitol), and radionuclides, such as chromium-51-labeled ethylenediaminetetraacetic acid (^{51}Cr-EDTA). Of these, ^{51}Cr-EDTA is the most widely-used for measuring the damage by NSAIDs. It is not degraded by intestinal bacteria, reflects some of the colon permeability, and is used in a relatively simple assay. Increased intestinal permeability is observed in about 50%-70% of long-term NSAID users. Although the clinical usefulness of the intestinal permeability test is low, it has been used in a clinical study that observed the effects of food or drugs on the inhibition of intestinal damage caused by NSAIDs[1,6].

Measurement of intestinal inflammation

Intestinal inflammation by NSAIDs can be measured by scintigraphy using ^{111}Indium-labeled neutrophils[21]. In 50%-70% of individual taking NSAIDs for more than six months, labeled white cells were found to accumulate in the terminal ileum 20 h after administration, and a slight increase of inflammation was observed compared to patients with inflammatory bowel disease (IBD). This can be measured up to 16 mo after the patient has stopped taking the drug. However, this method is very expensive and it is difficult to apply in clinical tests. The detection of calprotectin in feces is used for detecting intestinal inflammation caused by NSAIDs, and inflammation is found in 44%-70% of long-term NSAID users. Excretion of ^{111}Indium in the stool is proportional to fecal calprotectin. However, it is also increased in individuals with IBD and colon cancer, unlike the intestinal permeability test, and has the disadvantage of low specificity for NSAID enteropathy[3,6].

Endoscopy

The recently introduced wireless capsule endoscopy and double-balloon enteroscopy can diagnose lesions, such as inflammation, erosions, and ulcers and complications including bleeding and stenosis, which are caused by NSAIDs[20]. In particular, capsule endoscopy, which is a non-invasive examination, is very useful. It can diagnose small bowel lesions in 70% of NSAID users and shows a high correlation with the fecal calprotectin test in measuring intestinal inflammation. Erosions or ulcers, which are the endoscopic findings of NSAID-induced enteropathy, can be caused by many factors besides NSAIDs, and histological examination cannot determine the cause of these lesions. Diseases for differential diagnosis include infection, IBD, ischemia, radiation enteritis, vasculitides, and drugs such as potassium chloride (KCl). The history of NSAID use, biopsy, and improvement of clinical symptoms after stopping the drug use are required for diagnosis. A diaphragm-like stricture is a characteristic finding, which is a secondary scar reaction of ulcer injury, and has non-inflammatory mucosa. There are usually multiple strictures occurring in the mid-intestine, ileum, and colon[22]. Maiden *et al*[19] classified the findings of capsule endoscopy into five groups: reddened folds, the denuded area, red spots, mucosal breaks, and blood. Graham[7] divided capsule endoscopy findings into red spots, small erosions, large erosions, and ulcers. In contrast, double-balloon enteroscopy has the advantages of directly treating bleeding lesions and the ability to perform histological examinations; however, it is a time-consuming and an invasive test[23]. Unfortunately, both tests incur relatively high costs, so their use is limited.

CLINICAL MANIFESTATION

In 60%-70% of NSAID-induced enteropathy, it is subclinical. This disorder displays nonspecific symptoms, such as iron deficiency anemia, gastrointestinal bleeding, hypoalbuminemia, vitamin B12 or bile acid malabsorption, diarrhea, and acute abdominal pain. Complications such as massive bleeding, stricture, and perforation may occur. These complications are rare, but can be fatal[6].

Gastrointestinal bleeding

Small intestinal injury caused by NSAIDs, even when not severe, can cause persistent bleeding and iron deficiency anemia. In patients with NSAID enteropathy, the sites of inflammation and bleeding are identical when measured by scintigraphy using ^{111}Indium-labeled neutrophils to observe intestinal inflammation, and technetium-99 m labeled red blood cell scintigraphy to show bleeding. In patients taking NSAIDs for rheumatoid arthritis who had severe anemia but no bleeding lesions observed by gastroscopy and colonoscopy, small intestinal ulcers have been observed in 47% when enteroscopy was performed. Generally, there is 2-10 mL of daily blood loss[4,6,24]. Apparent acute gastrointestinal bleeding is relatively rare and is caused by ulcers and erosions.

Protein loss

Protein loss in inflamed intestinal mucosa caused by the prolonged use of NSAID leads to hypoalbuminemia[1,3,6,24,25]. Previously, the loss of protein was thought to be secondary to bleeding, but it may occur without anemia. A gross bleeding lesion may not be found in the intestine of patients with enteropathy accompanied by loss of protein[24]. Nowadays, it is thought that protein loss associated with enteropathy can occur without lesions, such as inflammation, erosions, or ulcers.

Perforation and obstruction

Perforation associated with NSAID use is an uncommon complication that has a risk similar to that of bleeding. A case of perforation in a patient treated with high doses of indomethacin was reported[6,24].

Chronic ulcers caused by NSAID result in fibrosis and diaphragm-like strictures. Multiple diaphragm-like septa of 1-4 mm-thickness form in the middle part of the small intestine. If the intestinal lumen is narrowed, obstruction of the small intestine occurs in 17% of patients with NSAID-induced small intestinal ulcers[6]. This is associated with the drug dosage and duration, and accompanied by diarrhea, weight loss, iron deficiency anemia, and protein loss[24].

PREVENTION AND TREATMENT

There is still no proven method of preventing or curing small intestine damage due to NSAIDs. The simplest method is to stop taking the drugs. NSAIDs in prodrug and enteric-coated forms, and ones with controlled release have been developed, but they do not inhibit damage to the small intestine. In addition, H2-blocking agents and sucralfate that have effects on upper gastrointestinal complications are not useful for treating or preventing NSAID-related small intestinal damage, and the effect of proton pump inhibitors (PPI) has not yet been proven[26].

COX-2 selective inhibitor

The development of COX-2 selective inhibitors was expected to significantly reduce gastrointestinal complications caused by NSAIDs. COX-2 selective inhibitor reduced NSAID-associated upper gastrointestinal complications, but the effect on complications of the small intestine has yet to be proven. Currently, short-term treatment with COX-2 selective inhibitors has shown no effect on small intestinal permeability[27]. There have been some reports that symptoms of enteropathy are not observed in patients treated for short periods of time with COX-2 selective inhibitors[27-30]. However, it was also reported that the symptoms of patients treated with COX-2 inhibitors for more than three months were no different to those of patients treated with traditional NSAIDs[31,32]. An underlying reason for this observation is that selective COX-2 inhibitors also have some inhibitory effects on COX-1, and COX-2 has a role in the regulation of mucosal blood flow in some tissues. In addition, COX-2 inhibition increases leukocyte adherence without changes in the bloodstream. COX-2 may have an anti-inflammatory role in the vasculature, and COX-2 selective inhibitor has the disadvantage of adverse cardiovascular side effects.

NO, hydrogen sulfide-releasing NSAID, and zinc-NSAID

It has been reported that COX inhibiting NO donator, hydrogen sulfide-releasing NSAID, and zinc-NSAID prevent NSAID-induced gastrointestinal damage by vasodilation, anti-inflammation, and some cytoprotective actions[33]. Exogenous NO plays a role in maintaining mucosal integrity in the gastrointestinal tract by modulating mucosal blood flow and mucus secretion. Combining an NO donor drug with naproxen or aspirin provides protection from damage by NSAID[34]. Hydrogen sulfide has vasodilation, anti-oxidant, and anti-inflammatory effects[35,36].

Metronidazole

Metronidazole is an antibiotics used to treat anaerobic pathogen infections. When administered (800 mg/d), this drug decreases intestinal inflammation and blood loss caused by NSAID, but does not affect intestinal permeability[37]. Microbes sensitive to metronidazole are major neutrophil chemoattractants in NSAID enteropathy. However, other antibiotics except metronidazole are not effective for treating small intestinal damage caused by NSAIDs. The impact of metronidazole is not achieved by the effect on intestinal bacteria but by the inhibition of oxidative phosphorylation in the mitochondria of the intestinal cells[3].

Sulfasalazine

Sulfasalazine reduces NSAID-induced inflammation and blood loss[38]. The beneficial effect of sulfasalazine on rheumatoid arthritis seems to be due to the sulphapyridine moiety not to its 5-aminosalicylic acid moiety[39]. However, its role is unclear in NSAID-related enteropathy. It is useful in the ileitis of ankylosing spondylitis or for treating long-term NSAID users with rheumatoid

arthritis[38,39]. However, additional research is needed.

Rebamipide

Rebamipide increases mucus and stimulates the production of prostaglandin[40]. It also has anti-inflammatory properties. Rebamipide is a free radical scavenger and produces its effects by inhibiting superoxide production and suppressing myeloperoxidase activity[41]. Therefore, rebamipide can be expected to have an effect on intestinal inflammation. In a recent study, rebamipide prevented diclofenac-induced small bowel injury compared to a placebo[42].

Lansoprazole

Lansoprazole prevents the indomethacin-induced small bowel injury by upregulating HO-1, which has anti-inflammatory and anti-oxidative effects. This compound shows a broader PPI role in addition to its acid production suppression[18].

Goldstein *et al*[30] divided healthy volunteers into the three groups: a celecoxib group, a naproxen plus omeprazole group, and control group, and performed capsule endoscopy. Small bowel lesions were found in 16%, 55% and 7% of the individuals in each group, respectively. This indicates that small bowel lesions cannot be prevented with omeprazole. In other words, in the sites unaffected by gastric acid secretion, such as the small intestine, the increased mucosal protective effect of lansoprazole is more important.

Misoprostol

Misoprostol is a synthetic prostaglandin (PGE1) analog. It has a mucosal protective effect and effectively suppresses NSAID gastrointestinal side effects[43]. However, there is conflicting evidence for its effect on small bowel complications. In one report, misoprostol inhibited NSAID-associated intestinal permeability changes and showed a significant effect on enteropathy[44,45]. It was also found to be effective for treating enteropathy induced by low doses of aspirin[12]. However, misoprostol showed no significant effect on intestinal permeability in patients administered indomethacin in randomized controlled trials[46]. In this study, however, low doses of misoprostol were given for only one week, so additional research to verify these results is required. Misoprostol also has common side effects such as diarrhea, abdominal pain, headache, and constipation[3,6].

Eupatilin

Song *et al*[47] reported that eupatilin protects cultured feline ileal smooth muscle cells against cell damage caused by indomethacin. These protective functions are apparently due to eupatilin-mediated HO-1 induction through extracellular signal-regulated kinase and NF-E2-related factor-2 signal. Therefore, eupatilin is expected to lower the risk of complications such as ulcers, bleeding, and obstruction through its mucosal protective actions in chronic NASID users, but more systematic research is necessary.

Nutritional intervention

A period of time is needed for the prophylactic use of a drug; therefore, it would be better to use foods such as pharmaconutrients, which have relatively low pharmacological risks compared with drugs that have many side effects. Recombinant human lactoferrin has bactericidal, anti-inflammatory, and antioxidant activities, and can be taken orally as a supplement[48]. Commercial fish protein hydrolysate is a fermented fish product that is beneficial for the intestine[49]. Both recombinant human lactoferrin and fish hydrolysate reduce NSAID-associated intestinal permeability compared to a placebo.

Glutamine is a non-essential amino acid and used as energy sources of intestinal mucosa cells. It has been reported that after short-term administration of NSAIDs, glutamine is effective for the prevention of increased intestinal mucosa permeability[50]. In bovine colostrum, there are plenty of growth factors, such as insulin-like growth factor, various immunoglobulins, and antimicrobial peptides. Administration of bovine colostrum with glutamine is effective in reducing gut injury and transbacterial location caused by short-term administration of NSAIDs[51-53].

Other drugs

It was reported that the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor fluvastatin has antioxidative activity and suppresses the formation of ileal ulcers caused by NSAIDs in rats[54]. Other HMG-CoA reductase inhibitors, pravastatin and atrovastatin, did not show these effects[54]. In addition, it was reported that the immunosuppressive drug tacrolimus (FK506) prevents small bowel ulcers caused by indomethacin in rats. This may be due to inhibition of iNOS induction by tacrolimus[55].

CONCLUSION

As capsule endoscopy and enteroscopy have recently become more widely used, NSAID-induced small intestinal damage has emerged as a clinically important issue. To reduce the risk of complications, such as ulcers, bleeding, and obstruction, in chronic NASID users, many researches have attempted to treat and prevent these disorders. To this end, metronidazole, sulfasalazine, COX-2 inhibitors, misoprostol, rebamipide, human lactoferrin, and fish protein hydrolate, have been examined, but there are currently no results of long-term administration. Thus, methods to effectively treat and prevent small bowel injury caused by NSAIDs are still lacking. Therefore, a long-term randomized study in chronic NSAID users is needed. In addition, careful monitoring and special attention for the indications of NSAIDs are required to avoid this disorder in individuals taking NSAIDs.

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TOPIC HIGHLIGHT

Sanjun Cai, Professor, *Series Editor*

Imaging diagnosis of colorectal liver metastases

Ling-Hui Xu, San-Jun Cai, Guo-Xiang Cai, Wei-Jun Peng

Ling-Hui Xu, San-Jun Cai, Guo-Xiang Cai, Wei-Jun Peng, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
Ling-Hui Xu, Wei-Jun Peng, Department of Radiology, Fudan University Shanghai Cancer Center, Shanghai 200032, China
San-Jun Cai, Guo-Xiang Cai, Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, China
Author contributions: Xu LH wrote the paper; Peng WJ wrote the outline and revised the manuscript; Cai SJ and Cai GX gave valuable advice on writing and helped to revise part of this manuscript.
Correspondence to: **Wei-Jun Peng, MD,** Department of Radiology, Fudan University Shanghai Cancer Center, 270 Dong An Road, Shanghai 200032, China. weijunpeng@yahoo.com
Telephone: +86-21-64433384 Fax: +86-21-64433384
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Abstract

Rapid advances in imaging technology have improved the detection, characterization and staging of colorectal liver metastases. Multi-modality imaging approach is usually the more useful in diagnosis colorectal liver metastases. It is well established that hepatic resection improves the long-term prognosis of many patients with liver metastases. However, incomplete resection does not prolong survival, so knowledge of the exact extent of intra-hepatic disease is crucially important in determining patient management and outcome. The diagnosis of liver metastases relies first and totally on imaging to decide which patients may be surgical candidates. This review will discuss the imaging options and their appropriate indications. Imaging and evaluating of colorectal liver metastases (CRLM) have been performed with contrast-enhanced ultrasound, multidetector computed tomography, magnetic resonance imaging (MRI) with extra-cellular contrast media and liver-specific contrast media MRI, and positron emission tomography/computed tomography. This review will concentrate on the imaging approach of CRLM, and also discuss certain characteristics of some liver lesions. We aim to highlight the advantages of each imaging technique, as well as underscoring potential pitfalls and limitations.

Key words: Liver metastasis; Colorectal cancer; Imaging-modality; Diagnosis

Peer reviewer: Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

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INTRODUCTION

Metastatic disease to the liver is a very common clinical situation in oncology. The liver is one of the most common sites of metastatic spread of epithelial cancers, second only to regional lymph nodes. Colorectal cancer is one of a few malignant tumors in which the presence of limited synchronous liver metastases or metachronous metastases need surgical resection[1]. Colorectal liver metastases (CRLMs) develop during the course of colorectal cancer in up to 50%-70% of patients[1-3]. Metastases are confined to the liver in 30%-40% of patients at the time of detection and are potentially resectable in about 20%-30% of the cases[4,5]. Hepatic resection is the only potentially curative treatment for these colorectal liver metastases and in selected groups, the 5-year median survival has been reported to be up to 30% (range 15%-67%)[5]. Patients with untreated but potentially resectable metastases show a median survival of 8 mo and the 5-year survival rate

of these patients is less than 5%[6,7]. Eligibility for surgical treatment requires strict criteria. Besides an adequate clinical condition, all liver lesions have to be completely resectable. The diagnosis of liver metastases relies first and totally on imaging to decide which patients may be surgical candidates. Thus, the imaging technique able to demonstrate the exact number, regional distribution, size of metastases and the volume of the remaining liver is crucial to determine resectability.

In many centers, imaging and evaluating of CRLM have been performed with contrast-enhanced ultrasound (CEUS), multi-detector computed tomography (MDCT), magnetic resonance imaging (MRI) with extra-cellular contrast media and liver-specific contrast media MRI, and positron emission tomography/computed tomography (PET/CT). This review will concentrate on the imaging approach of CRLM, and also discuss certain characteristics of some liver lesions. We aim to highlight the advantages of each imaging technique, as well as underscoring potential pitfalls and limitations.

IMAGING MODALITY PERFORMANCE

CEUS

The development of CEUS has dramatically increased the potential of sonography in the assessment of focal liver lesions. The use of contrast agents allows perfusion mapping of focal lesions, thus enabling characterization of focal lesions. Bernatik *et al*[8] investigated the diagnostic yield of CEUS *vs* helical CT in the detection of liver metastases (no histological diagnosis), CEUS showed 97% of lesions seen by CT.

Although CEUS is widely used to assess the liver, it has some limitations: it needs considerable operator expertise and often reveals equivocal results in patients with (chemotherapy-induced) fatty infiltration of the liver. Due to the limitations in the visualization of segmental distribution and 3D-shape of metastases, it is limited in the preoperative assessment of patients with colorectal liver metastases.

MDCT

Nowadays MDCT is the mainstay of staging and follow-up of these patients, because it provides good coverage of the liver and the complete abdomen and the chest in one session. MDCT scanner has the capability for high-resolution studies with sub-millimetre slice thickness resulting in isotropic pixel sizes, which enable images to be reformatted in various planes that still have the same resolution as the axial images. This may improve detection of small lesions. High-resolution scans with maximum intensity technique and volumetric three-dimensional rendering enable accurate segmental localization and delineation of tumour[9]. Vascular reconstruction enables the demonstration of the hepatic arterial and portal venous anatomy obviating the need for conventional angiography in surgical planning of tumour resection[10]. Volumetric measurement of tumour size and normal liver is also more accurate[11].

How many scans are necessary for a CT examination of the liver? In patients with colorectal cancer, liver metastases are calcified in 11% at initial presentation[12]. These lesions with calcification are much better seen on unenhanced scans than on portal-venous phase scans. Small CRLM often are hyperattenuating during the hepatic arterial phase whereas larger lesions will often show a hyperattenuating rim during the hepatic arterial phase and a hypoattenuating centre representing diminished vascularity and/or tumour necrosis[13], and larger lesions usually are detected as hypoattenuating lesions during the portal venous phase[14]. However the vascularity and therefore enhancement characteristics can be widely variable for reasons that are poorly understood[15-17].

Meijerink *et al*[18] concluded 50 patients suspected of CRLM, they found adding rigid-body co-registered subtraction CT images to a conventional 4-phase CT protocol for pre-operative detection and characterization of CRLM seems of no value. Wicherts *et al*[19] found Arterial and equilibrium phase have no incremental value compared to hepatic venous phase CT in the detection of CRLM. Venous phase is still the most significant timing to detect liver metastases.

Several studies have assessed the value of using thin slices to improve detection of small metastases. Two point five mm or 3.75 mm thick slices were significantly superior to 5, 7.5 and 10 mm thick slices[20,21]. When the slice thickness is decreased to 1 mm, no further improvement in lesion detection is seen, but there is a considerable increase in image noise with subsequent degradation of image quality[22].Therefore a slice thickness of 2-4 mm is recommended for axial viewing.

Although MDCT is the modality of choice for staging colorectal cancer, up to 25% of liver metastases may still be missed[23,24]. Extra care has to be taken for patients with contrast allergies or with renal impairment.

CT with arterioportography

In CT with arterioportography (CTAP), CT scanning of the liver is performed during contrast agent injection into either the superior mesenteric artery or splenic artery *via* a percutaneously placed catheter. It provides maximum tumor-to-liver contrast by enhancing the liver parenchyma alone as in the portal phase and depicts tumor deposits as areas of perfusion defects. This is based on the fact that metastases are almost exclusively fed *via* the hepatic artery. CTAP was usually reserved for imaging candidates prior to surgical hepatic resection as it provided an accurate segmental localization of liver metastases and excellent depiction of liver vasculature. This invasive technique is less routinely performed with the advent of MDCT and MRI with liver-specific contrast agents, which are as accurate in lesion detection but with much lower false positive rates[25].

MRI

The standard MRI protocol should always include un-

enhanced T1- and T2-weighted and contrast-enhanced pulse sequences. In liver MR imaging a set of T1-weighted in-phase and opposed-phase gradient-recalled echo gradient-recalled echo images is acquired to assess the parenchyma for the presence of fatty infiltration or focal sparing of diffuse fatty infiltration. For T2-weighted imaging, the turbo-spin echo (TSE) or the fast spin echo with fat suppression are preferred over the single-shot TSE pulse sequences. In addition, heavily T2-weighted pulse sequences with a time of echo of approximately 160-180 ms may help in differentiation between solid [metastasis, hepatocellular carcinoma (HCC), *etc.*] and non-solid lesions (e.g., haemangioma, cyst)[26,27].

After the acquisition of unenhanced pulse sequences, contrast-enhanced pulse sequences are always obtained. Nowadays, two different groups of MR contrast agents for liver imaging are available: first, the non-specific gadolinium chelates and second the liver-specific MR contrast agents. The latter group can be divided into two subgroups, the hepato-biliary contrast agents, and the reticulo-endothelial (or Kupffer cell) contrast agents.

NON-SPECIFIC GADOLINIUM CHELATES

The liver and liver-lesion enhancement patterns obtained with non-specific gadolinium chelates (extracellular contrast agents) are similar to those obtained with iodinated contrast agents used in CT. Several agents with similar properties are on the market, including gadopentetate dimeglumine (Schering, Berlin, Germany), Gd-DTPA-BMA (GE Healthcare, Oslo, Norway), Gd-DOTA (Guerbet, Aulnay-sous-Bois, France), and Gado-teridol (Bracco, Milan, Italy).

Extracellular gadolinium chelates are used extensively for liver MRI. Following intravenous injection of a gadolinium-based agent, typically three phases of contrast enhancement are imaged: the arterial, portal venous phase and the equilibrium phase. During the arterial phase, most of the liver does not enhance as the majority of the liver's blood supply is *via* the portal vein. Enhancement patterns of liver lesions are similar to those demonstrated on CEUS and contrast-enhanced CT. The equilibrium phase or delayed phase is useful for helping with lesion differentiation (e.g., haemangioma *vs* metastasis). In addition, washout of contrast from HCC and peripheral or heterogeneous washout from liver metastases are characteristic findings on delayed imaging[28,29].

LIVER-SPECIFIC CONTRAST AGENTS

Hepatobiliary agents

Hepatobiliary agents represent a heterogeneous group of paramagnetic molecules of which a fraction is taken up by hepatocytes and excreted into the bile. Mangafodipir trisodium (Teslascan®, GE Healthcare) is taken up by hepatocytes and results in signal intensity increase on T1-weighted images (a so-called "T1 enhancer")[30], and a fraction is also taken up by the pancreas, which has been used for pancreatic MR imaging[31,32]. Focal non-hepatocellular lesions (i.e., metastases) do not enhance post-contrast, resulting in improved lesion conspicuity Mangafodipir-enhanced MRI has been show to be superior to unenhanced MRI and helical CT for detection of liver metastases[1,32,33].

Gd-BOPTA (Multihance®, Bracco) is a liver-targeted paramagnetic contrast agent and unlike conventional Gadolinium chelates, has almost two-fold greater T1 relaxivity which improves image contrast and detection of liver lesions, due to its high T1-relaxing effect and hepatocyte binding capability[34].

Gd-EOB-DTPA (Primovist®, Schering) and Gd-BOPTA are hybrid contrast agents, which carry a lipophilic ligand[35]. After intravenous bolus injection these agents show biphasic liver enhancement with a rapid T1 enhancement of the liver similar to that seen with non-specific extracellular gadolinium agents. Then hepatic signal intensity continues to rise for 20-40 min (Gd-EOB-DTPA) and 60-90 min (Gd-BOPTA), reaching a plateau after about 2 h because of hepatocytic uptake. This results in increasing contrast between liver and non-hepatocellular tumors[36].

Reticuloendothelial agents

All reticuloendothelial system (RES) agents are superparamagnetic iron oxide-based contrast agents (SPIO). SPIO particles are taken up by RES cells (so-called Kupffer cells) of normal liver parenchyma, as also by macrophages of the spleen and lymph nodes. They shorten T2 and T2* relaxation times in the liver tissue, and resulting in a loss of signal intensity in normal liver parenchyma. Despite of this, malignant liver lesions do not have a substantial number of RES cells and appear as hyperintense lesions with distinct borders in contrast to the hypointense liver parenchyma after application of SPIO on T2-weighted MR images[37,38] .

There are some published studies comparing some methods reporting varying sensitivity. Some have reported that SPIO-enhanced MRI has better diagnostic efficacy for liver lesions over that of Gadolinium-enhanced MRI, Gd-BOPTA-enhanced MRI and dynamic CT imaging with high sensitivity values[39-41]. Another study has claimed equal sensitivity between SPIO-enhanced MRI and Gd-BOPTA-enhanced MRI in the delayed hepatocyte phase for the detection of LMs[42,43]. Mainenti *et al*[44] found Gd- and SPIO-enhanced MRI had equal performance and were shown to perform significantly better than the other modalities on a per lesion basis. These data were similar to previous studies comparing Gd- and SPIO-enhanced MRI each other[4,39] or with MDCT[39,45] or PET/CT[46]. Based on Zech *et al*[47] experience and the existing literature, imaging using Gd-EOB-DTPA-enhanced MR can be expected to be superior to the using standard gadolinium chelates or to spiral CT, especially for the differential diagnosis of hypervascular lesions.

Blyth *et al*[48] suggest that MRI is a highly sensitive method of pre-operative imaging of colorectal liver me-

tastases and should be considered the "gold standard". Except contraindications to MRI include pacemakers, implantable cardiac defibrillators, cochlear implants and metallic orbital foreign bodies, MR imaging is still limited in the anatomic coverage, although the recent introduction of multi-channel MR coils with wider coverage and the moving-table MR technique has re-established the "competitiveness" of MR with MDCT with regard to patient throughput. One of the advantages of MR in liver imaging is the better soft tissue contrast, which reveals better characterization of focal liver lesions in question. The development of a liver-specific MR contrast agent has further improved the diagnostic yield of MRI in lesion detection and characterization.

PET/CT

The recent introduction of PET/CT hybrid scanners enables seamless and accurate fusion of the high resolution anatomic localisation of CT with the functional data of FDG-PET. A combination of FDG-PET and CT scanning characteristics seems promising, and integrated PET/CT is becoming more widely available, although the exact clinical value and efficacy is not yet fully established. Due to restricted availability, high cost and an additional radiation exposure, PET/CT should be used in selected patients where the diagnosis is not clear following conventional diagnostic modalities.

TRUE ACCURACY OF IMAGING AND FUTURE DEVELOPMENTS

Twenty to twenty-five percent of patients with known solid malignant tumors have hepatic metastases at the time of diagnosis. The incidence of solid benign liver tumors is around 20%[49], thus in patients with known malignancy, 20%-25% of lesions under 2 cm are benign[50]. The most frequent benign lesion is hemangioma with a prevalence of 7%-21%, followed by focal nodular hyperplasia with a prevalence of up to 3%; other benign lesions are far rarer[28,49].

In two studies showed that 24%[39] and 18%[51] of lesions 1 cm or smaller were not detected by any imaging technique. Which imaging modality is the best model in detection of CRLM? The issue of when to use which imaging method is still not solved. The answer likely depends on local equipment, availability, and operator expertise.

Contrast-enhanced intra-operative US (CE-IOUS) is considered the gold standard thereby achieving universal usage and should arguably be considered the final diagnostic procedure[52-55]. Several studies have shown that IOUS still has a higher sensitivity and specificity than the noninvasive techniques, such as helical CT and MRI[54,56-58]. However, there have been few studies on CE-IOUS in literature and CE-IOUS is not widely used among hepatic surgeons.

CT liver imaging offers increased sensitivity and may also able to assess extrahepatic disease but is inferior to MRI scanning in direct comparisons[59-62].

CTAP is considered by many to be the "gold standard" for hepatic imaging but it is an invasive technique with a high (up to 15%) false-positive rate[60].

Hekimoglu *et al*[37] could detect lesions CRLM over 1 cm by all the 3 imaging modalities including SPIO-enhanced MRI, GbD-enhanced dynamic MRI and dynamic CT imaging also with a high sensitivity. However, only SPIO-enhanced MRI detected LMs less than 1 cm with 100% sensitivity which has not been reported until today. So, He therefore, recommend SPIO-enhanced MRI for patients with colorectal carcinoma with suspected small sized LMs. MRI provides a sensitive, non-invasive method of assessing liver lesions and direct comparison between CTAP and MRI shows that MRI is better at identifying and characterising liver lesions[63-65].

Kinkel *et al*[66] performed a meta-analysis including papers published between 1985 and 2000 and concluded that, at equivalent specificity, PET/CT is more sensitive than US, CT and MRI for the detection of hepatic metastases from gastro-esophageal and colo-rectal cancers. Subsequently Bipat *et al*[4] performed a meta analysis including papers published between 1990 and 2003 and concluded that PET/CT is the most sensitive diagnostic tool for the detection of hepatic metastases from colo-rectal cancer on a per patient basis, but not on a per lesion basis. Mainenti *et al*[44] found PET/CT shows a trend to perform better than the other modalities in the identification of patients with CRLM.

The combination of PET and CT is a perfect solution. On theoretical grounds, it is preferable to combine PET with (functional) MRI, for better soft tissue evaluation with a relatively low radiation burden. An excellent example of the application of PET/MRI fusion is accurate delineation of malignant lesions in the liver, to allow optimally guided locoregional therapeutic intervention[67]. It is expected that integrated PET/MRI scanners will become clinically available in the next few years.

Clearly, continuing improvements in imaging are allowing metastases to be identified at an earlier stage but a different approach is needed to improve the detection of metastases smaller. A multi-modality strategy is recommended since no single modality can accurately detect all colorectal liver metastases[68].

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GUIDELINES FOR CLINICAL PRACTICE

Systematic review of health-related quality of life after esophagectomy for esophageal cancer

Marco Scarpa, Stefano Valente, Rita Alfieri, Matteo Cagol, Giorgio Diamantis, Ermanno Ancona, Carlo Castoro

Marco Scarpa, Stefano Valente, Rita Alfieri, Matteo Cagol, Giorgio Diamantis, Carlo Castoro, Oncological Surgery Unit, Veneto Institute of Oncology, 35128 Padova, Italy
Ermanno Ancona, Department of Gastrointestinal and Surgical Sciences, University of Padova, 35128 Padova, Italy

Author contributions: Scarpa M, Alfieri R, Ancona E and Castoro C designed the research; Scarpa M, Valente S, Cagol M and Diamantis G performed the research; Scarpa M, Valente S, Alfieri R and Diamantis G analyzed the data; Ancona E and Castoro C coordinated the research group and obtained the funds; Scarpa M, Cagol M, Ancona E and Castoro C wrote the paper.
Correspondence to: Marco Scarpa, MD, Oncological Surgery Unit, Veneto Institute of Oncology, Via Gattamelata 64, 35128 Padova, Italy. marcoscarpa73@yahoo.it
Telephone: +39-049-8211693 Fax: +39-049-8211694
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Abstract

This study is aimed to assess the long-term health-related quality of life (HRQL) of patients after esophagectomy for esophageal cancer in comparison with established norms, and to evaluate changes in HRQL during the different stages of follow-up after esophageal resection. A systematic review was performed by searching medical databases (Medline, Embase and the Cochrane Library) for potentially relevant studies that appeared between January 1975 and March 2011. Studies were included if they addressed the question of HRQL after esophageal resection for esophageal cancer. Two researchers independently performed the study selection, data extraction and analysis processes. Twenty-one observational studies were included with a total of 1282 (12-355) patients. Five studies were performed with short form-36 (SF-36) and 16 with European Organization for Research and Treatment of Cancer (EORTC) QLQ C30 (14 of them also utilized the disease-specific OES18 or its previous version OES24). The analysis of long-term generic HRQL with SF-36 showed pooled scores for physical, role and social function after esophagectomy similar to United States norms, but lower pooled scores for physical function, vitality and general health perception. The analysis of HRQL conducted using the Global EORTC C30 global scale during a 6-mo follow-up showed that global scale and physical function were better at the baseline. The symptom scales indicated worsened fatigue, dyspnea and diarrhea 6 mo after esophagectomy. In contrast, however, emotional function had significantly improved after 6 mo. In conclusion, short- and long-term HRQL is deeply affected after esophagectomy for cancer. The impairment of physical function may be a long-term consequence of esophagectomy involving either the respiratory system or the alimentary tract. The short- and long-term improvement in the emotional function of patients who have undergone successful operations may be attributed to the impression that they have survived a near-death experience.

Key words: Health-related quality of life; Esophageal cancer; Esophagectomy; Short form 36; European Organization for Research and Treatment of Cancer QLQ C30; European Organization for Research and Treatment of Cancer OES18

Peer reviewer: Guy Douglas Eslick, PhD, Program in Molecular and Genetic Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Building II, 2nd Floor, Boston, MA 02115, United States

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INTRODUCTION

Esophageal cancer is an increasingly common cancer with a poor prognosis. During recent decades, the incidence of esophageal cancer has risen steadily, and it is now the fastest rising solid tumor in most Western countries. Moreover, moderate to high incidence rates have been reported in other areas, including parts of China, Central Asia, South and East Africa, South America, Northern France, and the United States among African-Americans[1]. Despite recent improvements in diagnosis, surgical treatment and (neo)-adjuvant therapy, the prognosis of patients with esophageal cancer remains poor, with overall 5-year survival rates of only 5%-15%[1,2]. Esophagectomy is the standard treatment for those patients who present with resectable esophageal cancer[3-5], but it offers a limited (25%-35%) chance of cure[5,6] and is associated with a considerable risk of serious complications[4,5,7]. Therefore, the use of chemotherapy or radiotherapy in combination with surgery has been tested. Nevertheless, meta-analyses of randomized trials of neoadjuvant chemotherapy and chemoradiation followed by surgery and surgery alone for patients with esophageal carcinoma showed only minor survival advantages[8,9]. Only patients with a complete pathologic response to neoadjuvant therapy enjoy a significantly better chance of survival, whereas non-responders have a worse prognosis than patients undergoing surgery alone[10,11].

For a long time, morbidity and mortality represented the main (and often the only) outcome measure that could be used to evaluate esophagectomy for esophageal cancer. The morbidity and mortality rates associated with the procedure and the poor patient survival rate left almost no space for further consideration. However, in recent years, along with the increase in the success of the therapy, health-related quality of life (HRQL) has generally become accepted as an important outcome parameter, along with long-term survival, mortality, and complication rates. In fact, knowledge of risk factors for poor postoperative HRQL may be relevant to clinical decision making. Moreover, these findings may be used to inform patients of the long-term consequences of surgery. On this basis, the aim of this systematic review was to analyze quality of life after curative surgery for esophageal cancer.

INCLUSION AND EXCLUSION CRITERIA

Since we expected to find only observational studies, the checklist proposed by Meta-analysis of Observational Studies in Epidemiology group 38 was used as a guideline to perform this systematic review[12]. We defined observational studies as reports that used data from existing databases, cross-sectional studies, case series, case-control studies, or studies with a historical control or a cohort design.

Studies were eligible for inclusion if they reported on a series of patients who underwent esophagectomy because of esophageal cancer and if post-operative quality of life was described and analyzed in the "material and methods" and "results" sections. Studies reporting on a mix of esophageal adenocarcinoma and squamous cell cancer patients were included. In contrast, those reporting on malignancies other than esophageal adenocarcinoma or squamous cell cancer were excluded. All studies eligible for inclusion in this systematic review also had to present detailed information on the methods used to assess quality of life and on when the questionnaire was administered. Studies that analyzed HRQL using questionnaires other than short form-36 (SF-36), European Organization for Research and Treatment of Cancer (EORTC) QLQ C30 and OES18/24 and those that only presented their results graphically were excluded. When studies were discovered to report (partially) similar patient data, only the most recent and complete data sets were considered.

SEARCH STRATEGY

Four medical databases were used in this research: Medline (January 1978 to March 2011), the Cochrane Database of Systematic Reviews, the Cochrane Central Register of Controlled Trials and Embase. These databases were searched with the help of a clinical librarian. The keywords and medical subject headings used were "esophageal cancer", "esophagectomy" and "quality of life", as indicated in Figure 1. Only clinical studies written in English were selected. A manual cross-reference search of the eligible papers was performed to identify additional relevant articles. Based on the initial search results, two researchers (Scarpa M and Valente S) independently selected the studies that matched the inclusion criteria. Data quoted as unpublished and data from abstracts were not used. Any disagreements between the two researchers regarding which studies should be included were resolved through discussion.

DATA EXTRACTION

Data were extracted only from original articles using a preformatted sheet with a set of pre-defined parameters: demographic data, histologic type, cancer stage, cancer site, type of surgery (two-way or three-way esophagectomy), type of reconstruction (esophagogastroplasty or esophagocoloplasty), neoadjuvant or adjuvant therapy, timing of follow-up and HRQL data gathering, type of questionnaire used, item and total results.

OUTCOME MEASURE: QUALITY OF LIFE INSTRUMENTS

Studies were included if at least one of the following validated quality of life instruments was used: the EORTC-QLQ-C30, the EORTC-QLQ-OES18 or 24, or the SF-36. A summary of these questionnaires appears below.

The EORTC-QLQ-C30 questionnaire was developed by the Quality of Life division of EORTC. This 30-item questionnaire explores the generic quality of life of patients affected by oncologic diseases. It is a self-report

Mesh terms: "esophageal cancer" and "esophagectomy" and "quality of life"

Key words:

"esophageal neoplasm" or "esophageal neoplasms" or "neoplasms, esophageal " or" neoplasm, esophageal" or "esophagus neoplasm" or "esophagus neoplasms" or "neoplasm, esophagus" or "neoplasms, esophagus" or "cancer of esophagus" or "cancer of the esophagus" or "esophagus cancer" or "cancer, esophagus" or "cancers, esophagus" or "esophagus cancers" or "esophageal cancer" or "cancer, esophageal" or "cancers, esophageal" or "esophageal cancers" or "esophageal adenocarcinoma" or "adenocarcinoma of the esophagus" or "esophagus adenocarcinoma" or "adenocarcinoma of esophagus" or "adenocarcinoma, esophagus" or "oesophageal neoplasm" or "oesophageal neoplasms" or "neoplasms, oesophageal "or" neoplasm, oesophageal" or "oesophagus neoplasm" or "oesophagus neoplasms" or "neoplasm, oesophagus" or "neoplasms, oesophagus" or "cancer of oesophagus" or "cancer of the oesophagus" or "oesophagus cancer" or "cancer, oesophagus" or "cancers, oesophagus" or "oesophagus cancers" or "oesophageal cancer" or "cancer, oesophageal" or "cancers, oesophageal" or "oesophageal cancers" or "oesophageal adenocarcinoma" or "adenocarcinoma of the oesophagus" or "oesophagus adenocarcinoma" or "adenocarcinoma of oesophagus" or "adenocarcinoma, oesophagus"

and

"esophagectomy" or "esophagectomies" or "esophageal resection" or "esophageal resections" or "resection of the esophagus" or "resections of the esophagus" or "esophagogastroplasty" or "esophagogastrectomy" or "oesophagectomy" or "oesophagectomies" or "oesophageal resection" or "oesophageal resections" or "resection of the oesophagus" or "resections of the oesophagus" or "oesophagogastroplasty" or "oesophagogastrectomy"

and

"quality of life" or "life qualities" or "life quality" or "health related quality of life" or "HRQL" or "QoL"

Figure 1 Key words. HRQL: Health related quality of life; QoL: Quality of life.

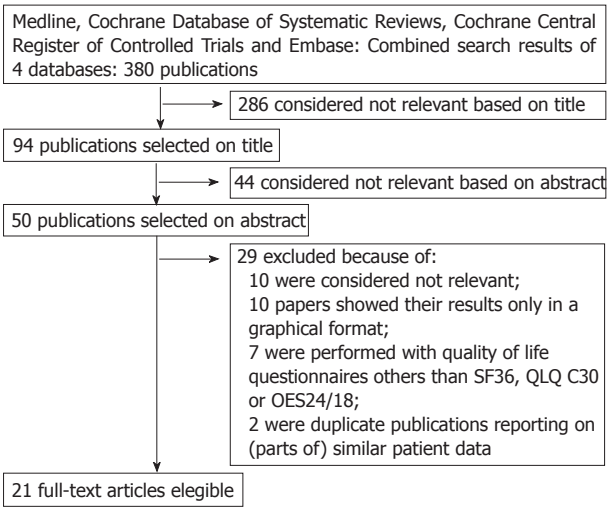


Figure 2 Study selection. SF-36: Short form-36.

instrument that includes five functional scales (physical, role, emotional, social and cognitive), three symptom scales (fatigue, nausea and vomiting, and pain), a global health status scale and six single items (dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties)[13]. The EORTC-QLQ-OES-18 and 24 are two extra modules that are used specifically for esophageal cancer. These questionnaires consist of 18 questions (or 24 in the previous version) assessing dysphagia, deglutition, abdominal/gastrointestinal symptoms, eating difficulties, pain, and emotional problems related to esophageal cancer and to the side effects of chemotherapy/radiotherapy[14]. The SF-36 consists of 36 items within 8 dimensions: psychological functioning, role limitations due to physical problems, pain, general health perceptions, energy/vitality, social functioning, and role limitations due to emotional problems and mental health[15].

STATISTICS

A clinical statistician was consulted to assess the accuracy of our analysis. The Review Manager 4.2 software (The Cochrane Collaboration, Copenhagen: Nordic Cochrane Centre, 2003) was used to process the data and conduct the analysis. For studies presenting HRQL results obtained from patients who had undergone the same treatment regimen and been presented with the same questionnaire at the same point in time with respect to their surgery, a meta-analysis of the HRQL scores was attempted. The results were presented as weighted mean differences [95% confidence interval (CI)]. Based on previous research, a difference of at least 10 mean score points between time points or comparison groups was considered clinically relevant, whereas a difference of 5-10 was considered weak[16]. $P < 0.05$ was considered to indicate that the results were significant.

STUDY SELECTION

The studies selected are shown in Figure 2. The initial search yielded 380 articles, of which 349 did not meet the criteria for inclusion. Based on their titles, 286 papers were excluded because they clearly covered a variety of unsuitable topics. Forty-four articles were then excluded on the basis of their abstracts; these were case reports and review articles without original data or articles on forms of neoplasm other than esophageal carcinoma. Of the 50 candidate papers, an additional 29 were then excluded, of which 10 were not focused on quality of life after esophagectomy for esophageal carcinoma[14,17-25], 10 presented their results only in a graphical format[26-35], 7 used quality of life questionnaires other than the SF36, QLQ C30 or OES24/18[36-42] and 2 reported data from the same dataset as other studies[43,44]. Although they reported the same dataset, three studies from the Karolinska Institute of Stockholm were included in the review because they analyzed different aspects of HRQL after esophagectomy[45,46]. However, the patients included in these studies were counted only once in all totals.

The 21 studies included in the analysis were all published between 1995 and 2010 and are listed according

Table 1 Studies characteristics: Aims of the studies and timing of health-related quality of life recording

Sudy	Study aim	Timing post op HRQL measures
McLarty *et al*[54]	Analysis of HRQL in long term survivors after surgery alone	A single assessment > 60 mo
De Boer *et al*[53]	Analysis of HRQL in long term survivors after transhiatal esophagectomy	A single assessment 3.5 (2.1-5.4) yr
Headrick *et al*[49]	Analysis of HRQL in long term survivors after esophagectomy for HGD or adenocarcinoma	A single assessment 5.3 (0.5-9) yr
Cense *et al*[52]	Analysis of HRQL in long term survivors after esophagocolonplasty	A single assessment 35 (7-97) mo
Moraca *et al*[50]	Analysis of HRQL in long term survivors after esophagectomy for HGD or Tis	A single assessment 4.9 (0.5-12) yr
Reynolds *et al*[55]	Comparison between HRQL after neoadjuvant CT-RT+ surgery and after surgery alone	Baseline, after CT-RT, 3, 6, 9, 12 mo po
Avery *et al*[56]	Comparison between HRQL after neoadjuvant CT-RT+ surgery and after definitive CT-RT	1, 5, 3, 6, 9 mo
van Meerten *et al*[57]	Analysis of HRQL after neoadjuvant CT-RT + surgery	Baseline, after CT-RT, 3, 6, 9, 12 mo po
Wang *et al*[59]	Comparison between open surgery and minimally invasive esophagectomy	2, 4, 12, 24 wk
Parameswaran *et al*[58]	Analysis of HRQL after minimally invasive esophagectomy	6, 12 mo
Viklund *et al*[45]	Analysis of HRQL predictors after esophagectomy for cancer (type of recstruction)	A single assessment 6 mo
Rutegard *et al*[46]	Analysis of HRQL predictors after esophagectomy for cancer (type of recstruction)	A single assessment 6 mo
Rutegard *et al*[47]	Analysis of HRQL predictors after esophagectomy for cancer (type of recstruction)	A single assessment 6 mo
Olsen *et al*[60]	Analysis of HRQL in long term survivors (surgery alone or neoadjuvant CT-RT + surgery)	A single assessmentat 24 mo po
Lagergren *et al*[61]	Analysis of HRQL in long term survivors (surgery alone or neoadjuvant CT-RT + surgery)	Baseline and 36 mo
Djarv *et al*[62]	Analysis of HRQL in long term survivors (surgery alone or neoadjuvant CT-RT + surgery)	6, 36 mo
Courrech Staal *et al*[63]	Analysis of HRQL in long term survivors (surgery alone or neoadjuvant CT-RT + surgery)	A single assessment at 54 (16–162) mo
Blazeby *et al*[64]	Comparison between HRQL after surgery alone and after palliative RT	A single assessment 16 (10-24) wk
Ariga *et al*[65]	Comparison between surgery alone and definitive CT-RT + salvage surgery	A single assessment 24 mo
Schneider *et al*[48]	Comparison between HRQL after emergency and elective esophagectomy	1 wk and 9 mo
Rosmolen *et al*[51]	Comparison between HRQL after endoscopical ablation and esophagectomy for early Barrett's neoplasms	A single assessment at 24 (17–35) mo

The 21 studies included in the analysis are listed according their aim and then chronologically. HRQL: Health-related quality of life; HGD: High grade dysplasia; CT-RT: Chemotherapy-radiotherapy; op: Operative; po: Post-operative.

their aim and then chronologically in Tables 1 and 2. Five studies were performed using SF36 and 16 using EORTC QLQ C30 (14 of them also utilized the disease-specific OES18 or the previous version, the OES24). Nine studies were observational cross-sectional studies and twelve were prospective ones. Patients were enrolled consecutively in 17 of them. Generic and disease-specific questionnaires were both used in 18 studies, and the HRQL was the primary focus for 15 of them. Five were population-based studies.

Study characteristics

There were 21 observational studies analyzed, and these studies included data for a total of 1282 patients. The number of patients ranged from 12 to 355 per study. The follow-up duration after esophagectomy, as reported in the articles, was between 4 and 63 mo. The range of mean ages reported by the different papers was 59 to 69 years. The indication for surgery was esophageal adenocarcinoma in 835 patients and squamous cell carcinoma in 395. However, in the series by Schneider *et al*[48], 5 out of 17 patients were operated on for esophageal perforation. Hendrick *et al*[49], Moraca *et al*[50] and Rosmolen *et al*[51] included 35, 24 and 7 patients, respectively, who underwent esophagectomy because of high-grade dysplasia. The characteristics of the patients included in each study are described in Table 3.

Long term generic HRQL after esophagectomy vs healthy subjects

Five studies analyzed the long-term generic HRQL of 246 patients using the SF-36 questionnaire (median follow-up range: 36-64 mo)[49,50,52-54]. The studies by Moraca *et al*[50] and Cense *et al*[52] were not used for the meta-analysis because the SF-36 scores in the first were not reported in a standard, comparable manner and because all patients reported on in the second underwent esophagocoloplasty. The studies by Hendrick *et al*[49], De Boer *et al*[53] and McLarty *et al*[54] were sufficiently homogenous, and thus, a meta-analysis of their results was attempted. The pooled scores for physical function, physical role, and social function after esophagectomy were similar to sex- and age-matched United States norms, whereas the pooled scores for physical function, vitality and general health perception were lower than the relevant norms ($P = 0.005$, $P < 0.001$ and $P = 0.006$, respectively). In contrast, scores for bodily pain and mental health in long-term survivors after esophagectomy were higher than the relevant norms ($P = 0.08$ and $P = 0.02$, respectively). The significant weighted mean differences that emerged based on the comparison between the long-term generic HRQL of patients who had undergone esophagectomy and that of healthy subjects are shown in Figure 3.

Generic and disease specific HRQL after neoadjuvant therapy and esophagectomy

Three studies analyzed generic and disease-specific HRQL for 255 patients using the QLC-30 and OES-18 questionnaires at different stages in the follow-up timeline (baseline, 3, 6, 9 and 12 mo) after neoadjuvant therapy and esophagectomy[55-57]. Reynolds *et al*[55] and Avery *et al*[56] compared the HRQL of patients who had undergone neoadjuvant therapy with that of patients who had un-

Table 2 Studies characteristics: Studies setting and feature

Study	Year	Country	Center	Prospective	Consecutive	HRQL as primary endpoint	Preoperative HRQL assessment	SF36	OES18 /OES24	QLQ C30	Population based study
McLarty *et al*[54]	1997	United States	Mayo Clinic, Rochester MI	No	Yes	No	No	Yes	No	No	No
De Boer *et al*[53]	2000	Netherlands	Academic Medical Centre, Amsterdam	No	No	Yes	No	Yes	No	No	No
Headrick *et al*[49]	2002	United States	Mayo Clinic, Rochester MI	No	Yes	No	No	Yes	No	No	No
Cense *et al*[52]	2004	Netherlands	Academic Medical Centre, Amsterdam	No	No	Yes	No	Yes	No	No	No
Moraca *et al*[50]	2006	United States	Virginia Mason Medical Centre, Seattle	No	Yes	No	No	Yes	No	No	No
Reynolds *et al*[55]	2006	Ireland	St James's Hospital, Dublin	Yes	Yes	Yes	Yes	No	No	Yes	No
Avery *et al*[56]	2007	United Kingdom	University of Bristol, Bristol	Yes	Yes	Yes	Yes	No	Yes	Yes	No
van Meerten *et al*[57]	2008	Netherlands	Erasmus University, Rotterdam	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Wang *et al*[59]	2009	China	Fudan University, Shanghai	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Parameswaran *et al*[58]	2010	United Kingdom	Royal Devon and Exeter NHS Trust, Exeter	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Viklund *et al*[45]	2005	Sweden	Karolinska Institute, Stockholm	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Rutegard *et al*[46]	2008	Sweden	Karolinska Institute, Stockholm	Yes	No	Yes	No	No	Yes	Yes	Yes
Rutegard *et al*[47]	2008	Sweden	Karolinska Institute, Stockholm	Yes	No	Yes	No	No	Yes	Yes	Yes
Olsen *et al*[60]	2005	Sweden	Sahlgrenska University Hospital, Goteborg	No	Yes	No	No	No	Yes	Yes	No
Lagergren *et al*[61]	2007	United Kingdom	University of Bristol, Bristol	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Djarv *et al*[62]	2008	Sweden	Karolinska Institute, Stockholm	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Courrech Staal *et al*[63]	2010	Netherlands	Netherlands Cancer Institute, Amsterdam	Yes	Yes	Yes	No	No	Yes	Yes	No
Blazeby *et al*[64]	1995	United Kingdom	University of Bristol, Bristol	No	Yes	Yes	No	No	No	Yes	No
Ariga *et al*[65]	2009	Japan	University of Yamaga, Yamaga	Yes	Yes	No	No	No	Yes	Yes	No
Schneider *et al*[48]	2010	Germany	University of Heidelberg, Heidelberg	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Rosmolen *et al*[51]	2010	Netherlands	Academic Medical Center, Amsterdam	No	Yes	Yes	No	Yes	Yes	Yes	No

The 21 studies included in the analysis are listed according their aim and then chronologically. HRQL: Health-related quality of life; SF36: Short form-36.

dergone esophagectomy alone. Based on these studies, data for the patients who had undergone neoadjuvant therapy and esophagectomy were identified. Data from the study by Reynolds *et al*[55] were uploaded for meta-analysis, but could not be used because the standard deviation values were missing. The baseline scores were compared to scores obtained after a 6-mo follow-up. The EORTC- QLQ-C30 global scale results tended to be better at the baseline than at the 6-mo follow-up ($P = 0.08$), and physical function was also better at the baseline ($P < 0.001$). Likewise, the symptom scales showed worsened fatigue, dyspnea and diarrhea 6 mo after the procedure ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). In contrast, emotional function had significantly improved after 6 mo ($P < 0.001$). The significant weighted mean difference results associated with the comparison between the baseline HRQL figures and the figures achieved 6 mo after neoadjuvant therapy and esophagectomy are shown in Figure 4.

Generic and disease specific HRQL after minimally invasive esophagectomy

Two studies prospectively analyzed the generic and disease-specific HRQL of 255 patients using the EORTC-QLQ-C-30 and OES-18 questionnaires at different points on the follow-up timeline (baseline, 3, 6, 9 and 12 mo) after minimally invasive esophagectomy[58,59]. The surgical techniques used in the two studies were similar and included thoracoscopic esophageal mobilization and mediastinal lymphadenectomy, followed by laparoscopic gastric mobilization and resection, and abdominal lymph-

Table 3 Patient characteristics

Study	Patients	Median/[1]mean age (range)	Recruitment period	Adenocarcinoma	Squamous cell carcinoma	Tis/ HGD/ pCR	Stage I	Stage II	Stage III	Stage IV	Lower esophagus or cardias	Medium or upper esophagus	Mean/[1]median follow up (mo)	Esophago-gastroplasty	Esophago-colonplasty	Esophago-jejeunoplasty	Neoadjuvant treatment	Adjuvant RT/CT/ CT+RT
McLarty *et al*[54]	107	62 (30-81)	1972-1990	72	28	0	34	73	0		62	45	> 60	99	3	4	0	9
De Boer *et al*[53]	35	66.4[1] (42-87)	1993-1996	27	6	2	14	1	18		32	3	> 24	35	0		0	
Headrick *et al*[49]	54	64 (36-83)	1991-1997	53	1	5/35	7	2	5		54	0	63.6[1] (6-108)					
Cense *et al*[52]	14	69.9 (51-81) ECP, 66.4 (42-87) EGP	1993-2002	6	7	1	8	3	2		10	3	35 (7-97)	0	14			
Moraca *et al*[50]	36	66[1] (43-88)	1991-2003	35	0	12/24	12						58.8 (6-144)	36	0	0	0	0
Subtotal	246 (14-107)			193	42	3	75	79	25	0	158	51	0	170	17	4	0	9
Reynolds *et al*[55]	107	61 (29-79)	1999-2004	34	68		25	63	83	3			3				87	87
Avery *et al*[56]	94	62.4[1]	2000-2004	64	17			14	67				1.5				69	69
van Meerten *et al*[57]	54	59 (40-75)	2001-2004	41	12	13	12	6	16	5	49	5	3				54	54
Subtotal	255 (54-107)			239	97	13	37	83	166	8	49	5	7.5	0	0	0	210	210
Wang *et al*[59]	56	60.7 ± 9.3 VATS 58.2 ± 11.5 open	2007-2008	3	52		11	37	8		10	46	6	56			0	
Parameswaran *et al*[58]	62	67 (49-80)	2005-2007	57	5	3	7	21	27	4			12 ± 0.5	62			48	
Subtotal	118 (56-62)			60	57	3	18	58	35	4	10	46	6	118	0	0	48	0
Viklund *et al*[45]		64 (34-84)[1]	2001-2003										6					16
Rutegard *et al*[46]		91 pts < 60, 127 pts 60-70, 137 pts >70	2001-2005										6				37	
Rutegard *et al*[47]	355	91 pts < 60, 127 pts 60-70, 137 pts > 70	2001-2005	271	84		81	120	133	18	302	53	6				37	
Subtotal	355																	
Olsen *et al*[60]	18	62.5 (20-76)	1997-2001			3	5	1	9				24	13	4	1	8	8
Lagergren *et al*[61]	47	63 (44-79)	2000-2003	35	9	8	10	23	6		23	24	> 36				29	
Djarv *et al*[62]	87	27 pts < 60, 28 pts 60-70, 32 pts > 70	2001-2007	68	19		34	36	13	3	75	12	> 36	69	2	16		
Courrech Staal *et al*[63]	36		2009	22	14	0	4	12	12	8	28	8	54 (range, 16–162)				21	
Subtotal	188 (18-87)			125	42	8	53	72	40	11	126	44		82	6	17	58	8
Blazeby *et al*[64]	33	64 (62-76)	1993-1994	19	14								4 (2.5-6)				0	
Ariga *et al*[65]	48	65.5 (46-78)	2001-2004	0	48		10	15	23		20	28	41.2 (95% CI: 35.1-47.2)					
Schneider *et al*[48]	12	59	2001-2005	1	11								> 9	10	2			
Rosmolen *et al*[51]	27	63.0 (9.5)	2001-2005	27	0	2	19	6					24 (IQR 17 – 35)	23	4		0	0
Subtotal	120 (12-48)			47	73	2	29	21	23	0	20	28		33	6	0	0	0

The 21 studies included in the analysis are listed according their aim and then chronologically. pts: Patients; ECP: Esophagocolonplasty; EGP: Esophagogastroplasty; VATS: Video assisted thoracoscopy; CI: Confidence interval; HGD: High grade dysplasia; pCR: Pathological complete response; RT: Radiotherapy; CT: Chemotherapy; IQR: Interquartile range.

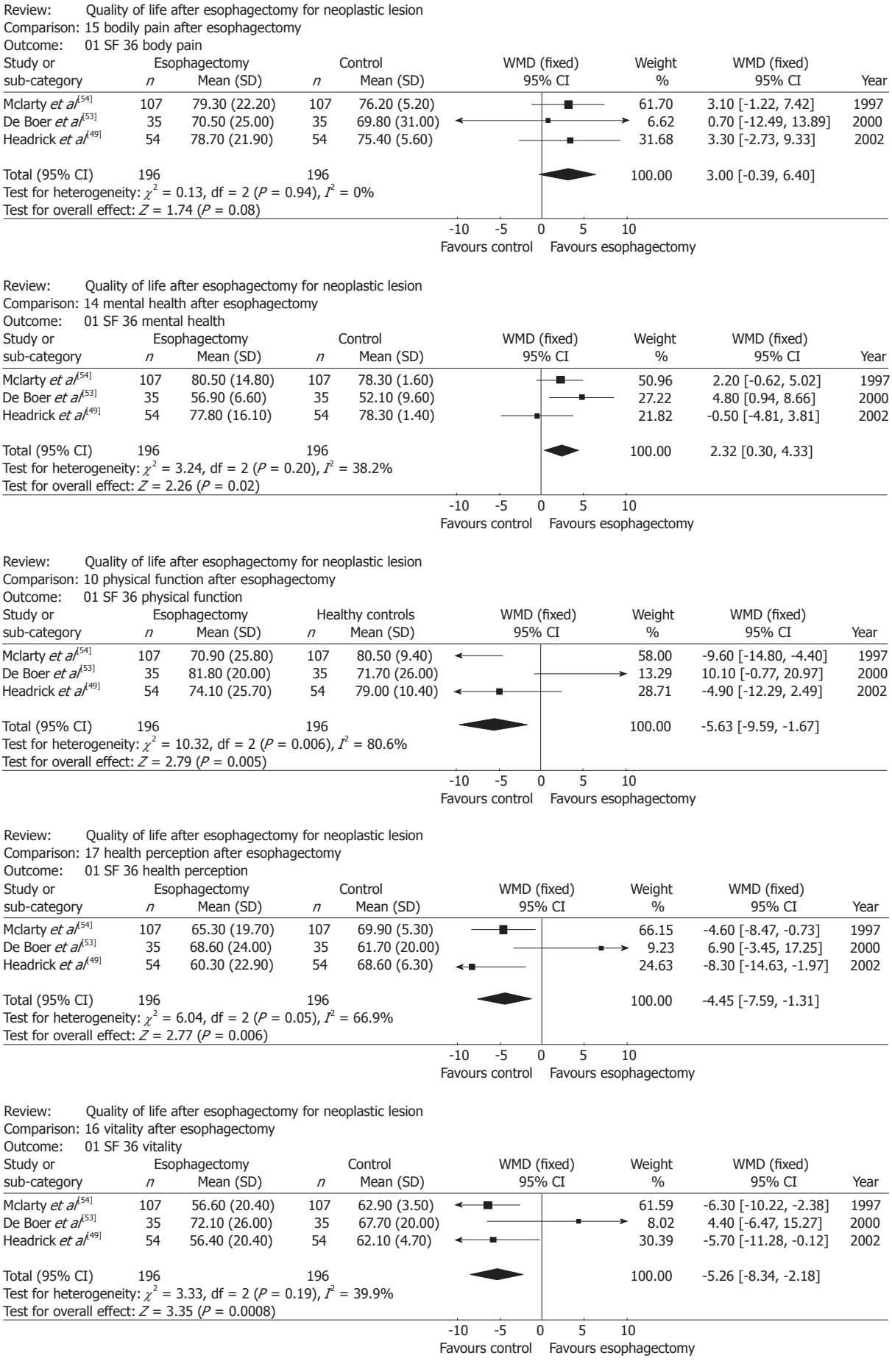
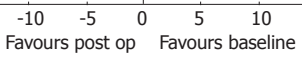


Figure 3 Long term generic health-related quality of life in patients after esophagectomy for esophageal cancer. WMD: Weighted mean difference; SF: Short form.

Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 18 global EORTC C30 scale in patients after neoadjuvant CT-RT and esophagectomy
Outcome: 01 EORTC C30 global scale

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Reynolds *et al*[55]	41	82.00 (0.00)	41	74.00 (0.00)			Not estimable	2006
Avery *et al*[56]	69	70.00 (21.00)	69	64.00 (23.00)		43.85	6.00 [-1.35, 13.35]	2007
van Meerten *et al*[57]	50	78.00 (15.00)	50	75.00 (18.00)		56.15	3.00 [-3.49, 9.49]	2008
Total (95% CI)	160		160			100.00	4.32 [-0.55, 9.18]	

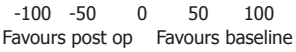
Test for heterogeneity: χ^2 = 0.36, df = 1 (P = 0.55), I^2 = 0%
Test for overall effect: Z = 1.74 (P = 0.08)



Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 19 physical function in patients after neoadjuvant CT-RT and esophagectomy
Outcome: 01 EORTC C30 physical function

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo post op *n*	6 mo post op Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Reynolds *et al*[55]	41	79.00 (0.00)	41	78.00 (0.00)			Not estimable	2006
Avery *et al*[56]	69	89.00 (20.00)	69	76.00 (18.00)		44.75	13.00 [6.65, 19.35]	2007
van Meerten *et al*[57]	50	95.00 (8.00)	50	83.00 (19.00)		55.25	12.00 [6.29, 17.71]	2008
Total (95% CI)	160		160			100.00	12.45 [8.20, 16.69]	

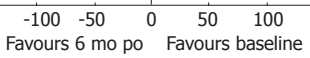
Test for heterogeneity: χ^2 = 0.05, df = 1 (P = 0.82), I^2 = 0%
Test for overall effect: Z = 5.74 (P < 0.00001)



Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 20 emotional function in patients after neoadjuvant CT-RT and esophagectomy
Outcome: 01 EORTC C30 emotional function

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Reynolds *et al*[55]	41	76.00 (0.00)	41	75.00 (0.00)			Not estimable	2006
Avery *et al*[56]	69	71.00 (25.00)	69	78.00 (25.00)		43.06	-7.00 [-15.34, 1.34]	2007
van Meerten *et al*[57]	50	74.00 (19.00)	50	85.00 (18.00)		56.94	-11.00 [-18.25, -3.75]	2008
Total (95% CI)	160		160			100.00	-9.28 [-14.75, -3.80]	

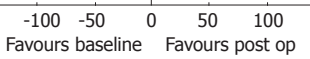
Test for heterogeneity: χ^2 = 0.50, df = 1 (P = 0.48), I^2 = 0%
Test for overall effect: Z = 3.32 (P = 0.0009)



Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 21 fatigue in patients after neoadjuvant CT-RT and esophagectomy
Outcome: 01 EORTC C30 fatigue

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo post op *n*	6 mo post op Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Reynolds *et al*[55]	41	36.00 (0.00)	41	36.00 (0.00)			Not estimable	2006
Avery *et al*[56]	69	27.00 (25.00)	69	39.00 (26.00)		38.33	-12.00 [-20.51, -3.49]	2007
van Meerten *et al*[57]	50	14.00 (15.00)	50	28.00 (19.00)		61.67	-14.00 [-20.71, -7.29]	2008
Total (95% CI)	160		160			100.00	-13.23 [-18.50, -7.96]	

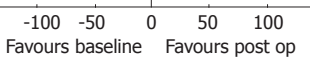
Test for heterogeneity: χ^2 = 0.13, df = 1 (P = 0.72), I^2 = 0%
Test for overall effect: Z = 4.92 (P < 0.00001)



Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 22 dyspnea in patients after neoadjuvant CT-RT and esophagectomy
Outcome: 01 EORTC C30 dyspnea

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo post op *n*	6 mo post op Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Reynolds *et al*[55]	41	11.00 (0.00)	41	27.00 (0.00)			Not estimable	2006
Avery *et al*[56]	69	6.00 (17.00)	69	28.00 (30.00)		48.18	-22.00 [-30.14, -13.86]	2007
van Meerten *et al*[57]	50	7.00 (15.00)	50	20.00 (24.00)		51.82	-13.00 [-20.84, -5.16]	2008
Total (95% CI)	160		160			100.00	-17.34 [-22.98, -11.69]	

Test for heterogeneity: χ^2 = 2.44, df = 1 (P = 0.12), I^2 = 58.9%
Test for overall effect: Z = 6.02 (P < 0.00001)



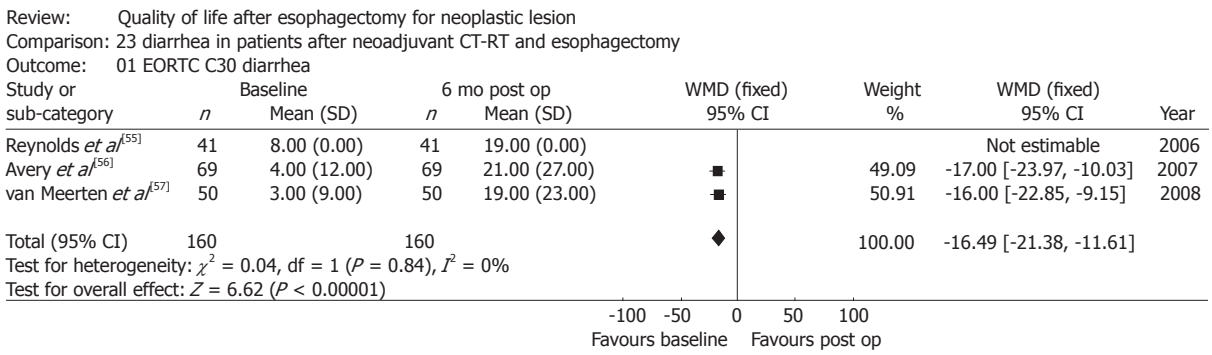


Figure 4 Generic and disease specific health-related quality of life in patients after neoadjuvant chemotherapy-radiotherapy and esophagectomy for esophageal cancer. WMD: Weighted mean difference; CT-RT: Chemotherapy-radiotherapy; EORTC: European Organization for Research and Treatment of Cancer; op: Operative; po: Post-operative.

adenectomy. Wang *et al*[59] compared the HRQL results achieved after minimally invasive esophagectomy with those achieved after open esophagectomy, and the study by Parameswaran *et al*[58] was an uncontrolled prospective study. Therefore, the baseline scores were compared to scores obtained during a 6-mo follow-up. Social function, cognitive function, emotional function and dysphagia proved to be significantly improved during the 6-mo follow-up ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). In contrast, physical function was better at the baseline ($P < 0.001$), and role function tended to appear worse at the 6-mo follow-up point. The significant weighted mean difference results associated with the comparison between the baseline HRQL figures and the figures from 6 mo after neoadjuvant therapy and esophagectomy are shown in Figure 5.

Predictors of generic and disease specific HRQL after esophagectomy

Three articles from the Karolinska Institute, Stockholm specifically investigated predictors of generic and disease-specific HRQL after esophagectomy for cancer by analyzing the data from the Swedish Esophageal and Cardia Cancer register, which had conducted a nationwide, prospective, population-based study of how esophageal surgery-related factors had influenced quality of life 6 mo after surgery[45-47]. Although they reported information from the same dataset, these studies were all included in the review because they analyzed different aspects of HRQL after esophagectomy. The first study, by Viklund *et al*[45], included 100 patients and indicated that surgery-related complications were the main predictor of reduced global quality of life 6 mo after surgery. Except for anastomotic strictures, each of the predefined complications (e.g., anastomotic leakage, infections, cardiopulmonary complications, and operative technical complications) contributed to a decrease in quality-of-life scores. Rutegard *et al*[46], using a larger study population, showed that extensive surgery (characterized as using the transthoracic approach, extensive lymphadenectomy, and wider resection margins and as being of a longer duration) was not associated with worse HRQL measures than less extensive operations. Moreover, they observed that the severity of dysphagia was similar in patients who had handsewn and stapled anastomoses. Technical surgical complications were confirmed to have a deleterious effect on several aspects of HRQL. Finally, the same group concluded that no clinically relevant differences in terms of generic and disease-specific HRQL were found to be correlated with differences in the volume of surgeries conducted at hospitals (low volume: 0-9 operations/year; high volume: > 9 operations/year) or by particular surgeons (low volume: 0-6 operations/year; high-volume: > 6 operations/year)[47].

Long-term generic and disease-specific HRQL after esophagectomy

Data from the articles that analyzed long-term generic and disease-specific HRQL after esophagectomy were collected, but their clinical heterogeneity was so high that it was impossible to pool them. Four studies analyzed the generic HRQL of 152 (18-87) patients using the QLC-30 questionnaire and evaluated disease-specific HRQL using the OES-18 questionnaire in conducting long-term follow-up[60-63]. Fagevik *et al*[60] evaluated 18 patients 2 years after thoraco-abdominal esophageal resection and observed that, 2 years after surgery, respiratory function was significantly lower than it was prior to surgery, as was physical performance. In contrast, HQRL was comparable to age- and sex-matched population norms for most other functions. After 2 years, diarrhea, dyspnea, appetite loss and fatigue were still clinically significant. Similarly, Lagergren *et al*[61] analyzed 47 patients who had survived for at least 3 years after esophagectomy for a malignant disease. In these long-term survivors, most HRQL items had returned to preoperative levels by the 3-year assessment; however, their scores for physical function, breathlessness, diarrhea, and reflux remained significantly worse than at the baseline. Nevertheless, patients reported significantly better emotional function 3 years after surgery than before treatment. Djärv *et al*[62] reported the results for a cohort of 87 patients who had survived for at least 3 years after esophagectomy for cancer, using data from the prospective Swedish nationwide population study. As in other studies, these patients reported significantly more problems with fatigue, diarrhea, appetite loss, nausea and

Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 05 emotional function pre and post minimally invasive esophagectomy
Outcome: 01 EORTC C30 emotional function

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Wang *et al*[59]	56	70.40 (0.00)	56	78.10 (6.80)		9.64	-7.70 [-11.00, -4.40]	2009
Parameswaran *et al*[58]	62	79.00 (3.06)	62	87.00 (3.06)		90.36	-8.00 [-9.08, -6.92]	2010
Total (95% CI)	118		118			100.00	-7.97 [-9.00, -6.95]	

Test for heterogeneity: χ^2 = 0.03, df = 1 (P = 0.87), I^2 = 0%
Test for overall effect: Z = 15.26 (P < 0.00001)

-10 -5 0 5 10
Favours treatment Favours control

Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 07 social function pre and post minimally invasive esophagectomy
Outcome: 01 EORTC C30 social function

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Wang *et al*[59]	56	62.60 (16.30)	56	69.30 (10.40)		7.28	-6.70 [-11.76, -1.64]	2009
Parameswaran *et al*[58]	62	73.00 (4.50)	62	79.00 (3.50)		92.72	-6.00 [-7.42, -4.58]	2010
Total (95% CI)	118		118			100.00	-6.05 [-7.42, -4.68]	

Test for heterogeneity: χ^2 = 0.07, df = 1 (P = 0.79), I^2 = 0%
Test for overall effect: Z = 8.68 (P < 0.00001)

-10 -5 0 5 10
Favours treatment Favours control

Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 08 dysphagia pre and post minimally invasive esophagectomy
Outcome: 01 EORTC OES18 dysphagia

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Wang *et al*[59]	56	39.60 (18.50)	56	4.60 (3.90)		7.98	35.00 [30.05, 39.95]	2009
Parameswaran *et al*[58]	62	25.00 (3.50)	62	18.00 (4.70)		92.02	7.00 [5.54, 8.46]	2010
Total (95% CI)	118		118			100.00	9.24 [7.84, 10.63]	

Test for heterogeneity: χ^2 = 113.02, df = 1 (P < 0.00001), I^2 = 99.1%
Test for overall effect: Z = 12.94 (P < 0.00001)

-100 -50 0 50 100
Favours control Favours treatment

Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 06 cognitive function pre and post minimally invasive esophagectomy
Outcome: 01 EORTC C30 cognitive function

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Wang *et al*[59]	56	73.00 (9.10)	56	77.00 (7.20)		8.02	-4.00 [-7.04, -0.96]	2009
Parameswaran *et al*[58]	62	81.00 (2.55)	62	84.00 (2.55)		91.98	-3.00 [-3.90, -2.10]	2010
Total (95% CI)	118		118			100.00	-3.08 [-3.94, -2.22]	

Test for heterogeneity: χ^2 = 0.38, df = 1 (P = 0.54), I^2 = 0%
Test for overall effect: Z = 7.01 (P < 0.00001)

-10 -5 0 5 10
Favours treatment Favours control

Review: Quality of life after esophagectomy for neoplastic lesion
Comparison: 04 physical function pre and post minimally invasive esophagectomy
Outcome: 02 EORTC C30 role function pre and post minimally invasive esophagectomy

Study or sub-category	Baseline *n*	Baseline Mean (SD)	6 mo po *n*	6 mo po Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI	Year
Wang *et al*[59]	56	68.50 (9.60)	56	70.40 (9.60)		19.04	-1.90 [-5.46, 1.66]	2009
Parameswaran *et al*[58]	62	73.00 (5.60)	62	71.00 (4.08)		80.96	2.00 [0.28, 3.72]	2010
Total (95% CI)	118		118			100.00	1.26 [-0.29, 2.81]	

Test for heterogeneity: χ^2 = 3.74, df = 1 (P = 0.05), I^2 = 73.3%
Test for overall effect: Z = 1.59 (P = 0.11)

-10 -5 0 5 10
Favours treatment Favours control

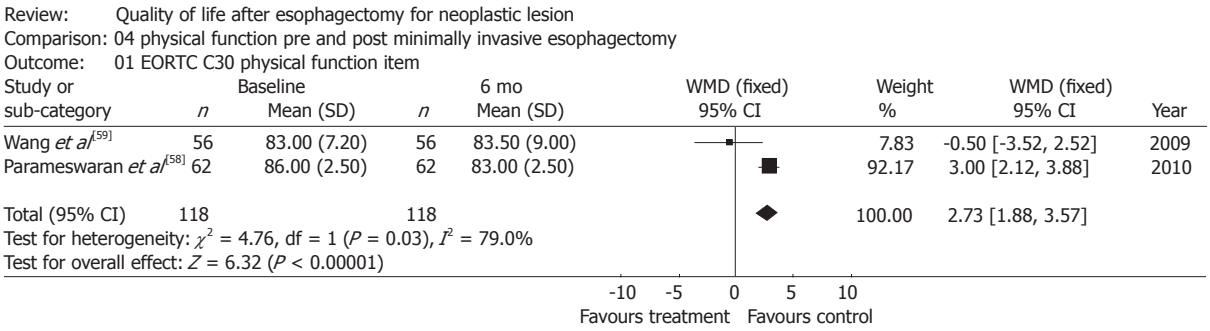


Figure 5 Generic and disease specific health-related quality of life after minimally invasive surgery for esophageal cancer. WMD: Weighted mean difference; EORTC: European Organization for Research and Treatment of Cancer; po: Post-operative.

vomiting than did those in the reference population, and they also reported significantly poorer role and social function. Finally, Courrech Staal *et al*[63] compared the generic and disease-specific HRQL of 36 patients with esophagectomy (who had or had not received neoadjuvant therapy) after a median follow-up of 54 mo with the data for a reference sample of patients with esophageal cancer (1031 patients) and with that of the general population (7802 subjects). These long-term survivors reported better health-related quality of life than the reference sample of patients with esophageal cancer, even if their HRQL appeared lower than that of the reference sample of individuals from the general population[63].

PECULIAR ASPECTS OF HRQL AFTER ESOPHAGECTOMY

Data from the four articles that analyzed peculiar aspects of HRQL after esophagectomy were collected, but although the EORTC questionnaires were used, the level of clinical heterogeneity was so high that it was impossible to pool them[48,51,64,65]. In 1995, in one of the first studies focused on HRQL, Blazeby *et al*[64] observed that patients treated using esophagectomy reported significantly better physical, emotional, cognitive, and global health scores than did those who had received palliative treatment. These patients also had significantly worse pain, fatigue, appetite loss, constipation, and dysphagia. Ariga *et al*[65] performed a prospective direct comparison of outcomes after treatment in patients with resectable esophageal cancer who had received definitive chemoradiation and those who had undergone surgery alone. They surveyed HRQL in patients who had survived more than 2 years using a cross-sectional approach, and concluded that the HRQL of patients who had definitive chemoradiation and that of patients who had undergone surgery alone were similar. Diarrhea, appetite loss and eating problem scores were worse in patients who had undergone surgery alone than in those who had undergone chemoradiation[65]. Moreover, Schneider *et al*[48] compared the HRQL of those patients who had undergone elective and emergency esophagectomy with those who had undergone collar reconstruction. They observed a temporary decrease in postoperative HRQL in both groups and a return to preoperative values during the follow-up except for physical functioning, which remained decreased in patients who had undergone elective esophagectomy for cancer. Finally, Rosmolen *et al*[51] compared HRQL and fear of cancer among 81 patients who had undergone endoscopic treatment and 27 patients who had undergone surgery for early Barrett's neoplasia. They observed that patients in the surgery group reported significantly more eating problems and reflux symptoms on the EORTC-OES18 questionnaire, whereas endoscopy patients reported more fear of recurrence than surgery patients. They concluded that preserving the esophagus after endoscopy, which is preferable from a clinical perspective, may induce fear of cancer recurrence[51].

CONCLUSION

Esophageal cancer is an increasingly common cancer with a poor prognosis. Esophagectomy is the standard treatment for those patients who present with resectable esophageal cancer[3-5], but it still offers a limited (25%-35%) chance of cure[5,6] and is associated with considerable risk of serious complications[4,5,7]. For a long time, morbidity and mortality represented the main, and often the only, outcome measures that could be used to evaluate esophagectomy for esophageal cancer. The morbidity and mortality rates associated with the procedure and the patients' poor survival rates due to the aggressive nature of the disease left almost no space for further analysis. However, in recent decades, along with the increased success of the therapy, HRQL has become an important outcome parameter in addition to survival, mortality, and complication rates[67]. In fact, postoperative HRQL can yield information that is relevant for clinical decision-making and help to inform patients about the long-term consequences of surgery. With that in mind, this systematic review was designed to collect and analyze data reflecting patterns in HRQL after curative surgery for esophageal cancer.

Twenty-one studies published between 1995 and 2011 were included in this analysis. One limitation of this review is the clinical heterogeneity of the studies included. To increase homogeneity, only studies performed using SF36 or with EORTC-QLQ-C30 and OES18 (or the pre-

vious version, OES24) were included. Nevertheless, virtually every study used a different surgical approach and a different means of comparison, and they also did not present exact data. Furthermore, data for the recruited patients were collected during a range of intervals after surgery. Therefore, it was very difficult and sometimes impossible to obtain sufficiently homogeneous data to recalculate the statistical analyses or perform a meaningful meta-analysis. For example, 5 studies analyzed long-term generic HRQL after esophagectomy in comparison with that of healthy subjects using SF-36 questionnaires distributed at roughly comparable intervals after esophagectomy[49,50,52-54] but two studies could not be used for the meta-analysis, one because the SF-36 scores were not reported in a standard or comparable way and the other because all patients underwent esophagocoloplasty[50,52].

On the other hand, the remaining three studies were sufficiently homogenous, and thus, a meta-analysis of their results was attempted[49,53,54]. In these three studies, the pooled scores for physical, role, and social function after esophagectomy were similar to sex- and age-matched United States norms. In contrast, in a group of patients with similar follow-up, Djärv *et al*[62] and Courrech *et al*[63] observed significantly poorer role and social function. Differences between the HRQL measurement tools (SF36 and QLQ C30) may have created this difference in the results, and cross-cultural differences among the different groups of patients may also have been a factor. Health-related quality of life may vary from one population to another according to differences in cultural heritage, value systems, family structure, medical systems, values and norms related to illness-related communication, and other factors[67].

In the three studies that analyzed long-term generic HRQL after esophagectomy *vs* in healthy subjects, the pooled physical function, vitality and general health perception scores were lower than the sex- and age-matched norms[49,53,54]. Similarly, in patients alive at 3 years analyzed using the EORTC-QLQ-C30 and OES18, Djärv *et al*[62] and Courrech *et al*[63] reported encountering significantly more problems with fatigue, diarrhea, appetite loss, nausea and vomiting than in the reference population. Moreover, Lagergren *et al*[61] and Fagevik *et al*[60] observed that scores for physical function, breathlessness, diarrhea, and reflux were significantly worse than at the baseline. Finally, also in the three studies that prospectively analyzed generic and disease-specific HRQL using QLC-30 and OES-18 questionnaires after neoadjuvant therapy and esophagectomy, the EORTC C30 global scale results tended to be worse 6 mo after esophagectomy, as were physical function, fatigue, dyspnea and diarrhea[55-57]. Physical function impairment is a long-term consequence of esophagectomy that can involve either the respiratory system (which can be impaired by the thoracotomy sequelae) or the alimentary tract (which can be affected by accelerated transit and functional sequelae).

In contrast, in the three studies that compared HRQL after esophagectomy to that of healthy subjects using SF-36, bodily pain and mental health in long-term survivors after esophagectomy was higher than normal[49,53,54]. Similarly, in the three studies that prospectively analyzed generic and disease-specific HRQL using QLC-30 and OES-18 questionnaires after neoadjuvant therapy and esophagectomy, emotional function was significantly better at the time of the 6-mo follow up[55-57]. Moreover, Lagergren *et al*[61] observed that long-term survivors reported significantly better emotional function even 3 years after surgery. The short- and long-term improvement in emotional function in patients successfully operated on for esophageal cancer may be attributed to their sensation of having been quite close to death and having survived. In our opinion, this experience is different to that of having survived a car accident because of the duration, which can give patients sufficient time to experience the challenge in a positive way. On the other hand, it should be noted that this improved emotional function was observed in survivors.

Several studies analyzed a specific aspect of HRQL after esophagectomy. Two studies prospectively analyzed generic and disease-specific HRQL after minimally invasive esophagectomy[58,59]. The surgical techniques reported on in both studies were similar, and thus, it was possible to compare the results. The results were similar to those report by larger studies performed with group of patients who had undergone open esophagectomy, in that these patients experienced significantly improved social function, cognitive function, emotional function and dysphagia. However, physical function worsened 6 mo after esophagectomy[58,59]. In their direct comparison, Wang *et al*[59] concluded that global quality of life and physical functioning were better in the minimally invasive group than in the open surgery group. Additional larger studies should explore the exact benefit of minimally invasive esophagectomy in terms of HRQL.

Data from a Swedish nationwide, prospective, population-based study were used in three studies by the Karolinska Institute, Stockholm that investigated the most important predictors of HRQL after surgery for esophageal cancer using data from 6 mo after surgery[45-47]. Unexpectedly, extensive surgery, as used in the transthoracic approach, was not associated with lower HRQL than less invasive operations[46]. No clinically relevant differences in generic or disease-specific HRQL were observed based on the volume of procedures done at hospitals[47]. Age, sex, and body mass index showed no association with HRQL 6 mo after surgery, but patients with comorbidity, tumors in a more advanced stage (Ⅲ to Ⅳ), or tumors located in the middle or upper esophagus exhibited an increased risk of poor HRQL. Moreover, the occurrence of surgery-related complications was the main predictor of reduced postoperative HRQL[45,47]. Except for anastomotic strictures, each of the predefined complications (e.g., anastomotic leakage, infections, cardiopulmonary complications, and operative technical complications) decreased the patients' HRQL scores. In patients with non-neoplastic diseases such as Crohn's disease or ulcerative

colitis, postoperative complications did not seem to have a long-term effect on HRQL[68,69], so the reasons why patients submitted to esophagectomy can experience postoperative complications that can heavily affect HRQL must be different. Firstly, a 6-mo follow-up period may be not sufficiently long enough, as the problems caused by the complications may be still ongoing. Secondly, the different type of surgery that implies usually has thoracotomy play a direct role. In fact, the complications of a thoracotomy may have direct implications for dyspnea, fatigue and pain.

Some articles analyzed more peculiar aspects of HRQL after esophagectomy[48,64,65]. The observation that patients who received palliative treatment had significantly worse pain, fatigue, appetite loss, constipation, and dysphagia might be expected[64]. In contrast, the results presented by Ariga *et al*[65] are much less expected. They observed that patients with squamous cell carcinoma who underwent definitive chemoradiation had similar general HRQL scores and lower diarrhea, appetite loss and eating problem scores than those who had undergone surgery alone[65]. These results could be mainly attributed to the effect of the loss of function of the stomach transposed in the thorax. Schneider *et al*[48] observed the persistence of decreased physical functioning in patients who had undergone elective esophagectomy for cancer, as compared to patients who had undergone emergency esophagectomy for benign conditions. The conclusions of this study may suggest that the long-term impairment of physical functioning could be due more to the cancer itself or to radiation/chemotherapy than to esophagectomy. Finally, the analysis of the HRQL after endoscopic treatment and surgery for early Barrett's neoplasia showed that conservative, non-definitive treatments such as endoscopic ablation may cause more fear of recurrence than more invasive but definitive treatments, such as esophagectomy[51]. Fear of cancer recurrence may negatively impact HRQL, and proper counseling may be advisable when patients elect such options[51].

In conclusion, short- and long-term generic and disease-specific HRQL is deeply affected by esophagectomy for cancer. The impairment of physical function may be a long-term consequence of esophagectomy and can involve either the respiratory system (which can be impaired by the thoracotomy sequelae) or the alimentary tract (which can be affected by accelerated transit and functional sequelae). The short- and long-term improvement in the emotional function of patients who have been successfully operated on may be attributed to their impression of having survived a near-death experience.

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ORIGINAL ARTICLE

Increased extrinsic apoptotic pathway activity in patients with hepatocellular carcinoma following transarterial embolization

Shih-Ho Wang, Li-Mien Chen, Wen-Kai Yang, Jane-Dar Lee

Shih-Ho Wang, Division of General Surgery, Department of Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University, College of Medicine, Kaohsiung 83301, Taiwan
Li-Mien Chen, Division of Hematology/Oncology, Department of Medicine, Taichung Armed Forces General Hospital, Taichung 41152, Taiwan
Li-Mien Chen, Jane-Dar Lee, Central Taiwan University of Science and Technology, Taichung 40601, Taiwan
Wen-Kai Yang, Jane-Dar Lee, Department of Surgery, Taichung Armed Forces General Hospital, Taichung 41152, Taiwan
Wen-Kai Yang, Department of Life Sciences, National Chung Hsing University, Taichung 40227, Taiwan
Author contributions: Wang SH, Chen LM and Lee JD designed the research; Yang WK performed the research and analyzed the data; Lee JD wrote the paper.
Supported by A grant from Taichung Armed Forces General Hospital, Taiwan
Correspondence to: Jane-Dar Lee, MD, PhD, Department of Surgery, Taichung Armed Forces General Hospital, No. 348, Sec 2, Chungshan Rd, Taiping Dist, Taichung 41152, Taiwan. janedar@hotmail.com
Telephone: +886-4-23925948 Fax: +886-4-23920451
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Abstract

AIM: To determine the apoptosis pathway in residual viable hepatocellular carcinoma (HCC) tissues following transarterial embolization (TAE).

METHODS: Ten patients with HCC who received surgical resection after TAE were enrolled in the study group, and 24 patients with HCC who received surgical resection only served as the control group. In the study group, we measured the changes in tumor size and α fetoprotein (AFP) levels after TAE. All tissue samples were taken from the residual tumors. The expression of various apoptotic proteins was evaluated *via* immunoblotting procedures. The results were analyzed using a Student's *t* test.

RESULTS: Tumor size and the AFP level decreased by 46.2% and 55.3% after TAE, respectively. There was no significant difference detected for the Bcl-2/Bax ratio or the cleaved caspase-9 expression levels in either group. However, extrinsic apoptopic pathway-related expression of Fas and cleaved caspase-8 expression were significantly higher in the study group than in the control group ($P < 0.05$). In addition, cleaved caspase-3 expression in the study group was higher (1.62-fold) than in control group ($P < 0.05$).

CONCLUSION: TAE is an effective palliative therapy that decreases tumor size and AFP levels *via* an increase in extrinsic apoptosis pathway in patients with unresectable HCC.

Key words: Apoptosis; Hepatocellular carcinoma; Transarterial embolization

Peer reviewer: Samir Ahboucha, PhD, Assistant Professor, Équipe Neurosciences, Pharmacologie et Environnement, Université Cadi Ayyad, Faculté des Sciences Semlalia, Avenue My Abdellah, BP 2390, Marrakech 40000, Morocco

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers in the world[1,2]. Generally, HCC is induced by chronic liver injury such as liver cirrhosis. The chronic injury leads to a change in the cellular properties of the liver and curtails the delivery of blood throughout the liver. Subsequently, this pathological change leads to a reduction in blood circulation throughout the liver and accompanying hypoxia. The increased cellularity associated with the highly proliferative tumor cells also induces local hypoxia inside the actual HCC[3]. Hypoxia can stimulate angiogenesis to support tumor growth by inducing angiogenic factors[3,4]. Many people with advanced HCC have tumors that are unresectable by the time of disease diagnosis. Transarterial embolization (TAE) appears to be as effective as transarterial chemoembolization (TACE) and plays a major role in the treatment of patients with advanced HCC[5,6]. However, the exact molecular changes and apoptotic pathway activity of cancerous tissues following embolization still needs to be elucidated.

Several studies have reported that TACE inhibits tumor angiogenesis and induces tumor cell apoptosis. Other studies have found that TACE stimulates tumor angiogenesis and increases the proliferative activity of the tumor cells due to anoxic stress-related Bcl-2 overexpression[7,8]. Unfortunately, most previous papers neglected to examine the counter factor of apoptotic protein (Bax) expression in residual viable cancerous tissues following TAE in HCC patients. We examined the expression of apoptotic proteins in the specimens taken from both groups (surgical resection only and surgery after TAE) of HCC patients. To determine whether the intrinsic or extrinsic apoptotic pathways were involved, we examined the Bcl-2/Bax ratio (anti-apoptosis protein/an inducer of apoptosis), cleaved caspase-9, Fas, cleaved caspase-8 and cleaved caspase-3 expression levels.

To our knowledge, this is the first report to study the apoptotic pathway in the residual viable cancerous tissues of HCC patients following TAE. A better understanding of the molecular changes and apoptotic pathways associated with HCC will aid in the development of more effective future therapies.

MATERIALS AND METHODS

Patients and tissue samples

From January 2006 to December 2009, we enrolled 10 patients (6 men and 4 women; average age, 53.6 years) with HCC as a study group. All 10 patients received TAE with 95% alcohol and Gelform due to poor liver function and relative high initial risks for operation. The study group patients then underwent hepatic resection three weeks after the TAE. During the same period, we enrolled 24 patients (16 men and 8 women; average age, 54.4 years) who only received surgical resection for HCC as a control group. All liver tumor tissues were stored at -80 ℃ for immunoblotting (including Bcl-2/Bax ratio, cleaved caspase-9, Fas, cleaved caspase-8 and cleaved caspase-3) or fixed in formalin for immunohistochemical (IHC) staining (including caspase-9, caspase-8 and cleaved caspase-3). Simultaneously, we measured the changes in tumor size and α fetoprotein (AFP) in the study group after TAE. The specimens were removed only after written informed consent was obtained from the patients. This study was approved by the Institute Ethics Committee of Taichung Armed Forces General Hospital.

Antibodies

Nine types of primary antibodies were used in the present study: (1) Bcl-2, a mouse monoclonal antibody (sc-7382, Santa Cruz, Santa Cruz, CA, United States; dilution 1:500 for immunoblotting); (2) Bax, a mouse monoclonal antibody (sc-7480, Santa Cruz; dilution 1:200 for immunoblotting); (3,4) caspase-9, two rabbit polyclonal antibodies for immunoblotting (No. 9502, Cell Signaling, Beverly, MA, United States; dilution 1:1000) and IHC staining (sc-8355, Santa Cruz; dilution 1:100); (5) Fas, a rabbit polyclonal antibody (sc-7886, Santa Cruz; dilution 1:400 for immunoblotting); (6,7) caspase-8, a mouse monoclonal antibody for immunoblotting (No. 9746, Cell Signaling; dilution 1:1500) and a goat polyclonal antibody (sc-6134, Santa Cruz; dilution 1:50 for IHC staining); (8) cleaved caspase-3, a rabbit monoclonal antibody (No. 9664, Cell Signaling; dilution 1:1000 and 1:50 for immunoblotting and IHC staining, respectively); and (9) β-actin, a mouse monoclonal antibody (No. 8226, Abcam, Cambridge, MA, United States; dilution 1:8000 for immunoblotting). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (No. 0031430, Pierce, Hercules, CA, United States; for detection of Bcl-2, Bax, caspase-8 and β-actin) and goat anti-rabbit IgG (No. 0031460, Pierce; for detection of caspase-9, Fas and cleaved caspase-3) for immunoblotting or a commercial kit (PicTure™; Zymed, South San Francisco, CA, United States) for IHC staining.

Immunoblotting

The immunoblotting method was modified from our previous studies[9,10]. In brief, aliquots containing 100 μg of sample supernatants were added to the sample buffer and heated at 95 ℃ for 5 min. After electrophoresis and transformation, blots (polyvinylidene fluoride membranes; Millipore, Bedford, MA, United States) were preincubated in phosphate buffer saline with 0.05% Tween 20 (PBST) buffer containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding. According to the manufacturer's manual, Bcl-2, Bax, cleaved caspase-9, Fas, cleaved caspase-8, cleaved caspase-3 and β-actin antibodies should bind to proteins with molecular weights of about 26, 23, 35-37, 48, 43, 17-19 and 44 kDa, respective-

Table 1 Tumor size and α-fetoprotein levels in the study group

Items	Pre-TAE	Post-TAE	Decreased ratio (%)[1]
Tumor size (cm^3)			
Mean	70.15	34.2	46.2
Range	10.4-172.3	6.0-67.5	33.0-60.8
AFP (ng/mL)			
Mean	120.5	53.7	55.3
Range	11.7-319.0	3.6-159.7	8.5-92.3

[1]1-(post-TAE/pre-TAE); **AFP:** α-fetoprotein; TAE: Transarterial embolization.

Table 2 Apoptotic protein expression in both groups (mean ± SE)

Pathways	Proteins	Study group	Control group	*P* value
Intrinsic	Bcl-2/Bax	1.07 ± 0.21	1.31 ± 0.14	0.350
	Cleaved caspase-9	38.45 ± 14.09	35.06 ± 6.29	0.801
Extrinsic	Fas	70.51 ± 14.02[a]	35.84 ± 8.36	0.042
	Cleaved caspase-8	78.30 ± 13.23[a]	47.88 ± 6.09	0.031
Common	Cleaved caspase-3	123.41 ± 26.46[a]	76.12 ± 8.45	0.048

[a]$P < 0.05$ *vs* control group.

ly. The blots were cut into upper and lower parts at feasible sites for the incubations. The blots were incubated with the primary antibodies diluted in 1% bovine serum albumin and 0.05% sodium azide in PBST. The blots were then incubated with HRP-conjugated secondary antibody diluted in PBST. The blots were developed using a SuperSignal West Pico Detection Kit (No. 34082, Pierce). After development, the immunoblots were scanned and imported as TIFF files. All immunoreactive bands were analyzed using MCID software version 7.0 (Imaging Research, Ontario, Canada). The results were converted to numerical values to compare the relative protein abundance of the immunoreactive bands.

Immunohistochemical staining

The immunohistochemical (IHC) staining method used was modified from our previous study[9]. In brief, the sections were stained with primary antibody (caspase-9, caspase-8 or cleaved caspase-3) or PBS (negative control) before being analyzed with the commercial kit. The sections were observed under light microscope (model BX50; Olympus, Tokyo, Japan), and the micrographs were reviewed.

Statistical analysis

All data were analyzed using the Student's *t* test with $P < 0.05$ being considered statistically significantly different. Values were expressed as the mean ± SE.

RESULTS

The tumor size and AFP levels decreased by about 46.2% and 55.3%, respectively, following TAE (Table 1). The Bcl-2/Bax ratio and cleaved caspase-9 expression levels, both indicative of the intrinsic apoptosis pathway, demonstrated no statistical differences between the groups ($P > 0.05$; Figures 1 and 2A; Table 2). However, the expression levels of Fas and cleaved caspase-8, both involved in the extrinsic apoptosis pathway, were significantly higher in the study group ($P < 0.05$; Figure 2C and D; Table 2). Cleaved caspase-3 expression in the study group was also higher than in the control group ($P < 0.05$; Figure 2B; Table 2). Representative sections from study group patients (Figure 3A-D) and control group patients (Figure 3E-H) for IHC staining (caspase-9, caspase-8 and cleaved caspase-3) supported the immunoblotting results.

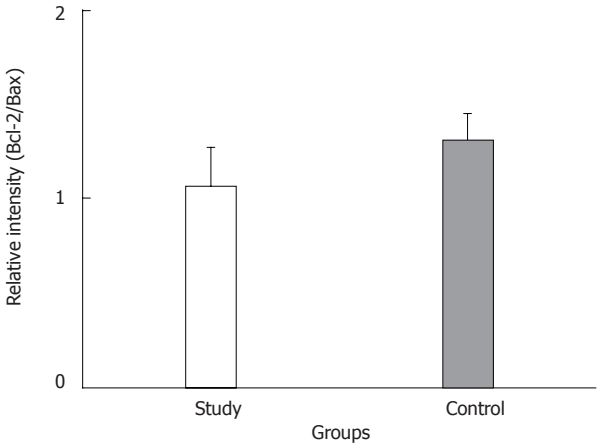

Figure 1 Relative intensity (ratio) of protein abundance of Bcl-2 to Bax in the study and control groups. Study: HCC post-TAE; Control: HCC. HCC: Hepatocellular carcinoma; TAE: Transarterial embolization.

These findings demonstrated that the decreased tumor size and AFP levels in HCC patients following TAE resulted from increased activity of the extrinsic apoptosis pathway.

DISCUSSION

HCC is one of the most common and lethal malignant tumors worldwide. The late diagnosis and advanced underlying liver cirrhosis responsible for HCC generally makes complete resection non-viable. TACE is one of the most validated treatments for unresectable HCC in patients with preserved liver function[11-13]. Several studies have reported that TACE inhibits tumor angiogenesis and induces tumor cell apoptosis[7,14]. Conversely, other studies indicate that TACE stimulates tumor angiogenesis and increases proliferative activity of the tumor cells due to hypoxic stress-induced Bcl-2 overexpression[7,8]. Hypoxia enhances proliferation, angiogenesis, metastasis, chemoresistance and radioresistance to suppress differentiation and apoptosis of HCC[15]. Therefore, combining therapies that induce and target hypoxia may be beneficial to HCC patients. TAE appears to be as effective as TACE in improving survival rates of cirrhotic HCC patients[5,6,16]. TAE allows local tumor response to reach 88%, and in addition, bleeding control can be increased

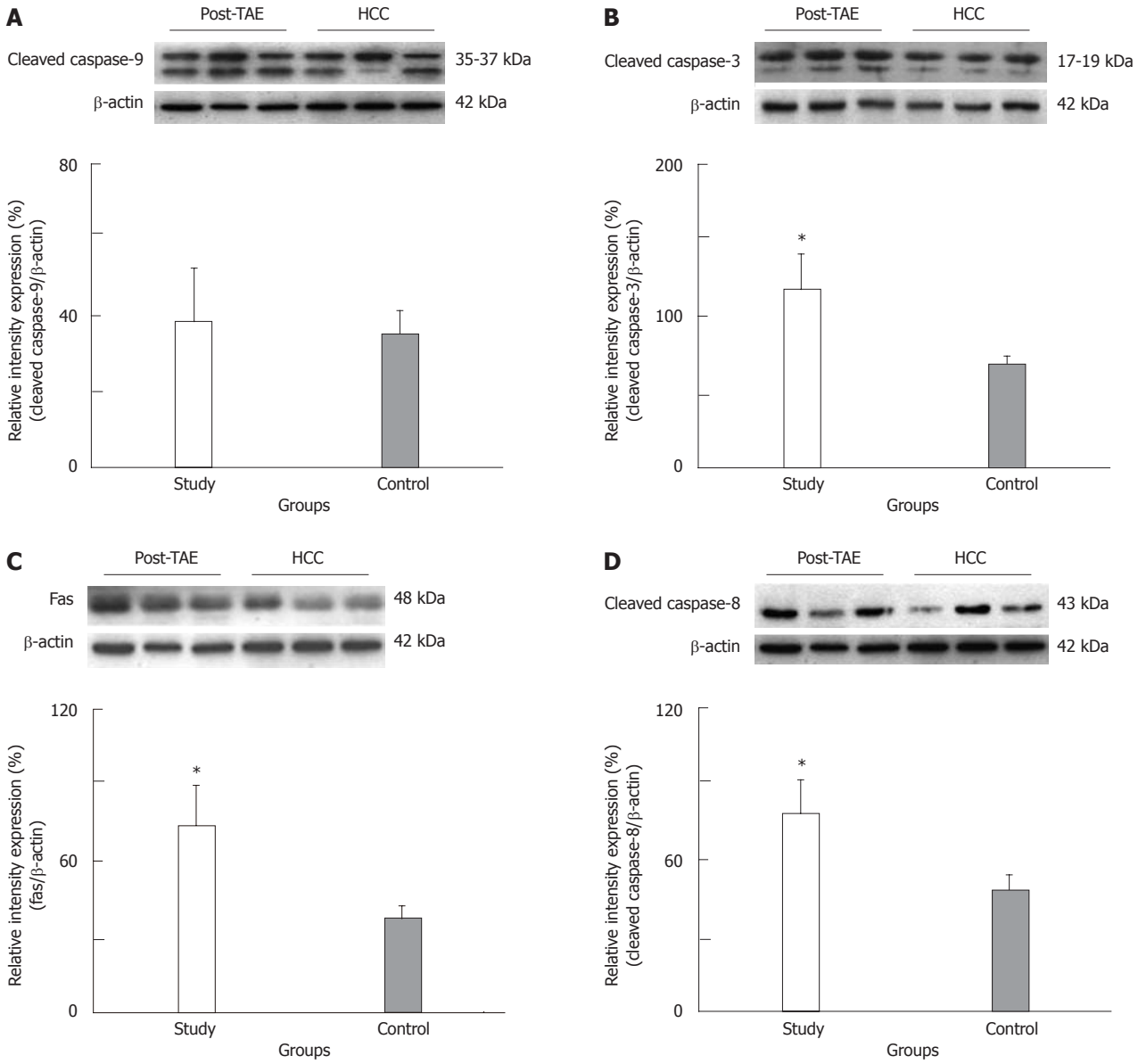

Figure 2 Representative immunoblot and relative intensities of cleaved caspase-9 (A), cleaved caspase-3 (B), Fas (C) and cleaved caspase-8 (D) proteins in the study and control groups. β-actin was used as a loading control. The asterisk indicates a significant difference between the study (HCC post-TAE) and control (HCC) groups. **HCC: Hepatocellular carcinoma; TAE: Transarterial embolization.**

from 83% to 100%[17,18]. However, there have been no further studies investigating the molecular changes and apoptotic pathways of residual viable HCC tissues following TAE.

Apoptosis, in multicellular organisms, plays an important role in development and tissue homeostasis[19-21]. The two major mechanisms of cell death are referred to as the intrinsic and extrinsic pathways. The intrinsic (or mitochondrial) pathway is induced by cellular stress, which involves Bcl-2, Bax, mitochondrial outer-membrane permeability and caspase-9 protein. The extrinsic (or death receptor) pathway is induced by specific ligands that engage death receptors, which generally involves Fas and the binding and activation of caspase-8 protein[22-24]. In the human apoptotic pathway cascade, 14 caspases (cysteinyl aspartate-specific proteinases) have been discovered to date[25,26]. Among them, caspase-3 is considered the most crucial executioner protease, as it is essential for apoptotic death in mammalian cells[27].

In this study, tumor size and AFP level were decreased by 46.2% and 55.3% following TAE, respectively. There was no significant difference of Bcl-2/Bax ratio and cleaved caspase-9 expression in either group. However, the expression levels of Fas and cleaved caspase-8, both involved in the extrinsic pathway, were significantly higher in the study group than in control group. In addition, increased cleaved caspase-3 expression was also observed in the TAE group (1.62-fold over the control group level). These findings demonstrate increased apoptosis through the extrinsic pathway in HCC patients following TAE. Previous studies had not examined the expression of proliferative and apoptotic proteins (Bcl-2

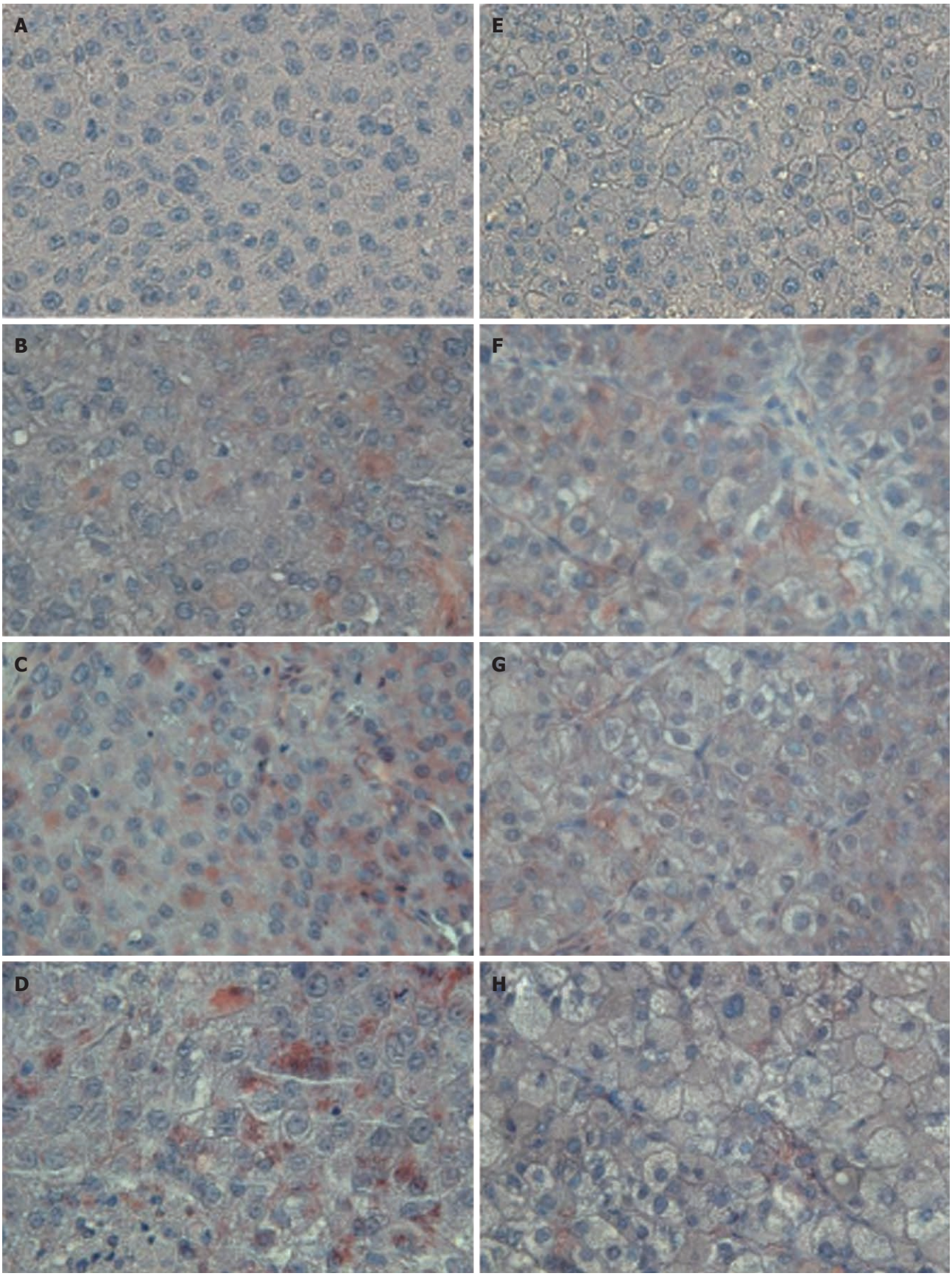

Figure 3 Representative sections of patients from the study (A-D) and control (E-H) groups after hematoxylin staining (negative control, A and E), caspase-9 immunostaining (B and F), caspase-8 immunostaining (C and G) or cleaved caspase-3 immunostaining (D and H). Study: HCC post-TAE; Control: HCC. Higher immunoreactivity of caspase-8 and cleaved caspase-3 stain was observed in the study group than in the control group. Magnification, **400 ×**. **HCC:** Hepatocellular carcinoma; **TAE: Transarterial embolization**.

and Bax) in residual viable HCC tissues simultaneously.

In conclusion, our data indicate that TAE is an effective palliative therapy that decreases tumor size and AFP levels *via* increasing the extrinsic apoptosis pathway in patients with unresectable HCC. Thus, it would be of interest to investigate whether increasing the expression of extrinsic apoptotic proteins could provide more effective treatment for patients with HCC.

COMMENTS

Background

Transarterial embolization (TAE) plays a major role in the treatment of patients with unresectable hepatocellular carcinoma (HCC). However, the apoptotic pathway of residual viable HCC tissues following TAE remains unclear. Elucidating the apoptosis pathway associated with residual viable HCC following a TAE procedure should aid in the development of more effective therapies in the future.

Research frontiers

To evaluate potential therapeutic effects, the authors examined the changes in tumor size and α fetoprotein (AFP) levels following TAE. There are two major mechanisms of cell death, and the intrinsic and extrinsic pathways. The intrinsic (or mitochondrial) pathway is induced by cellular stress, which involves Bcl-2, Bax and caspase-9 protein. The extrinsic (or death receptor) pathway is induced by specific ligands that engage death receptors, which involves Fas and the binding and activation of caspase-8 protein. Caspase-3 is considered as the crucial executioner protease because it is essential for apoptotic death in mammalian cells. All of these apoptotic proteins were examined during this study.

Innovations and breakthroughs

This is the first report to study the apoptotic pathway in the residual viable cancerous tissues of patients with HCC following TAE. A better understanding of the molecular changes in and apoptotic pathways of HCC should lead to the development of more efficacious therapies in the future.

Applications

TAE is an effective therapy for decreasing tumor size and AFP levels *via* increasing the extrinsic pathway apoptosis in patients with unresectable HCC. Thus, it would be of interest to investigate whether further increasing the expression of extrinsic apoptotic proteins could provide more effective treatment for patients with HCC.

Peer review

This is a study showing different expression of apoptotic proteins in residual viable HCC tissues in patients subjected to TAE or not. The authors demonstrated that in TAE group there is a significant increase of apoptosis through extrinsic pathway.

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BRIEF ARTICLE

Diagnostic accuracy of tests for *Helicobacter pylori* in an Alaska Native population

Dana L Bruden, Michael G Bruce, Karen M Miernyk, Julie Morris, Debby Hurlburt, Thomas W Hennessy, Helen Peters, Frank Sacco, Alan J Parkinson, Brian J McMahon

Dana L Bruden, Michael G Bruce, Karen M Miernyk, Julie Morris, Debby Hurlburt, Thomas W Hennessy, Helen Peters, Alan J Parkinson, Brian J McMahon, Arctic Investigations Program, Division of Preparedness and Emerging Infections, National Center for Emerging Zoonoses and Infectious Diseases, Centers for Disease Control and Prevention, Anchorage, AK 99508, United States

Karen M Miernyk, Frank Sacco, Brian J McMahon, Alaska Native Tribal Health Consortium, Anchorage, AK 99508, United States

Author contributions: McMahon BJ, Parkinson AJ and Sacco F designed this research study; Hurlburt D and Peters H collected the data; Morris J and Miernyk KM conducted the laboratory testing; Bruden DL analyzed the data; Bruden DL, Bruce MG, Miernyk KM and Morris J wrote the paper; Hennessy TW and McMahon BJ provided critical review of the paper.

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Correspondence to: Dr. Dana L Bruden, MS, Arctic Investigations Program, Division of Preparedness and Emerging Infections, National Center for Emerging Zoonoses and Infectious Diseases, Centers for Disease Control and Prevention, 4055 Tudor Centre, Anchorage, AK 99508, United States. dbruden@cdc.gov
Telephone: +1-907-729-3431 Fax: +1-907-729-3429

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Abstract

AIM: To evaluate the accuracy of two non-invasive tests in a population of Alaska Native persons. High rates of *Helicobacter pylori* (*H. pylori*) infection, *H. pylori* treatment failure, and gastric cancer in this population necessitate documentation of infection status at multiple time points over a patient's life.

METHODS: In 280 patients undergoing endoscopy, *H. pylori* was diagnosed by culture, histology, rapid urease test, ^{13}C urea breath test (UBT), and immunoglobulin G antibodies to *H. pylori* in serum. The performances of ^{13}C-UBT and antibody test were compared to a gold standard defined by a positive *H. pylori* test by culture or, in case of a negative culture result, by positive histology and a positive rapid urease test.

RESULTS: The sensitivity and specificity of the ^{13}C-UBT were 93% and 88%, respectively, relative to the gold standard. The antibody test had an equivalent sensitivity of 93% with a reduced specificity of 68%. The false positive results for the antibody test were associated with previous treatment for an *H. pylori* infection [relative risk (RR) = 2.8]. High levels of antibodies to *H. pylori* were associated with chronic gastritis and male gender, while high scores in the ^{13}C-UBT test were associated with older age and with the *H. pylori* bacteria load on histological examination (RR = 4.4).

CONCLUSION: The ^{13}C-UBT outperformed the antibody test for *H. pylori* and could be used when a non-invasive test is clinically necessary to document treatment outcome or when monitoring for reinfection.

Key words: Urea breath test; Antibody test; Sensitivity; Specificity

Peer reviewer: Dr. Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, Tartu 51014, Estonia

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection has been associated with peptic ulcer disease, gastric cancer, and acute gastritis[1]. Alaska Native persons have a high seroprevalence of *H. pylori* (75% all ages)[2], along with high rates of gastric cancer[3]. In rural Alaska, *H. pylori* seroprevalence is as high as 69% by the ages of 5-9 years and 87% among 7-11 year olds, as measured by the urea breath test (UBT)[4]. These findings have led to research investigations on *H. pylori* treatment outcome, reinfection rates after treatment, and the association of *H. pylori* infection with anemia in this population[4-8]. In Alaska, antimicrobial resistance rates in *H. pylori* are as high as 63% for metronidazole, 31% for clarithromycin, and 9% for levofloxacin[5,9,10]. Along with high levels of antimicrobial resistance, treatment failure rates approaching 30% in urban Alaska and 45% in rural Alaska have been demonstrated. The rate of *H. pylori* reinfection in Alaskan adults after two years was 14.5%[6]. In rural Alaskan children, aged 7 to 11 years, the reinfection rate exceeded 50% 32 mo after documented successful treatment[11]. Tests are needed after esophagogastroduodenoscopy (EGD) to document cure and continued infection-free status because of high rates of treatment failure and reinfection for *H. pylori*. Tests dependent on an EGD are not feasible for sequential follow-up or for longitudinal research studies. In rural and remote study populations, EGD testing is not available. Additionally, the cost and invasiveness of an EGD make *H. pylori* tests that are dependent upon them impractical in some settings.

This investigation was conducted as part of an Alaskan *H. pylori* reinfection study in which we enrolled persons scheduled for EGD over a three year period, treated them for *H. pylori*, and then followed them for two years with the ^{13}C-UBT test. As part of a secondary objective, we enrolled persons both positive and negative for *H. pylori* infection who were undergoing EGD for clinical indications. We aimed to determine the accuracy of non-invasive *H. pylori* tests compared to the invasive gold standard tests, based on samples obtained during EGD. The non-invasive tests that were considered in this evaluation were the ^{13}C-UBT and the detection of immunoglobulin G (IgG) antibodies to *H. pylori* (anti-HP) in serum. The invasive tests evaluated in this study were culture, histology and rapid urease test [campylobacter-like organism (CLO) test®]. We also sought to determine if the performance of the ^{13}C-UBT and the antibody assay could be improved through use of different cut-off points. Additionally, we examined whether the quantitative level of anti-HP or the ^{13}C-UBT were associated with clinical characteristics of the *H. pylori* infection, such as the presence of a peptic ulcer and the severity of gastritis, in this Alaskan population.

MATERIALS AND METHODS

Patients

Persons ⩾ 18 years of age undergoing EGD for clinical indications at the Alaska Native Medical Center (ANMC) in Anchorage, Alaska gave their consent to participate in an *H. pylori* reinfection study between September 1998 and December 2000. A description of this study cohort has been previously published[6]. From this cohort, we conducted a cross-sectional analysis to determine the sensitivity and specificity of five tests for *H. pylori*: serology, culture, CLO test® (Ballard Medical Products, Draper, UT, United States), histology and ^{13}C urea breath test (BreathTek™ UBT; Meretek Diagnostics Inc., Lafayette, CO, United States). The result of the breath test is measured as the delta over baseline (DOB), which is the difference between the ratio $^{13}CO_2/^{12}CO_2$ after and before consumption of a Pranactin-Citric solution containing ^{13}C-urea. The participants were recruited prior to EGD; therefore, the cohort consisted of persons both positive and negative for *H. pylori*. Upon enrollment, a medical chart review was performed at ANMC to determine the participants' history of: peptic ulcer disease, previous EGD procedures, and previous treatment for an *H. pylori* infection. Endoscopic findings documented during EGD included location and type of ulcer and presence of antral and fundal gastritis.

This study was approved by the Centers for Disease Control and Prevention Institution Review Board (IRB), the Alaska Area IRB of the Indian Health Service, the Southcentral Foundation Board, as well as the Alaska Native Tribal Health Consortium Board of Directors. Written informed consent was obtained from all participants.

Laboratory methods

At the time of EGD (initial enrollment), blood was drawn and a ^{13}C-UBT test was administered. Sera were tested for *H. pylori*-specific IgG by an in-house enzyme-linked immunosorbent assay (ELISA), as described previously[7]. Sera were negative if the optical density (OD) was ⩽ 0.3, positive if ⩾ 0.5, and indeterminate if in between. In-house ELISA cut-offs were determined using 254 sera collected from a previous study[7].

All participants had up to three gastric biopsy specimens taken for testing for *H. pylori* from the antrum and the fundus of the stomach. One biopsy was taken, as per the manufacturer's instructions for the CLO test®, for the detection of urease. Biopsies were stained with Diff-Quik® (Mercedes Medical, Sarasota, FL, United States) stain, for identification of *H. pylori*, and with hematoxylin and eosin stain for histological evaluation. A research pathologist examined both histological slides for the presence of intestinal metaplasia, acute and chronic gastritis, and the amount of *H. pylori* present, according to the Updated Sydney System[12]. The final one or two biopsies were used to culture *H. pylori*. Cultures were incubated at 37 ℃, 12% CO_2, and 98% humidity for up to 10 d. Isolates were identified as *H. pylori* on the basis of positive catalase, oxidase, and urease reactions, typical colony morphology, and curved gram-negative bacilli on gram-stained smears.

Table 1 Characteristics of 280 patients enrolled in Anchorage, Alaska undergoing esophagogastroduodenoscopy during 1999 and 2000

Characteristic	% (*n*)
Mean age (min, max)	48 yr (19, 88)
Sex (female)	66% (184)
Medical chart review	
History of peptic ulcer disease	19% (53)
Previous EGD	32% (90)
Previously treated for *H. pylori*	23% (63)
Endoscopist evaluation during EGD	
Moderate-severe gastritis	41% (115)
Mild-no gastritis	59% (165)
Ulcer	9% (25)

EGD: Esophagogastroduodenoscopy; *H. pylori*: *Helicobacter pylori*.

Table 2 Percent positive for *Helicobacter pylori* by test type among 280 patients enrolled in Anchorage, Alaska for an *Helicobacter pylori* reinfection study, 1999-2000

Test type		% *H. pylori* positive (*n*/*N*)
Histology	Antrum	49% (135/278)
	Fundus	46% (124/271)
	Combined	50% (140/280)
Culture	Antrum	48% (133/275)
	Fundus	50% (136/273)
	Combined	51% (144/280)
CLO test®[1]		49% (138/280)
Gold standard[2]		53% (149/280)
^{13}C-UBT[3]		55% (155/280)
Anti-HP IgG	Indeterminates considered negative	60% (168/280)
	Indeterminates considered positive	67% (188/280)
	Indeterminates removed	65% (168/260)

[1]Rapid urease test, Ballard Medical Products. [2]A positive culture or in the case of a negative culture, a positive histology result and a positive campylobacter-like organism (CLO) test®. [13]C urea breath test (UBT)[3], Breath-Tek™, Meretek Diagnostics Inc. *H. pylori*: *Helicobacter pylori*; Anti-HP IgG: Antibodies to *H. pylori* immunoglobulin G.

Statistical analysis

The gold standard used to compare test accuracies was a positive result by culture or in the case of a negative culture, a positive result by both histology and CLO test®. Test accuracy was the number of true positives plus true negatives divided by the total sample size. The manufacturer's recommended cut-off (DOB ≥ 2.4) for the ^{13}C-UBT test was used for definition of a positive *H. pylori* result for analysis.

RESULTS

The characteristics of the 280 patients who underwent EGD are shown in Table 1. The mean age of participants was 48 years and 66% were female. Of the 280 participants, 155 (55%) were positive by the ^{13}C-UBT test (Table 2). Ninety-two persons tested positive for anti-HP IgG (OD value ≥ 0.5), 168 persons tested negative (OD value ≤ 0.3), and 20 were indeterminate. If persons with indeterminate results by the antibody test are considered negative for *H. pylori* infection, then 60% of the entire 280 persons were positive by serology (Table 2). However, if those with indeterminate results were removed, then 65% were positive for *H. pylori* antibodies (Table 2). Of the 280 participants, 50%, 51% and 49% of persons were positive for *H. pylori* by histology, culture and CLO test®, respectively. Using the gold standard of either a positive culture, or a positive result of both histology and CLO test®, 53% (149/280) of persons were positive for *H. pylori*.

The performance of the non-invasive tests in comparison to the invasive tests and the gold standard are shown in Table 3. The sensitivity of the ^{13}C-UBT test in relationship to invasive tests and the gold standard ranged from 93% to 97%. The specificity was somewhat lower, and ranged from 78% to 88% when compared against invasive tests and the gold standard. The accuracy of the ^{13}C-UBT test ranged from 86% to 90%. The sensitivity of the serological assay (detection of anti-HP) in relation to the invasive tests and the gold standard was between 92% and 93%, and the specificity ranged from 58% to 68%. The concordance of anti-HP and ^{13}C-UBT was 81%. For the two invasive tests of histology and culture, there was concordance on 89% of persons. Finally, the CLO test® had a concordance of 90% and 92% with respect to histology and culture, respectively.

The sensitivity and specificity of the anti-HP assay presented in Table 3 were calculated with the indeterminate (0.3 ≤ anti-HP OD < 0.5) results removed. The overall accuracy of the anti-HP assay against the gold standard was 81.5%. Of 118 persons negative for *H. pylori* by the gold standard, 32% (n = 38) were positive by anti-HP (representing the false positives) and 68% (n = 80) were negative by anti-HP. In these 118 persons, 55% (21/38) of persons positive by anti-HP had a previous treatment for *H. pylori* documented in their medical record compared to 20% (16/80) of persons negative for anti-HP [relative risk (RR) = 2.8, 95% CI: 1.6-4.7]. In these 118 persons, the magnitude of the anti-HP OD was positively associated with previous treatment for *H. pylori* (P < 0.0001, Figure 1). Persons with an anti-HP OD ≥ 0.8 were 4.9 times (95% CI: 2.1-11.7) more likely to have had a previous treatment for *H. pylori* than persons with an anti-HP OD < 0.15.

The receiver operating curves for the quantitative anti-HP OD measurements against the gold standard are shown in Figure 2, with indeterminate results included (n = 280). The accuracy of the serological assay was between 78%-80% for cut-off points between anti-HP OD values of 0.3 and 0.7. The accuracy of the serological test diminishes using cut-off points above an anti-HP OD value of 0.7. The sensitivity and specificity of the anti-HP assay at a cut-off point of 0.3 are 94% and 61%, respectively. At a higher cut-off point of 0.7, the specificity of the test improves to 82%, but the sensitivity decreases from 94% to 76%. At no cut-off point does the accuracy of the anti-HP assay exceed 80% when the indeterminate results are included. The optimal cut-off point when including the indeterminate results was an

Table 3 Sensitivity and specificity of non-invasive tests in relation to invasive tests and a gold standard in 280 patients from Anchorage, Alaska, 1999-2000[1]

Test type 1	Test type 2	Sensitivity	Specificity	NPV[2]	PPV[2]	Accuracy
^{13}C-UBT[3] *vs*	Histology	93.6% (131/140)	82.9% (116/140)	92.8% (116/125)	84.5% (131/155)	88.2% (247/280)
	Histology and CLO test®[4]	96.8% (121/125)	78.1% (121/155)	96.8% (121/125)	78.1% (121/155)	86.4% (242/280)
	Culture	93.1% (134/144)	84.6% (115/136)	92.0% (115/125)	86.5% (134/155)	89.0% (249/280)
	Culture and CLO test®	94.6% (123/130)	78.7% (118/150)	94.4% (118/125)	79.4% (123/155)	86.1% (241/280)
	Gold standard	93.3% (139/149)	87.8% (115/131)	92.0% (115/125)	89.7% (139/155)	90.7% (254/280)
Anti-HP IgG *vs*	Histology	91.5% (118/129)	61.8% (81/131)	88.0% (81/92)	70.2% (118/168)	76.5% (199/260)
	Histology and CLO test®	93.1% (108/116)	58.3% (84/144)	91.3% (84/92)	64.3% (108/168)	73.8% (192/260)
	Culture	93.3% (126/135)	66.4% (83/125)	90.2% (83/92)	75.0% (126/168)	80.3% (209/260)
	Culture and CLO test®	93.4% (113/121)	60.4% (84/139)	91.3% (84/92)	67.3% (113/168)	75.8% (197/260)
	Gold standard	92.9% (130/140)	68.3% (82/120)	89.1% (82/92)	77.4% (130/168)	81.5% (212/260)
Anti-HP IgG *vs*	^{13}C-UBT	91.0% (131/144)	68.1% (79/116)	85.9% (79//92)	78.0% (131/168)	80.8% (210/260)
Histology *vs*	Culture	87.5% (126/144)	89.7% (122/136)	87.1% (122/140)	90.0% (126/140)	88.6% (248/280)
CLO test® *vs*	Histology	89.3% (125/140)	90.7% (127/140)	89.4% (127/142)	90.6% (125/138)	90.1% (252/280)
CLO test® *vs*	Culture	90.3% (130/144)	94.1% (128/136)	90.1% (128/142)	94.2% (130/138)	92.1% (258/280)

[1]The gold standard was a positive *Helicobacter pylori* (*H. pylori*) test by culture or, in the case of a negative culture result, a positive histology result and a positive campylobacter-like organism (CLO) test. [2]Negative and positive predictive value (NPV and PPV). [3]^{13}C urea breath test (UBT), BreathTek™, Meretek Diagnostics Inc. [4]Rapid urease test, Ballard Medical Products. Anti-HP IgG: Antibodies to *H. pylori* immunoglobulin G.

Table 4 Among *Helicobacter pylori* positive persons (according to the gold standard, *n* = 149), the relationship between antibodies to *Helicobacter pylori* immunoglobulin G optical density and delta over baseline for the ^{13}C urea breath test and demographics, histology and endoscopy outcomes

Factors	Anti-HP IgG OD[1]			^{13}C-UBT DOB[2]		
	High, OD ⩾ 1.1, (*n* = 76)	Low, OD < 1.1, (*n* = 73)	*P* value	High, ⩾ 10%, (*n* = 71)	Low, < 10%, (*n* = 78)	*P* value
Demographics						
% Male	54% (41)	19% (14)	< 0.0001	31% (22)	42% (33)	0.15
% ⩾ 50 years of age	33% (25)	40% (29)	0.39	53% (27)	29% (27)	0.002
Factors from histological examination						
% Intestinal metaplasia	11% (8)	12% (9)	0.73	8% (6)	14% (11)	0.27
% Acute gastritis	55% (42)	49% (36)	0.47	49% (35)	55% (43)	0.48
% Chronic gastritis	93% (71)	77% (56)	0.003	86% (61)	85% (66)	0.82
% Numerous *H. pylori*	42% (32)	36% (26)	0.42	54% (38)	26% (20)	0.0005
Endoscopic factors						
% Ulcer	10% (7)	11% (8)	0.76	9% (6)	12% (9)	0.57
% Gastritis	58% (42)	40% (29)	0.03	49% (34)	48% (37)	0.88

[1]Antibodies to *Helicobacter pylori* (*H. pylori*) immunoglobulin G optical density (anti-HP IgG OD) was associated with male gender [$P < 0.0001$, odds ratio (OR) = 6.2] and chronic gastritis ($P = 0.003$, OR = 5.8) on multivariate analysis. [2]Delta over baseline (DOB) from ^{13}C urea breath test (UBT) test was associated with numerous *H. pylori* ($P = 0.0005$, OR = 3.6) and older age ($P = 0.01$, OR = 2.5) on multivariate analysis.

anti-HP OD value of 0.5, which resulted in a test accuracy of 79.6%. A high anti-HP OD among persons positive for *H. pylori* by the gold standard was associated with male gender and chronic gastritis on histological examination (Table 4). The association with chronic gastritis was stronger in the antral specimen ($P = 0.02$) than the fundal specimen ($P = 0.25$).

The overall accuracy of the ^{13}C-UBT test against the gold standard was 91%, with an area under the curve value of 0.97 (Figure 2). The accuracy of the ^{13}C-UBT test was above 90% for all values in between a DOB of 2.0 and 4.0 and was highest, at 93%, between 2.8 and 3.0. Using a lower value of 2.0 as the cut-off point (91% accuracy) resulted in false negative and positive rates of 5% and 12%, whereas use of a higher value of 4.0 (90% accuracy) resulted in false negative and positive rates of 15% and 5%, respectively. The ^{13}C-UBT had a specificity and positive predictive value of 100% for cut-offs ⩾ 7.0 DOB. Additionally, among persons positive for *H. pylori* by the gold standard, a high ^{13}C-UBT DOB was associated with the number of *H. pylori* organisms seen on histological slides and age ⩾ 50 years (Table 4). Persons with a ^{13}C-UBT ⩾ 9 DOB were 4.4 (95% CI: 1.7-11.3) times more likely to have a large number of *H. pylori* bacteria on histological examination than persons with a ^{13}C-UBT < 9 DOB (Figure 3).

DISCUSSION

Our study provides important information about the accuracy of five tests commonly used in clinical practice for the diagnosis of *H. pylori* infection. Clinicians have a

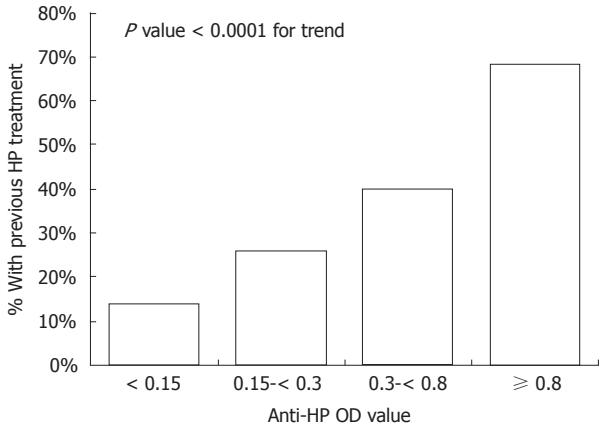

Figure 1 Among persons negative for *Helicobacter pylori* by the gold standard, the percentage of persons with a documented previous treatment for *Helicobacter pylori* according to the antibodies to *Helicobacter pylori* immunoglobulin G optical density (*n* = 118). HP: *Helicobacter polori*; OD: Optical density.

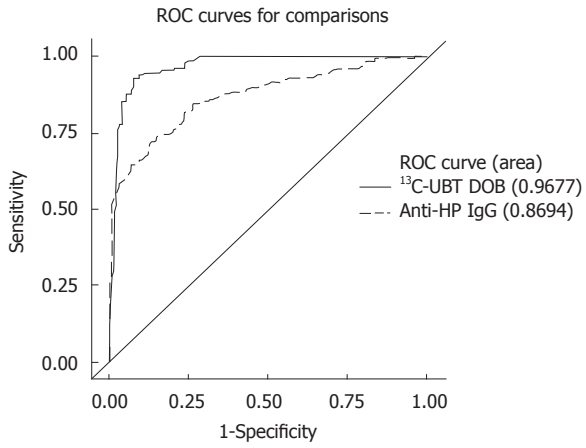

Figure 2 The receiver operating characteristic curves for anti-*Helicobacter pylori* level (serological test) and ^{13}C urea breath test delta over baseline *vs* the gold standard for *Helicobacter pylori* infection in a group of 280 Alaskans undergoing esophagogastroduodenoscopy. Gold standard was a positive *Helicobacter pylori* test by culture or, in the case of a negative culture result, a positive histology result and a positive campylobacter-like organism test. ROC: Receiver operating characteristic; UBT: Urea breath test; DOB: Delta over baseline; HP: *Helicobacter polori*; IgG: Immunoglobulin G.

variety of tests to choose from to diagnose *H. pylori* infection in patients with abdominal symptoms. The data comparing the sensitivity, specificity, and accuracy of each test are incomplete and can be confusing to clinicians. We evaluated the performance of *H. pylori* tests in a population of Alaskan native adults with a high prevalence of *H. pylori* infection (53% using the gold standard). The ^{13}C-UBT test accurately diagnosed 91% of persons relative to our gold standard, whereas the serological assay had a reduced accuracy of 81%. Our study suggests that in populations with high rates of sequelae from *H. pylori* (gastric cancer, duodenal ulcer disease), as well as high *H. pylori* treatment failure rates, and high reinfection rates after treatment, the ^{13}C-UBT test may be the best non-invasive test option available for longitudinal evaluation of *H. pylori* infection.

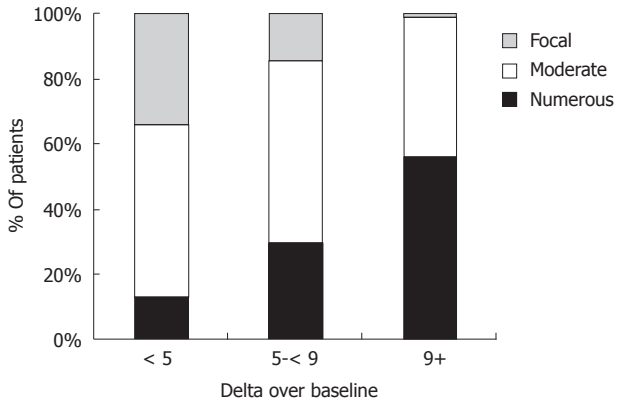

Figure 3 The amount of *Helicobacter pylori* (numerous, moderate, focal) on histological examination according to the delta over baseline value for the ^{13}C urea breath test among *Helicobacter pylori* positive persons (*n* = 149).

The antibody assay had low specificity and positive predictive value because this test can be positive in persons with a previous successfully treated *H. pylori* infection and in those with a current active infection. In this study population of urban Alaska Native adults evaluated by EGD, over 1 in 5 persons were found to have had a treatment for *H. pylori* documented in their medical records prior to entering the study. We found some persons who were negative by the gold standard tests for active infection, among whom we were able to document that the presence of anti-HP IgG was associated with previous treatment for *H. pylori*. Indeed in this group, persons with a high level anti-HP IgG were close to five times more likely to have been previously treated for *H. pylori* compared to those with low levels of anti-HP. Previously published data from this study found that the mean anti-HP IgG level was 0.64 OD units (above the assay's positive breakpoint) two years after documented successful treatment for *H. pylori*[7], demonstrating that anti-HP antibodies persist long after eradication. The antibody assay may still be suitable in treatment naïve populations in epidemiological investigations aimed at establishing baseline estimates of *H. pylori* prevalence. However, for the clinical purpose of identifying persons with active *H. pylori* infection, the antibody test has limited utility, because persons recovered from *H. pylori* infection might be mistakenly identified as harboring an active infection. In populations with a high prevalence of, and treatment for *H. pylori* infection, this assay is not optimal for use in a "test and treat" strategy in patients with dyspeptic symptoms. Patients identified by elevated antibody levels who are not actively infected may unnecessarily receive additional treatment for *H. pylori*.

We found that the anti-HP OD ⩾ 1.1 was associated with male gender and chronic gastritis as determined by histological evaluation. The finding of an association with gastritis has been documented in other studies[13-16]. Sheu *et al*[13] found the titer level of *H. pylori* to be associated with antral gastritis but not presence of ulcer,

similar to our study. In contrast, Chen *et al*[17] did not find an association with antral gastritis when restricting the analysis to *H. pylori* positive persons, an analysis similar to ours. Although the serological assay is less accurate in predicting active *H. pylori* infection in this Alaska Native population, high levels of anti-HP IgG increased the odds by 4-fold that chronic gastritis will be diagnosed by histology. The lower anti-HP level found in women could be attributable to inadvertent treatment of the *H. pylori* infection in the use of metronidazole for treating vaginal infections in women. Indeed, in this study group, women had higher levels of metronidazole use[8], and higher levels of metronidazole use were associated with lower anti-HP levels (CDC unpublished data).

With a sensitivity and specificity of 93% and 97%, respectively, using the manufacturer's recommended cut-off point, the ^{13}C-UBT provided the best non-invasive test for documentation of infection-free status. The accuracy of the test in our population compares well with performance of the UBT (both ^{13}C-UBT and ^{14}C-UBT) in other studies[18-20], and is in contrast to a recent study that found much lower specificity in Spain[21]. In our study, we found that the accuracy of the ^{13}C-UBT was > 90% with cut-off points between 2.0 and 4.0 DOB, similar to findings in other reports[22]. Additionally, we found that the DOB value was positively associated with the amount of *H. pylori* present on histological examination. Persons with very high DOB values were over four times more likely to have a high concentration of *H. pylori* bacteria than persons with low DOB values, an association documented elsewhere[23-25]. The association between the concentration of *H. pylori* organisms on histology and other pathological outcomes (gastritis, intestinal metaplasia), as well as treatment outcome, is being investigated further.

In summary, in a population in the United States with a high background prevalence of *H. pylori*, we found that the ^{13}C-UBT test outperforms the serological assay in the detection of active *H. pylori* infection. The ^{13}C-UBT avoids the problem associated with the serological test of incorrectly identifying persons who have recovered from *H. pylori* as having active infection. For clinical purposes, in persons with gastrointestinal symptoms in whom an EGD procedure is not planned, the ^{13}C-UBT appears to be useful to rule out or confirm *H. pylori* infection, and to test for a cure in persons who have been treated for *H. pylori* infection.

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COMMENTS

Background

High rates of *Helicobacter pylori* (*H. pylori*) infection and antimicrobial resistance have been documented in Alaska Native persons. High infection pressure and antimicrobial resistance have led to elevated rates of treatment failure of and reinfection with *H. pylori*. Non-invasive tests, not dependent on an esophagogastroduodenoscopy (EGD), are necessary to document cure and continued infection-free status.

Research frontiers

The performances of five tests [^{13}C urea breath test (UBT), detection of immunoglobulin G (IgG) antibodies to *H. pylori* in serum, culture, histology and rapid urease test] that are commonly used in clinical practice for *H. pylori* were evaluated in 280 patients undergoing EGD at the Alaska Native Medical Center in Anchorage, Alaska.

Innovations and breakthroughs

The overall accuracy for diagnosing *H. pylori* infection was 91% for the ^{13}C-UBT test and 81% for the antibody assay. False positive results for the antibody assay were associated with previous successful treatment of *H. pylori* infection. A high antibody level was associated with histological gastritis. An elevated level for the ^{13}C-UBT test was associated with a high bacterial load of *H. pylori* on histological examination.

Applications

In a population with a high background rate of *H. pylori* infection, the authors found the ^{13}C-UBT to be the most clinically useful noninvasive test for the diagnosis and follow-up of patients with gastrointestinal symptoms.

Terminology

The result of the ^{13}C-UBT test is measured as the delta over baseline, which is the difference between the ratio $^{13}CO_2/^{12}CO_2$ after and before the consumption of a Pranactin-Citric solution containing ^{13}C-urea. Test accuracy was the number of true positives plus true negatives divided by the total sample size.

Peer review

This study is a well-done population study for the evaluation of the accuracy of two non-invasive tests (^{13}C-UBT) and the detection of IgG antibodies to *H. pylori* in serum) in a population of Alaska Native persons with high prevalence of *H. pylori* infection.

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BRIEF ARTICLE

Low-volume plus ascorbic acid *vs* high-volume plus simethicone bowel preparation before colonoscopy

Stefano Pontone, Rita Angelini, Monica Standoli, Gregorio Patrizi, Franco Culasso, Paolo Pontone, Adriano Redler

Stefano Pontone, Rita Angelini, Monica Standoli, Gregorio Patrizi, Paolo Pontone, Adriano Redler, Department of Surgical Sciences, "Sapienza" University of Rome, 00161 Roma, Italy
Franco Culasso, Department of Medical Statistics, "Sapienza" University of Rome, 00161 Roma, Italy
Author contributions: Pontone S contributed to acquisition, analysis and interpretation of data, and study concept and design; Pontone P, Angelini R, Standoli M and Patrizi G contributed to acquisition of data; Culasso F contributed to statistical analysis; Pontone P and Redler A contributed to critical revision of the manuscript for important intellectual content.
Correspondence to: Dr. Stefano Pontone, Department of Surgical Sciences, "Sapienza" University of Rome, V le Regina Elena 324, 00161 Rome, Italy. stefano.pontone@uniroma1.it
Telephone: +39-06-49972446 Fax: +39-06-49972446
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Abstract

AIM: To investigate the effectiveness of low-volume plus ascorbic acid [polyethylene glycol plus ascorbic acid (PEG + Asc)] and high-volume plus simethicone [polyethylene glycol plus simethicone (PEG + Sim)] bowel preparations.

METHODS: A total of one hundred and forty-four outpatients (76 males), aged from 20 to 84 years (median age 59.5 years), who attended our Department, were divided into two groups, age and sex matched, and underwent colonoscopy. Two questionnaires, one for patients reporting acceptability and the other for endoscopists evaluating bowel cleansing effectiveness according to validated scales, were completed. Indications, timing of examination and endoscopical findings were recorded. Biopsy forceps were used as a measuring tool in order to determine polyp endoscopic size estimation. Difficulty in completing the preparation was rated in a 5-point Likert scale (1 = easy to 5 = unable). Adverse experiences (fullness, cramps, nausea, vomiting, abdominal pain, headache and insomnia), number of evacuations and types of activities performed during preparation (walking or resting in bed) were also investigated.

RESULTS: Seventy-two patients were selected for each group. The two groups were age and sex matched as well as being comparable in terms of medical history and drug therapies taken. Fourteen patients dropped out from the trial because they did not complete the preparation procedure. Ratings of global bowel cleansing examinations were considered to be adequate in 91% of PEG + Asc and 88% of PEG + Sim patients. Residual Stool Score indicated similar levels of amount and consistency of residual stool; there was a significant difference in the percentage of bowel wall visualization in favour of PEG + Sim patients. In the PEG + Sim group, 12 adenomas $\leqslant$ 10 mm diameter (5/left colon + 7/right colon) *vs* 9 (8/left colon + 1/right colon) in the PEG + Asc group were diagnosed. Visualization of small lesions seems to be one of the primary advantages of the PEG + Sim preparation.

CONCLUSION: PEG + Asc is a good alternative solution as a bowel preparation but more improvements are necessary in order to achieve the target of a perfect preparation.

Key words: Bowel preparation; Polyethylene glycol; Ascorbic acid; Colonoscopy; Simethicone

Peer reviewer: Haruhiko Sugimura, MD, PhD, Professor, Department of Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers diagnosed in Western countries and it is the major cause of cancer-associated morbidity and mortality[1]. The increased demand for colonoscopy can be attributed to widespread CRC screening and surveillance[2,3]. A screening procedure, to be effective, must ensure high sensitivity and it must be both safe and tolerable in order to warrant adequate compliance in asymptomatic individuals[4].

Colonoscopy has been accepted as the gold standard for colon exploration and is considered the most effective method for assessing colonic lesions. In fact, this procedure performed in asymptomatic individuals $\geqslant$ 50 years old with no history of CRC or adenomas, and in younger high-risk patients[1,5], permits an early detection of CRC.

Several specific pre-procedure quality indicators were selected in 2006 by the American Society for Gastrointestinal Endoscopy (ASGE)/American College of Gastroenterology (ACG) taskforce on quality in endoscopy with the aim of establishing competence in colonoscopy performance. They are: (1) appropriate indication; (2) informed consent obtained; (3) use of recommended surveillance intervals; (4) use of recommended ulcerative colitis and Crohn's colitis surveillance; and (5) patient preparation[5].

An inadequate preparation can be costly in terms of missed lesions, increased risk of complications, time required for procedure, and need for repeated colonoscopies[5,6]. Moreover, patient compliance to the preparation process is often poor[7] and it remains a deterrent for patients in whom colonoscopy is required[8]. Independent predictors of an inadequate colon preparation include a later colonoscopy starting time, failure to follow preparation instructions, inpatient status, indication of constipation, use of tricyclic antidepressants, male gender, and a history of cirrhosis, stroke, or dementia[9].

The ideal colon cleansing for diagnostic and surgical procedures would reliably empty the colon of fecal material, have no effect on the gross or microscopic appearance of the colon, require a short period for ingestion and evacuation, cause no discomfort and produce no significant shifts of fluids or electrolytes[10-13]. Moreover, the cleansing regimen should be simple and suitable for inpatients and outpatients. Nowadays, available methods do not completely meet these criteria, and problems with patient compliance, safety, and adequacy of cleansing prompt continued investigation for alternative forms of cleansing.

Polyethylene glycol (PEG)-based gut lavage is an isosmotic solution that passes through the bowel without absorption or secretion. PEG has been safely used in patients with serum electrolyte imbalances, advanced hepatic dysfunction, acute and chronic renal failure and congestive heart failure[14,15]. PEG does not alter the histological features of colonic mucosa and may be used in patients suspected of having inflammatory bowel disease without obscuring the diagnostic capabilities of colonoscopy or biopsy analysis. Several PEG lavage solutions have added simethicone which is an oral antifoaming agent that decreases bloating, abdominal discomfort and abdominal pain by promoting the clearance of excessive gas along the gastrointestinal tract by reducing the surface tension of air bubbles. This combination is safe and effective (significant fluid and electrolyte shifts are avoided) but requires the consumption of large volumes of fluid in order to achieve a cathartic effect[16]. Nowadays, a low-volume PEG oral solution for colon cleansing that combines PEG with electrolytes plus ascorbic acid and sodium sulphate is gaining popularity over large volume oral lavage solutions[17]. The megadose of ascorbic acid that is not completely absorbed remains in the colonic lumen where it exerts an osmotic effect so a smaller quantity of PEG is required.

The aim of our randomized trial was to compare the PEG + ascorbic acid and sodium sulphate preparation (MoviPrep®; Norgine BV; PEG + Asc) with a PEG + simethicone preparation (Selg®-Esse 1000, Promefarm Srl, IT; PEG + Sim) in terms of cleansing effectiveness, patient compliance, physical tolerability, endoscopic findings and costs.

MATERIALS AND METHODS

A total of one hundred and forty-four outpatients (76 males), aged from 20 to 84 years (median age 59.5 years), who attended our Department of Surgical Sciences of "Sapienza" University of Rome over the period May 2009 to October 2010 and who underwent elective colonoscopy for routine clinical indications were randomized. Patients were 1:1 randomized to receive the commercially available bowel cleansing regimens: (1) 2 L of PEG + ASC (MoviPrep®; Norgine BV); and (2) 4 L of PEG + Sim (Selg®-Esse 1000, Promefarm Srl, IT). A computer-generated randomization chart was used to determine allocation. Allocation was concealed with an opaque envelope. The envelope was opened when the patient met the inclusion criteria and provided informed consent. Exclusion criteria were as follows: hospitalized patients, allergy or hypersensitivity to any constituent of both lavage solutions, and inability to fill in a questionnaire. Patient demographics, mean time of examination, indications and colonoscopy findings are shown in Table 1.

Written instructions on how to prepare and ingest the bowel preparation solution (Table 2) and dietary advice, randomly alternating between PEG + Asc and PEG + Sim, were given and explained by the endosco-

Table 1 Patient demographics, indications and colonoscopy findings

	PEG + Asc	PEG + Sim
ITT patients	72	72
Compliant patients (%)	69 (96)	61 (85)
Cecal intubation (%)	62 (86)	68 (94)
Median age (range)	60.1 (20-84)	57.6 (33-81)
Male (%)	40 (55)	36 (50)
Median timing of colonoscopy (min)	22	21
Indications (%)		
Follow-up	27 (37)	18 (25)
Surveillance	8 (11)	11 (15)
CRC sreening	15 (21)	8 (11)
Hematochezia	13 (18)	16 (22)
Change in bowel habits	3 (4)	7 (10)
Anemia	2 (3)	1 (2)
Abdominal pain	4 (6)	11 (15)
Findings (%)		
No abnormalities	40 (55)	24 (33)
Diverticular disease	14 (19)	14 (19)
Polyps/Malignancy	13/2 (18/3)	22/0 (31/0)
IBD	1 (2)	3 (4)
Other	2 (3)	9 (13)

ITT: Intention to treat; CRC: Colorectal cancer; IBD: Inflammatory bowel disease; PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

Table 2 Colonoscopy preparation schedules

PEG + Asc	2 L from 6:00 to 8:00 PM (250 mL every 15 min) plus 500 mL of clear fluid for every L of solution, evening before colonoscopy Each liter of PEG + Asc (MoviPrep®) contains 100 g macrogol 3350, 7.5 g sodium sulfate, 2.7 g sodium chloride, 1 g potassium chloride, 4.7 g ascorbic acid, 5.9 g sodium ascorbate, and lemon flavoring
PEG + Sim	2 L from 3:00 to 5:00 PM and 2 L from 6:00 to 8:00 PM (250 mL every 15 min), evening before colonoscopy Each liter of PEG + Sim (Selg®-Esse 1000) contains 58.3 g macrogol 4000, 0.08 g simethicone, 5.68 g sodium sulfate, 1.68 g sodium bicarbonate, 1.46 g sodium chloride and 0.74 g potassium chloride and mandarin aroma

A low-fiber diet (mainly the avoidance of fruits and vegetables) was recommended for three days before the endoscopy to all subjects and, the day before, they were advised to have regular breakfast, a light lunch and only clear liquids for dinner. PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

pists or paramedical staff at the time of exam scheduling. The coordinator told all patients not to reveal to the physicians performing the colonoscopy which preparation they had taken. An informed consent form was obtained from each study subject.

Upon arrival at the endoscopy suite, patients filled in a questionnaire and were interviewed about their compliance to the assigned bowel preparation method (Appendix A). Feasibility of instructions and willingness to retake the exam in the future if needed was recorded. Difficulty in completing the preparation was rated in a 5-point Likert scale (1 = easy to 5 = unable). Adverse

Table 3 Colonoscopy preparation assessments

Aronchick scale	
1 Excellent	Small volume of clear liquid or greater than 95% of surface seen
2 Good	Large volume of clear liquid covering 5% to 25% of surface but greater than 90% of surface seen
3 Fair	Some semi-solid stool that could be suctioned or washed away but greater than 90% of surface seen
4 Poor	Semi-solid stool that could not be suctioned or washed away and less than 90% of surface seen
5 Inadequate	Repreparation needed
Residual stool score (total in sum of three score)	
Amount of residual stool	0 = none 1 = small 2 = moderate 3 = large
Consistency of residual stool	0 = none 1 = clear liquid 2 = colored liquid 3 = stool particles 4 = semi-solid stool 5 = solid stool
Percent bowel wall visualized	0 ⩾ 75% 1 = 50%-75% 2 = 25%-49% 3 ⩽ 25%

experiences (fullness, cramps, nausea, vomiting, abdominal pain, headache and insomnia), number of evacuations and types of activities performed during preparation (walking or resting in bed) were also investigated. The exams, performed by experienced endoscopists, were scheduled between 8:30 AM and 2:00 PM. Standard colonoscopies (EVIS EXERA Ⅱ video colonoscope CF-Q165I®, Olympus Europa Holding GmbH) were used for colonoscopic examinations. A minimum 6-min withdrawal time was spent. After the procedure, endoscopists filled in a questionnaire in order to evaluate the global bowel cleansing score with an Aronchick scale, as indicated in Table 3[18].

A Residual Stool Score (Table 3), based on the amount and consistency of residual stool and on the percent of bowel wall visualization[19,20], was recorded for each of five colon segments: cecum, right colon, transverse colon, left colon/sigmoid, and rectum. The three component scores from each colon segment were averaged and then summed to calculate a total residual stool score for each subject (range 0-11 for total score, 0 = best). Overall, colon cleansing efficacy was considered adequate if the ranking was 1-3 Aronchick scale score. Indications, timing of examination and endoscopical findings were recorded. Biopsy forceps were used as a measuring tool in order to determine polyp endoscopic size estimation.

χ^2 test including Yates' continuity correction was used as appropriate. A significant difference was considered when the P value was $\leqslant 0.05$. All analyses were performed using GraphPad InStat version 2.04a.

Table 4 Patient drop-out: global bowel cleansing, side effects and findings

Drop out gut cleansing	PEG + Asc	PEG + Sim
No. of patients	3	11
Global bowel cleansing (%)		
Aronchick 1	(1)	(2)
Aronchick 2	(1)	(5)
Aronchick 3	-	(2)
Aronchick 4	(1)	(2)
Aronchick 5	-	-
Cecal Intubation (patients)	2	11
Findings		
No abnormalities	1	9
Polyps/malignancy	0/1	2/0
Diverticular disease	1	-
Other	-	

PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

Table 5 Side effects in compliant patients ([1]multiple side effects possible)

	PEG + Asc	PEG + Sim
No. of patients	72	72
Side effects patients[1]	14	21
Nausea	7	16
Vomiting	4	5
Headache	3	1
Insomnia	1	1
Abdominal pain	2	1

PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

RESULTS

Seventy-two patients were selected for the PEG + Asc group and seventy-two for the PEG + Sim group. The two groups were age and sex matched as well as comparable in terms of medical history and drug therapies taken. Fourteen patients dropped out from the trial because they did not complete the preparation procedure (Table 4). Among these patients, some were unable to complete their preparations because of nausea (13 patients) and others because of vomiting. There were no significant differences in reported side effects between the PEG + Asc and the PEG + Sim groups. The most common reported side effects were nausea and vomiting (Tables 4 and 5). In 14 cases, endoscopists were unable to achieve cecal intubation: 6 patients due to lack of bowel cleansing, 6 due to a poor tolerance and 2 patients because of the presence of a malignant stricture (both in the PEG + Asc group). Rating global bowel cleansing using the Aronchick scale (Table 6): examinations were considered to be adequate in 91% of PEG + Asc and 88% of PEG + Sim patients. Residual Stool Score indicated similar levels of amount and consistency of residual stool; there was a significant difference in the percentage of bowel wall visualization in favour of PEG + Sim patients (Figure 1).

Table 6 Overall gut cleansing and cecal intubation performed

Overall gut cleansing	PEG + Asc	PEG + Sim
No. of patients	69	61
Aronchick 1	8/5	17/16
Aronchick 2	29/27	13/12
Aronchick 3	26/26	24/24
Aronchick 4	5/2	5/5
Aronchick 5	1/0	2/0

PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

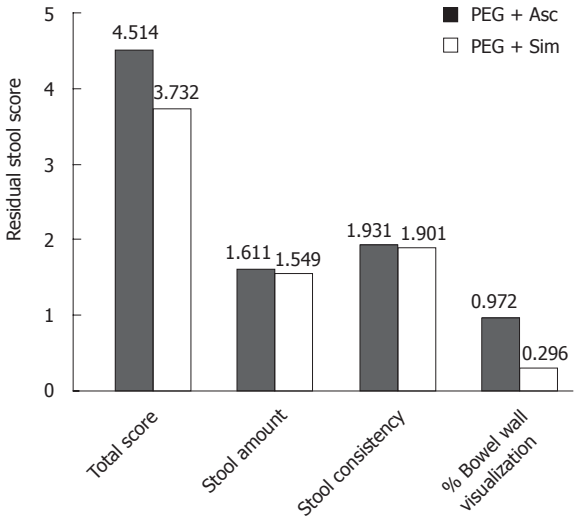

Figure 1 Residual stool score. A lower score indicates better bowel cleansing. Subjects in the PEG + Sim group demonstrated significantly lower scores for percentage of colon visualization. PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

In the PEG + Sim group, 12 adenomas ⩽ 10 mm in diameter (5/left colon + 7/right colon) *vs* 9 (8/left colon + 1/right colon) in the PEG + Asc group were diagnosed (Figure 2). Furthermore, in the PEG + Sim group, 12 adenomas ⩽ 5 mm in diameter *vs* 5 (left colon only) in the PEG + Asc group were diagnosed.

The average time of examination was about 22 min. Moreover, the median timing of colonoscopy was longer in negative tests (24 min, range 20-40) than in colonoscopies with polyp diagnosis (19 min, range 18-25). In patients with a score of 4 or 5 on the Aronchick scale of bowel preparation, the average time for colonoscopy completion was approximately 27 min. The average number of bowel movements obtained during the preparation did not appear to be related to the degree of cleanliness achieved, with 13 movements for 1 or 2 Aronchick scale, 11 movements for 3 Aronchick scale and 11 movements for 4 or 5 Aronchick scale score. Conversely, the presence of clear liquid at the time of the last evacuation is a reliable parameter of effective colonic cleansing. In fact, 93% of patients who achieved a 1, 2 or 3 Aronchick scale score reported the presence of clear liquids during the last evacuation, while patients with 4 or 5 Aronchick scale score reported this in only

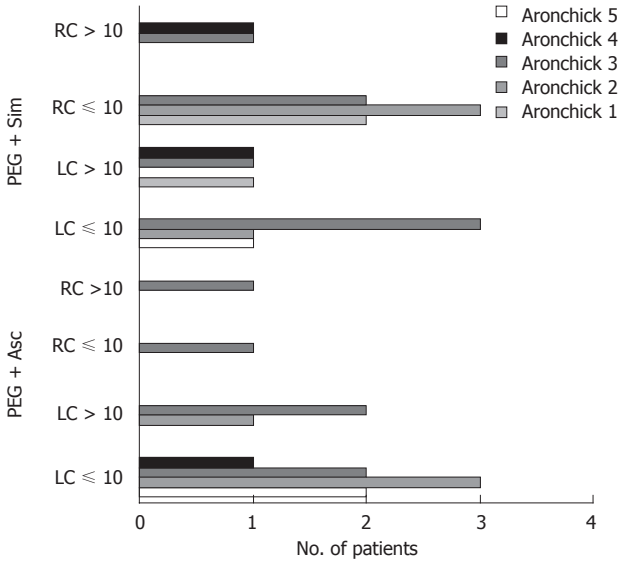

Figure 2 Patients with at least one newly diagnosed polyp in relation to the level of cleanliness achieved (LC = Left colon; RC = Right colon). PEG + Asc: Polyethylene glycol plus ascorbic acid; **PEG + Sim: Polyethylene glycol** plus simethicone.

Table 7 Patient questionnaire findings by preparation group tolerability

Question	PEG + Asc	PEG + Sim
Clear liquid at the time of the last evacuation		
Yes	51	62
No	21	10
Is this the first time you took a preparation for colonoscopy?		
Yes	33	43
No	39	29
Discomfort:		
None	18	28
Slightest	32	36
Moderate	17	6
Severe	5	2
How much would you be prepared to repeat this preparation for colonoscopy?		
A little	18	15
Fairly	32	42
Much	15	11
I would never repeat	7	4

PEG + Asc: Polyethylene glycol plus ascorbic acid; PEG + Sim: Polyethylene glycol plus simethicone.

40% of cases. Sixty-three percent of the subjects taking PEG + Sim and 39% of the subjects taking PEG + Asc ($P = 0.005$) reported that they would rather try another preparation for a future colonoscopy. Other patient questionnaire findings rated by preparation group tolerability are reported in Table 7.

DISCUSSION

Currently, the most significant disadvantage of performing colonoscopies is the need for adequate bowel preparation and poor bowel preparation impacts on the efficiency of colonoscopy[21]. Moreover, the major obstacle preventing a large scale implementation of CRC screening is the low level of patient compliance[21], and patient compliance is limited because of embarrassment[20,21], the bowel preparation procedure[8] and the fear of pain and discomfort associated with the examination[22-24].

Cleansing methods for colonoscopy have evolved by attempting to achieve a high efficiency together with a high patient compliance. A consensus of the American Society for Gastrointestinal Endoscopy, the American Society of Colon and Rectal Surgeons and the Society of American Gastrointestinal and Endoscopic Surgeons, indicated that PEG is the gold standard for colonoscopic bowel preparation (Grade IA), and aqueous sodium phosphate (NaP) is an alternative regimen to PEG solutions (Grade IA)[24].

Several meta-analyses on the available bowel preparations have favored NaP, concluding that it was effective and better tolerated by patients than PEG solutions[25-28]. However, the disadvantages of NaP are the associated side effects. Significant changes in serum electrolyte levels[29], even in patients without renal failure, have prompted recommendations for serum electrolyte evaluation prior to the administration of sodium phosphate[30,31]. On the other hand, osmotically balanced electrolyte lavage solutions (PEG-ELS, SF-ELS) offer safe and effective cleansing[22-24,32-34] but volume related discomfort and adverse experiences have decreased the percentage of patients completing the pre-examination preparation. This is mainly due to the large volumes of fluid required for bowel preparation, the unpleasant taste and an increase in the incidence of side effects[15]. In order to bypass volume and taste problems, a PEG electrolyte lavage solution containing ascorbic acid was developed. This low-volume formulation has satisfied many of our requirements. In fact, in our study the subjects enrolled were outpatients, and it was not possible to carry out a complete clinical history and serum electrolyte evaluation. Thus, one of our major considerations was patient safety in colonic preparation, which is well documented for PEG solutions.

To help ensure compliance, the paramedical staff explained to the patient in detail the instructions containing the correct procedures to follow, with special attention paid to explaining the importance of additional fluid consumption with this procedure. Patients were then asked if they completely understood the procedure they had to follow.

Our study has limitations, such as number of patients, single center, lack of a practice calibration on the bowel preparation scoring system for all physicians involved before the study, and full-dose *vs* split-dose regimen comparisons. However, some conclusions on efficacy of bowel wall cleansing, adenoma detection rate and patient compliance can be made.

Bowel cleansing can be evaluated using different scoring systems such as the Aronchick[18], the Ottawa[35] and the Boston scale[36]. In our study, we decided to use the Aronchick scale assisted by a residual stool score to evaluate effectiveness, as previously adopted by Bala-

ban *et al*[19] and Harenwood *et al*[20]. Our data on the bowel cleansing evaluation showed similar levels in both groups, that in most cases was found to be "excellent-fair" (Aronchick 1-3) allowing cecal intubation in 90% of cases. However, we noted better colonic wall visualization in the PEG + Sim group, probably due to the effect of the simethicone.

Only a small number of studies have compared the adenoma detection rate to the quality of bowel preparation[6,37,38]. Froehlich *et al*[6] and Harewood *et al*[37], however, demonstrated that a better bowel preparation led to a higher rate of colon lesion detection, enhancing the ability to discern smaller lesions and thus improved the thoroughness of colonoscopy. In our trial, only 3 out of 30 polyps (Aronchick 4 only) were diagnosed in the presence of inadequate bowel preparation and two of them were > 10 mm in diameter. Thus, we focused on the diagnosis of adenoma in relation to the degree of colonic preparation, paying attention to adenomas ⩽ 10 mm or 5 mm and their distribution. Indeed, while there is no significant difference in total adenoma detection rates between groups, looking at the number of adenomas ⩽ 10 mm and ⩽ 5 mm in diameter and their distribution, there was significant evidence of a greater number of microadenomas diagnosed in favor of the PEG + SIM group. This result reinforces our observations that PEG + Sim has a better ability to clean the colon wall as represented by the residual stool score. Although the impact of detection and removal of micro-adenomas on CRC incidence or mortality is debated, this parameter, which is strongly influenced by the quality of bowel preparation, could be objectively representative of the view of the intestinal wall.

However, since colonoscopy is the best screening test for CRC, we cannot underestimate the importance of patient compliance which directly affects its acceptance and distribution. We must therefore consider whether it is more important to have a highly effective or highly popular test and search for a compromise. So, from the aspect of patient compliance, the majority of patients in both groups completed the bowel preparation in the specified schedule (96% for PEG + Asc and 85% for PEG + Sim). Both groups contained patients who reported side effects and did not finish the pre-procedure preparation. However, this occurred predominantly in the PEG + Sim group, although colonoscopy was still able to be performed and it did not affect the results of the bowel cleansing score. Thus, in this study, the inability to completely drink the PEG + Sim solution (75% of the total was always drunk) did not significantly reduce the effectiveness of the pre-procedure preparation. It is difficult to say the same for the PEG + Asc group considering the small number of patients with adverse events (n = 3). However, our data have shown, in agreement with Ell *et al*[16], that the PEG + Asc formulation was more acceptable to patients and a greater number of them finished the recommended dose.

In conclusion, we agree that PEG + Asc is a good alternative solution, in particular addressing patient compliance, but some improvements seem to be necessary in order to achieve the target of a perfect preparation. One area could be the visualization of small lesions. This seems to be one of the primary advantages of the PEG + Sim solution. Based on the data, the low-volume preparation represents a valid alternative to high-volume preparations, especially with regard to patient compliance. However, improvements are needed to reduce the side effects in both types of preparation and further studies should be carried out, giving the patient the choice of preparation to be taken.

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COMMENTS

Background

Colonoscopy has been accepted as the gold standard for colon exploration and is considered the most effective method for assessing colonic lesions. An inadequate preparation can be costly in terms of missed lesions, increased risk of complications, time required for procedure and need for repeated colonoscopies.

Research frontiers

A bowel cleansing regimen should be simple and suitable for inpatients and outpatients. Nowadays, available methods do not completely meet these criteria, and problems with patient compliance, safety, and adequacy of cleansing prompt continued investigation for alternative forms of cleansing.

Innovations and breakthroughs

Our randomized trial compared the polyethylene glycol plus ascorbic acid (PEG + Asc) and sodium sulphate preparation with a polyethylene glycol plus simethicone (PEG + Sim) preparation in terms of cleansing effectiveness, patient compliance, physical tolerability, endoscopic findings and adenoma detection rate.

Applications

The low-volume preparation represents a valid alternative to high-volume preparations, especially with regard to patient compliance. On the other hand, the optimal visualization of colonic wall seems to be one of the primary advantages of the PEG + Sim solution. Through this study, the authors suggest different preparation regimens for different indications.

Peer review

This randomized trial compared the polyethylene glycol plus ascorbic acid and sodium sulphate preparation (MoviPrep®; Norgine BV, PEG + Asc) with a polyethylene glycol plus simethicone preparation (Selg®-Esse 1000, Promefarm Srl, IT, PEG + Sim) in terms of cleansing effectiveness, patient compliance, physical tolerability, endoscopical finding. Bowel preparation is a specific quality indicator and it is a critical point in clinical practice.

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BRIEF ARTICLE

Optimizing management of pancreaticopleural fistulas

Marek Wronski, Maciej Slodkowski, Wlodzimierz Cebulski, Daniel Moronczyk, Ireneusz W Krasnodebski

Marek Wronski, Maciej Slodkowski, Wlodzimierz Cebulski, Daniel Moronczyk, Ireneusz W Krasnodebski, Department of General, Gastroenterological and Oncological Surgery, Medical University of Warsaw, 02-097 Warsaw, Poland

Author contributions: Wronski M designed the study, collected the data and drafted the manuscript; Slodkowski M and Cebulski W collected the data and revised the manuscript; Moronczyk D collected the data, reviewed the literature and revised the manuscript; Krasnodebski IW revised the manuscript and approved its final version.

Correspondence to: Dr. Marek Wronski, Department of General, Gastroenterological and Oncological Surgery, Medical University of Warsaw, ul. Banacha 1A, 02-097 Warsaw, Poland. mwronski@vp.pl

Telephone: +48-22-5992482 Fax: +48-22-5992057
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Abstract

AIM: To evaluate the management of pancreaticopleural fistulas involving early endoscopic instrumentation of the pancreatic duct.

METHODS: Eight patients with a spontaneous pancreaticopleural fistula underwent endoscopic retrograde cholangiopancreatography (ERCP) with an intention to stent the site of a ductal disruption as the primary treatment. Imaging features and management were evaluated retrospectively and compared with outcome.

RESULTS: In one case, the stent bridged the site of a ductal disruption. The fistula in this patient closed within 3 wk. The main pancreatic duct in this case appeared normal, except for a leak located in the body of the pancreas. In another patient, the papilla of Vater could not be found and cannulation of the pancreatic duct failed. This patient underwent surgical treatment. In the remaining 6 cases, it was impossible to insert a stent into the main pancreatic duct properly so as to cover the site of leakage or traverse a stenosis situated downstream to the fistula. The placement of the stent failed because intraductal stones (n = 2) and ductal strictures (n = 2) precluded its passage or the stent was too short to reach the fistula located in the distal part of the pancreas (n = 2). In 3 out of these 6 patients, the pancreaticopleural fistula closed on further medical treatment. In these cases, the main pancreatic duct was normal or only mildly dilated, and there was a leakage at the body/tail of the pancreas. In one of these 3 patients, additional percutaneous drainage of the peripancreatic fluid collections allowed better control of the leakage and facilitated resolution of the fistula. The remaining 3 patients had a tight stenosis of the main pancreatic duct resistible to dilatation and the stent could not be inserted across the stenosis. Subsequent conservative treatment proved unsuccessful in these patients. After a failed therapeutic ERCP, 3 patients in our series developed superinfection of the pleural or peripancreatic fluid collections. Four out of 8 patients in our series required subsequent surgery due to a failed non-operative treatment. Distal pancreatectomy with splenectomy was performed in 3 cases. In one case, only external drainage of the pancreatic pseudocyst was done because of diffuse peripancreatic inflammatory infiltration precluding safe dissection. There were no perioperative mortalities. There was no recurrence of a pancreaticopleural fistula in any of the patients.

CONCLUSION: Optimal management of pancreaticopleural fistulas requires appropriate patient selection that should be based on the underlying pancreatic duct abnormalities.

Key words: Pancreaticopleural fistula; Pancreatitis; Surgery; Endoscopic retrograde cholangiopancreatography; Magnetic resonance cholangiopancreatography

Peer reviewers: Sundeep Singh Saluja, MS, MCh Assistant Professor, Department of Gastrointestinal Surgery, GB Pant Hospital and Maulana azad Medical College, Bahadur Shah Zafar Marg, New Delhi 110002, India; Maha Maher Shehata, Professor, Department of Internal Medicine, Gastroenterology and Hepatology Unit, Medical Specialized Hospital, Mansoura 35516, Egypt

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INTRODUCTION

The pancreaticopleural fistula is a rare type of internal pancreatic fistula. Although the precise incidence is unknown, pancreaticopleural fistulas are reported to occur in approximately 0.4% of patients with pancreatitis[1]. These fistulas are most commonly associated with alcoholic chronic pancreatitis[1-3]. The pancreaticopleural fistula results from a disruption of a major pancreatic duct usually due to an underlying pancreatic disease. A ductal disruption on the anterior surface of the pancreas usually leads to pancreatic ascites, whereas the posterior ductal leakage might result in thoracic fluid collections. The pancreatic juice spreads retroperitoneally through the paths of least resistance, commonly through the aortic or esophageal hiatus.

The management of pancreaticopleural fistulas remains controversial. The timing of surgical and endoscopic intervention for a pancreaticopleural fistula is disputable. There are no definitive criteria that would allow accurate prediction which patients are likely to benefit from medical treatment and which patients should be offered early surgical intervention. Medical therapy of pancreaticopleural fistulas fails in 59%-69% of cases[1,3,4]. Additionally, unsuccessful conservative treatment is associated with an increased rate of complications[3,5]. Recently, endoscopic instrumentation has become the preferred treatment in pancreaticopleural fistulas and an apparently high rate of fistula resolution has been reported in the literature[4,6]. In light of these observations we used endoscopic retrograde cholangiopancreatography (ERCP) with stenting of the pancreatic duct as an initial treatment in patients with a pancreaticopleural fistula.

In this article we present our experience in the management of patients with pancreaticopleural fistulas who initially underwent endoscopic therapy.

MATERIALS AND METHODS

Patients

Between January 2000 and February 2010, 10 patients with a spontaneous pancreaticopleural fistula presented to our department. Two patients who received primary surgical treatment for a pancreaticopleural fistula due to the surgeon's preference were excluded from the study. The patient group consisted of 7 males and 1 female. The mean age of the patients was 48 years (range: 34-63 years).

Methods

The pancreaticopleural fistula was defined as a pleural effusion with amylase content above 1000 IU/L and protein level above 3.0 g/dL. The fistulous tract was additionally demonstrated with computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP) or ERCP in all the cases.

Medical records were reviewed retrospectively for each patient. The clinical manifestations, underlying pancreatic pathology, imaging appearance and management of the pancreaticopleural fistula were evaluated. Early response to therapy and long-term outcome were assessed.

We used ERCP with an intention to stent the main pancreatic duct as an initial treatment. ERCP was performed as early as possible after the recognition of amylase-rich pleural effusion. ERCP was carried out by the interventional endoscopists skilled in therapeutic endoscopy with several years of experience in pancreatic procedures. Failure of endoscopic treatment was deemed an indication to operative intervention. Our medical management of pancreaticopleural fistulas included diet restriction and enteral or parenteral nutrition. Enteral feeding was delivered through a feeding tube inserted endoscopically or under fluoroscopic guidance into the proximal jejunum. Somatostatin analogs were administered in selected patients. Medical treatment before the trial of ERCP was not used uniformly, but most patients received it following the endoscopic procedure. The pleural effusion was drained in the patients with severe dyspnea or infected fluid. The details of conservative treatment are summarized in Table 1.

Time to fistula healing was calculated from the date of the initial ERCP till the day of normalization of amylase level in the pleural fluid or disappearance of pleural effusion on a chest X-ray or ultrasound.

Statistical analysis

Descriptive statistics were used including mean and range.

RESULTS

The pancreaticopleural fistula was most commonly a complication of underlying chronic pancreatitis or a late consequence of acute pancreatitis. Chronic pancreatitis had been symptomatic for a period between 2 mo and 10 years. In one case, a pancreaticopleural fistula developed in a patient who had a longitudinal pancreaticojejunostomy (a modified Puestow procedure) for chronic pancreatitis 10 mo earlier. All the patients presented with a thoracic symptomatology and most patients had left-sided pleural effusion. The diagnosis was established by means of thoracentesis in each case showing a high amylase level in the pleural fluid sample. The demographic and clinical characteristics of the patients with a pancreaticopleural fistula are summarized in Table 2.

The fistulous tract was additionally visible in CT in 6 cases, in ERCP in 5 cases and in MRCP in 2 cases (Figures 1-3). Imaging investigations revealed peripancreatic fluid collections in all patients. These collections were located along the fistulous tract that dissected into the mediastinum through the esophageal or aortic hiatus

Table 1 The details of conservative management of pancreaticopleural fistulas

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
NPO[1] (d)	19 (6)	0 (22)	7 (27)	0 (26)	6 (9)	0 (25)	8 (0)	14 (22)
TPN[1] (d)	-	0 (17)	0 (27)	0 (25)	4 (7)	0 (26)	-	0 (16)
EN[1] (d)	16 (6)	-	-	-	-	-	5 (17)	7 (12)
Somatostatin analog[1] (d)	-	0 (11)	0 (25)	0 (43)	-	-	-	0 (11)
Pleural fluid management	TD-25 d	TD-4 d	T-twice, TD-11 d	T-1 time, TD-25 d	TD-3 d	T-4 times	TD-30 d	Diagnostic thoracentesis alone

[1]Length of treatment after endoscopic retrograde cholangiopancreatography given in the parenthesis. NPO: Nothing per os; TPN: Total parenteral nutrition; EN: Enteral nutrition; T: Thoracenthesis; TD: Tube drainage.

Table 2 Demographic and clinical features of the patients with a pancreaticopleural fistula

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Age (yr)	41	63	42	34	50	46	59	52
Gender	M	F	M	M	M	M	M	M
Etiology	CP	CP	AP	CP	CP	CP	CP	CP
Alcohol abuse	+	-	+	+	+	+	+	+
Presentation	Dyspnea	Dyspnea, abdominal pain, chest pain	Dyspnea	Dyspnea, abdominal pain, chest pain	Dyspnea	Dyspnea, abdominal pain	Dyspnea	Dyspnea
Location of pleural effusion	Left	Left	Right	Left	Left	Left	Left	Left
Pleural amylase (IU/L)	6715	34 512	9716	13 000	2072	26 977	10 158	1830
Serum amylase (IU/L)	519	300	251	155	144	248	30	234
Duration of symptoms	4 wk	2 wk	2 wk	8 wk	1 wk	1 wk	6 d	4 d

M: Male; F: Female; CP: Chronic pancreatitis; AP: Acute pancreatitis.

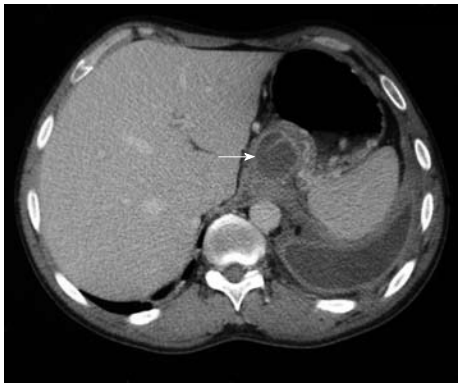

Figure 1 Computed tomography scan demonstrates left pleural effusion and a fluid collection extending through the esophageal hiatus that corresponds to a pancreaticopleural fistula (arrow).

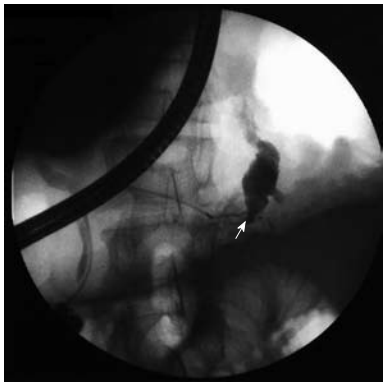

Figure 2 Endoscopic retrograde cholangiopancreatography shows a ductal leakage (arrow) in the tail of the pancreas with the contrast spreading into the chest.

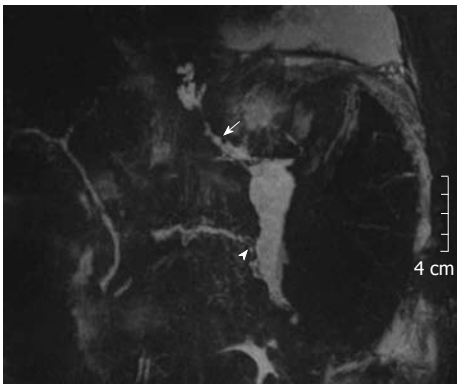

Figure 3 Magnetic resonance cholangiopancreatography demostrates a pancreaticopleural fistula (arrow) extending from a ductal disruption (arrowhead) at the tail of the pancreas into the chest. A ductal blockage in the body of the pancreas precluded evaluation of the anatomy of the upstream duct in endoscopic retrograde cholangiopancreatography.

(Figure 1). The pancreas was usually of normal size with features of chronic pancreatitis. The imaging findings in the patients with a pancreaticopleural fistula are shown in Table 3.

ERCP with an intention to insert a stent so as to bridge the site of leak was performed as early as possible after the recognition of amylase-rich pleural effusion. In only one case, the stent bridged the site of leakage. The fistula in this patient closed within 3 wk following the procedure. The main pancreatic duct in this case appeared normal, except for a leak located in the body of the pancreas. In another patient, the papilla of Vater could not be found and cannulation failed. This patient underwent surgical

Table 3 Imaging features of pancreaticopleural fistula

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Demonstration of fistulous tract	CT	ERCP, CT	CT	ERCP, CT	CT, MRCP	ERCP	ERCP, CT	ERCP, MRCP
Location of pleural effusion	Left	Left	Right	Left	Left	Left	Left	Left
CT	Enlarged pancreatic head, parenchymal calcifications, small peripancreatic fluid collections near the tail	Enlarged body and tail of the pancreas, peripancreatic fluid collections near the body and tail, dilated PD up to 6 mm	Pancreas not enlarged, PD dilated, small pseudocyst in the body	Pancreas not enlarged, small peripancreatic fluid collections near the tail, PD normal	Pancreas not enlarged, parenchymal calcifications, PD mildly dilated, small pseudocyst near the body and tail	Pancreas not enlarged, parenchymal calcifications in the head, PD dilated up to 3 mm, small pseudocyst near the tail	Pancreas not enlarged, PD normal, small pseudocyts near the pancreatic tail	Pancreas not enlarged, PD dilated up to 4 mm in the tail, pseudocyts near the pancreatic body and tail, ascites
MRCP	-	-	-	-	Pancreas not enlarged, PD dilated up to 3 mm in the body upstream to a stone, small pseudocysts near the tail	-	-	Pancreas not enlarged, PD normal, pseudocyts near the pancreatic body and tail, ascites, leak in the body

CT: Computed tomography; MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; PD: Pancreatic duct.

Table 4 The results of primary endoscopic intervention in pancreaticopleural fistulas

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Location of fistula	Tail	Body	Body	Body/tail	Tail	Body/tail	Body	Body/tail
ERCP	Dilated proximal PD, blockage of PD in the head due to intraductal stones	Ductal stricture and leak in the body	Cannulation not possible	No ductal stricture, leak in the tail	Normal proximal PD, blockage of PD in the body	No ductal stricture, leak in the body/tail	Ductal stricture in the body, leak within the stenotic duct	No ductal stricture, two leaks in the body/tail
ERCP	Failure (ductal blockage)	Failure (ductal stricture could not be dilated, stent not reached the stenosis and leak)	Failure (ampulla cannulation not possible)	Failure (stent not reached the site of leak, early stent migration)	Failure (ductal blockage precluding stenting)	Successful (stent bridging the site of leak)	Failure (ductal stricture could not be dilated, stent inserted up to the stricture)	Failure (stent bridging the site of one leak, but could not reach the site of the second leak)
Post-ERCP complications	-	Pleural empyema, infected peripancreatic fluid collection	-	Pleural empyema, pancreatitis flare-up	-	-	Pleural empyema, pneumonia, pancreaticobronchial fistula, infected peripancreatic fluid collection	-
Post-ERCP treatment	Surgery	Surgery	Surgery	Conservative	Conservative	Conservative	Surgery	Percutaneous drainage of peripancreatic fluid collection
Time to fistula closure (d)	30	25	28	11	24	17	26	34

PD: Pancreatic duct; ERCP: Endoscopic retrograde cholangiopancreatography.

treatment. In the remaining 6 cases, it was impossible to insert a stent into the main pancreatic duct properly so as to cover the site of leakage or traverse a stenosis situated downstream to the fistula. The placement of the stent failed because intraductal stones (n = 2) and ductal strictures (n = 2) precluded its passage or the stent was too short to reach the fistula located in the distal part of the pancreas (n = 2). In 3 out of these 6 patients, the pancreaticopleural fistula closed on further medical treatment. In these cases, the main pancreatic duct was normal or only mildly dilated, and there was a leakage at the body/tail of the pancreas. In one of these 3 patients, additional percutaneous drainage of the peripancreatic fluid collections allowed better control of the leakage and facilitated resolution of the fistula. This patient had two ductal disruptions in the body/tail of the pancreas and otherwise a normal main pancreatic duct. The stent was inserted so that it bridged the proximal ductal leak, but could

not cover the second site of leakage which still supplied the fistula. The remaining 3 patients had a tight stenosis of the main pancreatic duct resistible to dilatation and the stent could not be inserted across the stenosis. Subsequent conservative treatment proved unsuccessful in these patients.

After a failed ERCP, 3 patients in our series developed superinfection of the pleural or peripancreatic fluid collections. Moreover, one of these patients developed a pancreaticobronchial fistula manifesting as left lobar pneumonia probably due to a prolonged chest tube drainage. The details of endoscopic treatment are summarized in Table 4.

Half of the patients in our series required surgical intervention after an unsuccessful attempt of endoscopic treatment. Distal pancreatectomy with splenectomy was performed in two cases. The patient after the Puestow operation underwent distal pancreatectomy with an end-to-end pancreaticojejunostomy. In one case, only external drainage of the pancreatic pseudocyst was done because of diffuse peripancreatic inflammatory infiltration. There were no perioperative mortalities. Postoperatively, two patients developed a pancreaticocutaneous fistula which closed spontaneously in one case and after the placement of an additional endoscopic stent in the other case.

The median follow-up was 13.5 mo (range: 5 mo-10 years). There were no recurrences of the pancreaticopleural fistula in any of the patients. The only patient in our series successfully managed with endoscopic treatment alone underwent a pancreaticoduodenectomy 2 years later for intractable abdominal pain due to underlying chronic pancreatitis.

DISCUSSION

The diagnosis of a pancreaticopleural fistula is established when an amylase-rich fluid is drained on thoracentesis. There is not any established threshold of amylase level that is diagnostic of a pancreaticopleural fistula. We arbitrarily defined the pancreaticopleural fistula as a pleural effusion with an amylase content above 1000 IU/L. However, pleural effusions due to a pancreaticopleural fistula commonly show amylase level of many thousand units[1].

It is essential to evaluate the ductal anatomy and pancreatic morphology before planning further management. ERCP and MRCP are the commonly used modalities in the assessment of pancreatic fistulas. We discourage the use of ERCP as a first-line tool for confirmation of a pancreaticopleural fistula because of considerable risk of introducing infection. MRCP seems to be a better choice for the visualization of a pancreaticopleural fistula. This method enables recognition and outlining of both a fistula and the anatomy of the pancreatic ducts. Moreover, MRCP is a non-invasive study without the risk of superinfection or acute pancreatitis. It has been shown that MRCP detects pancreatic duct abnormalities and calculi with a similar accuracy to ERCP[7]. In contrast to ERCP, this study has the advantage of demonstrating the pancreatic duct upstream to the site of a complete obstruction, as it was the case in one of our patients. MRCP was helpful in the diagnosis of pancreaticopleural fistula in 80% of the cases, while ERCP and CT were useful in 78% and 47% respectively[2].

The role of conservative treatment and the timing of surgical or endoscopic intervention for a pancreaticopleural fistula is disputable. A 2-3-wk trial of medical therapy is traditionally recommended[1,5]. Failure of conservative treatment is considered an indication for endoscopic or surgical intervention. The success rate in resolution of a pancreaticopleural fistula on medical therapy alone has been reported to be of 31%-65%[1-3]. However, conservative treatment requires prolonged hospitalization and is costly. Moreover, failed medical treatment results in a higher rate of complications and significantly longer hospital stay[3]. In a series reported by Lipsett *et al*[5], 80% of deaths during non-operative management of internal pancreatic fistulas occurred in patients who had been treated conservatively for over 3 wk. Furthermore, pleural effusions left undrained for a long period might lead to the formation of lung entrapment, sequestrated pleural fluid collections or a pancreaticobronchial fistula. Therefore, the potential risks of prolonged medical treatment should always be weighted against the morbidity and mortality associated with operative treatment. The appropriate patient selection according to the ductal morphology visualized in MRCP might spare these 2-3 wk of conservative treatment in the cases with a poor chance of spontaneous fistula closure. In our series, only the fistulas that developed in patients with fairly normal pancreatic ducts responded well to medical treatment.

ERCP was initially used as a diagnostic tool. Nowadays, various therapeutic procedures can be performed at the time of ERCP. The armamentarium of interventional endoscopists includes pancreatic sphincterotomy, ductal stenting, nasopancreatic drainage and lithotripsy. Saeed *et al*[8] were first to report resolution of a pancreaticopleural fistula after endoscopic stenting of the pancreatic duct. Since then, there have been published multiple reports of successful closure of pancreaticopleural fistulas after endoscopic instrumentation of the pancreatic duct[4,6,9-12]. Nevertheless, endoscopic techniques carry the risk of serious complications such as acute pancreatitis, bleeding, perforation or septic complications. The complication rate of pancreatic endotherapy varies considerably and ranges from 7% to 25%[13-15]. Moreover, pancreatic endotherapy is technically demanding and requires substantial experience to avoid potentially life-threatening complications. Therefore, careful selection of patients is essential before endoscopic therapy is recommended.

We reviewed the management of pancreaticopleural fistulas based on the case reports and case series published between 1993 and 2009. Our search of the literature provided 30 cases of pancreaticopleural fistulas with adequately described ductal anatomy in ERCP and endoscopic treatment. Eight cases were treated medically following only a diagnostic ERCP. Most of these pa-

tients received total parenteral nutrition and a pancreatic enzyme inhibitor or somatostatin analog. Four out of the 8 pancreaticopleural fistulas healed on conservative treatment alone. The main pancreatic duct was normal or irregular without any obvious stricture in two of these cases and there was a ductal stenosis with a leak in the pancreatic tail in the remaining two cases. ERCP in all the 4 fistulas resistant to medical treatment revealed a downstream stricture of the pancreatic duct. Twenty cases were treated successfully with endoscopic instrumentation such as stent insertion (n = 16) or nasopancreatic drainage (n = 4). Sixteen out of 20 fistulas were located in the head or body of the pancreas. In 11 cases, there was only a ductal disruption without any significant stenosis of the main pancreatic duct. These patients were treated with a stent or nasopancreatic catheter placed across the site of leakage. In the remaining 9 cases, there was a concomitant ductal stricture that could be dilated and stented, although the stent or nasopancreatic catheter did not bridge the site of a ductal disruption in two cases. Another two patients underwent endoscopic instrumentation combined with extracorporeal lithotripsy due to intraductal stones. The reports of the pancreaticopleural fistulas after unsuccessful endotherapy did not provide adequate descriptions of the pancreatic duct anatomy.

The success rate of therapeutic endoscopy in pancreaticopleural fistulas is varied. Khan *et al*[6] successfully treated all 5 of their patients with a pancreaticopleural fistula by means of intraductal stenting. Similarly, Pai *et al*[14] reported a 96.4% success rate of endotherapy in the treatment of internal pancreatic fistulas, including 13 pancreaticopleural fistulas. However, most ductal disruptions in this series were located in the head and body of the pancreas (64.2%) and no leakage at the time of ERCP was found in 28.6% cases. Such proximal ductal disruptions are, however, more easily managed endoscopically. Varadarajulu *et al*[13] reported only a 55% success rate in resolution of the pancreatic duct disruption using a transpapillary stenting technique, although placement of a stent was possible in 95% of patients. However, it is noteworthy that 92% of the ductal disruptions that closed were in the cases where the stent bridged the site of leakage. In the remaining cases, the stent was put only close to the site of disruption (2%) or just across the ampulla (6%). In a series published by O'Toole *et al*[16] endotherapy used in internal pancreatic fistulas was successful in a third of their patients and there was a similar rate of both failed ductal cannulations and unsuccessful endoscopic treatment. Kaman *et al*[17] attempted placement of nasopancreatic drainage in six patients with an internal pancreatic fistula, but it failed in four patients due to ductal pathology. In their series, the fistula closed in only one patient. Similarly, endotherapy alone proved successful in just one case also in our series, and a partial success was seen in another patient. On the other hand, in some case series therapeutic endoscopy was not possible in most patients because of failed cannulation or stenting of the main pancreatic duct[18]. The duration of stent or nasopancreatic drainage is unknown, but most ductal disruptions resolve with a median time of 4-6 wk[14,15].

The majority of the pancreaticopleural fistulas that closed after endotherapy had a stent inserted so that it bridged the site of disruption. Shah *et al*[19] reported resolution of a pancreaticopleural fistula after exchange of a stent for a longer one that could bridge the site of disruption. Initial trial of a stent inserted only up to the site of disruption was unsuccessful in this case. It follows that a stent not only decreases the ductal pressure, but also plays a certain mechanical role in sealing the leakage. Decreasing ductal pressure may not be sufficient for resolution of a ductal disruption and coverage of the leak with a stent is required in some cases.

In our series, unsuccessful endotherapy resulted from the inability to cannulate the ampulla and failure to stent across the site of leakage or a downstream stenosis within the main pancreatic duct. The appropriate placement of a pancreatic stent for the treatment of a pancreaticopleural fistula requires coverage of the site of leakage or stenting across a stricture situated downstream to the fistula. Otherwise, endotherapy has to be considered unsuccessful and operative treatment should be performed early to prevent septic complications. In this series, the stent could not be inserted properly into the pancreatic duct because the papilla of Vater was not found (n = 1), intraductal stones (n = 2) and ductal strictures (n = 2) precluded passage of a stent or the stent was too short to reach the fistula located in the distal part of the pancreas (n = 2). In 3 out of these patients, the fistula closed on further medical treatment. In these cases, the main pancreatic duct was normal or only mildly dilated, except for a leakage located at the body/tail of the pancreas, what suggests a fairly good outflow of the pancreatic juice into the duodenum. In one of these 3 patients, additional percutaneous drainage of the peripancreatic fluid collections allowed better control of the leakage and facilitated resolution of the fistula. Therefore, the patients with a normal or mildly dilated main pancreatic duct without any downstream stenosis can receive a trial of conservative treatment, especially when the leakage is located in the pancreatic tail. The fistulas originating from the tail of the pancreas are usually low-output and more prone to spontaneous resolution. Endotherapy used in this patient group might further promote resolution of the fistula if a stent is inserted across the site of leakage. The patients with a downstream stricture or a fistula located in the head and body of the pancreas should preferably undergo endotherapy. However, a high rate of failure can be expected because of intraductal stones and strictures inherent to chronic pancreatitis that are often hard to dilatate due to severe fibrosis. In our series, the 3 patients with a tight stenosis of the main pancreatic duct could not be properly stented and conservative treatment used after ERCP was unsuccessful. After a failed ERCP, 3 patients in our series developed superinfection of the pleural or peripancreatic fluid collections. The pancreaticopleural fistula usually has a tortuous fistulous tract

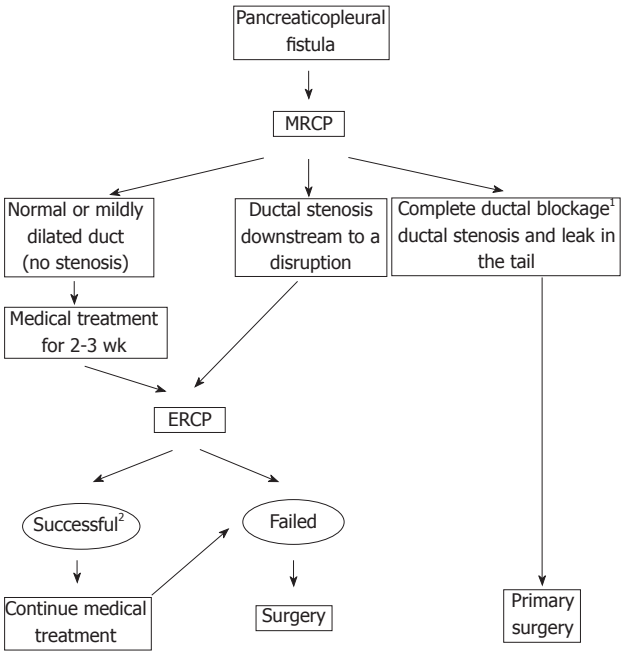

Figure 4 The algorithm for the treatment of pancreaticopleural fistulas. [1]Complete ductal blockage due to stones may be approached with endoscopic or extracorporeal lithotripsy. [2]Successful endotherapy: The stent inserted across the site of a ductal disruption or traverses a ductal stricture. MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography.

that connects with complex intraabdominal or thoracic fluid collections what renders them especially susceptible to infection. The patients after a failed ERCP should be treated operatively soon after the endoscopic procedure, preferably within the following 24-48 h. Any longer delay might lead to serious septic complications such as intraabdominal abscess or pleural empyema and continued medical treatment usually fails. On the other hand, infected peripancreatic fluid collections after a failed therapeutic ERCP further complicate surgical dissection and sometimes external drainage is all that can be done in such a situation. Antibiotic prophylaxis might prevent some septic complications after ERCP. However, prophylactic antibiotics were not used in our series.

Before the era of therapeutic endoscopy, surgery was frequently used as a primary treatment in patients with pancreaticopleural fistulas. Nowadays, surgery is often seen as a treatment of the last resort and used only after failure of medical and endoscopic therapy. However, a delay in the definitive treatment of the cases having a poor chance of healing without surgical intervention might result in increased morbidity and mortality. On the other hand, pancreatic operations performed for a pancreaticopleural fistula have now acceptably low morbidity and mortality rates in the high-volume centers[18,20]. Moreover, early operative treatment is recommended in the institutions where there are no endoscopists experienced in the treatment of pancreatic diseases. Primary and early surgical treatment of pancreaticopleural fistulas might prove cost- and time-saving in appropriately selected patients and prevent life-threatening complications due to a failed ERCP, repeated thoracenteses or prolonged pleural drainage. In our series, 4 patients underwent surgical treatment after a failed endotherapy. Two of these patients had uncomplicated postoperative course. The other 2 patients developed a pancreaticocutaneous fistula. In contrast to internal pancreatic fistulas, the external fistulas can be managed conservatively for a long time because of a low risk of septic complications, and they often heal spontaneously.

The pancreaticopleural fistulas usually occur in patients with chronic pancreatitis. Even though endotherapy may be initially successful, progression of the disease often leads to subsequent complications that frequently require surgical intervention. Therefore, surgical treatment might prove more effective for a longterm amelioration of the symptoms. In our series, one patient needed pancreatic resection because of intractable pain syndrome, although endotherapy had allowed resolution of the pancreaticopleural fistula 2 years earlier.

In conclusion, the choice of primary management in patients with pancreaticopleural fistulas should be tailored to the pancreatic ductal abnormalities. The patients with a normal or mildly dilated pancreatic duct without a downstream stenosis visualized on MRCP are optimally managed with a trial of medical treatment, especially when the leakage is located in the pancreatic tail. Ductal disruptions in the head and body of the pancreas and presence of a ductal stricture favor endoscopic treatment as a first-line therapy. Early surgical intervention is recommended whenever a stent cannot bridge the site of a ductal disruption or there is a downstream ductal stricture which can not be stented. Complete ductal obstruction anywhere along the main pancreatic duct or both a stricture and leakage within the pancreatic tail favor primary surgical treatment due to a poor chance of appropriate endoscopic stent placement. We propose an algorithm for the optimal management of pancreaticopleural fistulas (Figure 4).

COMMENTS

Background

The pancreaticopleural fistula is a rare type of internal pancreatic fistula. These fistulas are most commonly associated with alcoholic chronic pancreatitis and result from a disruption of a major pancreatic duct. The management options in pancreaticopleural fistulas include conservative treatment and endoscopic or surgical intervention.

Research frontiers

The management of pancreaticopleural fistulas remains controversial. The timing of surgical and endoscopic intervention for a pancreaticopleural fistula is still disputable. There are no definitive criteria that would allow accurate prediction which patients are likely to benefit from medical treatment and which patients should be offered early surgical or endoscopic intervention. In the study, the role of early therapeutic endoscopy with stenting of the pancreatic duct was evaluated in regard to the pancreatic ductal anatomy.

Innovations and breakthroughs

Endoscopic retrograde cholangiopancreatography with therapeutic intervention has recently become the preferred treatment in pancreaticopleural fistulas and an apparently high rate of fistula resolution has been reported in the literature. This study suggests that the choice of primary management in patients with pancreaticopleural fistulas should be tailored to the pancreatic ductal abnormalities.

Applications

The management of patients with pancreaticopleural fistulas can be improved with appropriate patient selection based on the pancreatic duct abnormalities. This selection is essential for the optimal treatment of pancreaticopleural fistula with low morbidity rate.

Peer review

The paper addresses the important issues in management of pancreaticopleural fistulas.

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BRIEF ARTICLE

Epidemiological aspects of Budd-Chiari in Egyptian patients: A single-center study

Mohammad Sakr, Eman Barakat, Sara Abdelhakam, Hany Dabbous, Said Yousuf, Mohamed Shaker, Ahmed Eldorry

Mohammad Sakr, Eman Barakat, Sara Abdelhakam, Hany Dabbous, Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Abbassia, Cairo 11341, Egypt
Said Yousuf, Department of Hematology, Faculty of Medicine, Ain Shams University, Abbassia, Cairo 11341, Egypt
Mohamed Shaker, Ahmed Eldorry, Department of Radiodiagnosis and Interventional Radiology, Faculty of Medicine, Ain Shams University, Abbassia, Cairo 11341, Egypt
Author contributions: Sakr M and Eldorry A contributed equally to the work; Sakr M, Eldorry A, Barakat E, Abdelhakam S, Dabbous H and Yousuf S designed the research; Abdelhakam S, Dabbous H and Shaker M performed the research; Sakr M, Eldorry A, Barakat E, Abdelhakam S, Dabbous H, Yousuf S and Shaker M contributed analytic tools; Sakr M, Eldorry A, Barakat E, Abdelhakam S, Dabbous H and Yousuf S analyzed the data; Barakat E, Abdelhakam S, Dabbous H and Shaker M wrote the paper.
Correspondence to: Sara Abdelhakam, MD, Lecturer of Tropical Medicine, Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Abbassia, Cairo 11341, Egypt. saratropical@yahoo.com
Telephone: +2-02-01001601548 Fax: +2-02-22598751
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Abstract

AIM: To describe the socio-demographic features, etiology, and risk factors for Budd-Chiari syndrome (BCS) in Egyptian patients.

METHODS: Ninety-four Egyptian patients with confirmed primary Budd-Chiari syndrome were presented to the Budd-Chiari Study Group (BCSG) and admitted to the Tropical Medicine Department of Ain Shams University Hospital (Cairo, Egypt). Complete clinical evaluation and laboratory investigations, including a thrombophilia workup and full radiological assessment, were performed to determine underlying disease etiologies.

RESULTS: BCS was chronic in 79.8% of patients, acute or subacute in 19.1%, and fulminant in 1.1%. Factor V Leiden mutation (FVLM) was the most common etiological cause of disease (53.1%), followed by mutation of the gene encoding methylene tetrahydrofolate reductase (MTHFR) (51.6%). Current or recent hormonal treatment was documented in 15.5% of females, and BCS associated with pregnancy was present in 17.2% of females. Etiology could not be determined in 8.5% of patients. Males had significantly higher rates of MTHFR gene mutation and Behçet's disease, and females had significantly higher rates of secondary antiphospholipid antibody syndrome. A highly significant positive relationship was evident between the presence of Behçet's disease and inferior vena caval occlusion, either alone or combined with occlusion of the hepatic veins ($P < 0.0001$).

CONCLUSION: FVLM is the most common disease etiology and MTHFR the second most common in Egyptian BCS patients. BCS etiology tends to vary with geographic region.

Key words: Budd-Chiari syndrome; Epidemiological aspects; Etiology; Factor V Leiden mutation; Methylene tetrahydrofolate reductase gene mutation

Peer reviewers: Can Gonen, MD, Department of Gastroenterology, Kutahya State Hospital, 43100 Kutahya, Turkey; Dr. Herwig R Cerwenka, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

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INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare, but potentially life-threatening, hepatic disorder caused by obstruction of hepatic venous outflow at any level from the hepatic venules to the right atrium[1]. The exact prevalence of BCS is unknown, but has been estimated as 1 per 100 000 of the general population worldwide[2], with a higher prevalence being evident in developing countries such as China, India, Nepal, and South Africa[3]. BCS affects all races, usually during the third or fourth decade of life, and is more common in females[4]. The etiology of BCS can be classified as either primary, attributable to intrinsic intraluminal thrombosis or development of venous webs; or secondary, caused by intraluminal invasion by a parasite or a malignant tumor, or extraluminal compression by an abscess, cyst, or solid tumor[5].

At least one hereditary or acquired procoagulative disorder is present in 74% of BCS patients; intravascular thrombosis, mostly encountered in patients with primary myeloproliferative disorders (MPD), is the most common etiological factor[4]. Polycythemia vera is present in 10%-40% of BCS patients, whereas essential thrombocythemia and myelofibrosis are less common[6]. Hepatic vein thrombosis occurs in up to 12% of patients with paroxysmal nocturnal hemoglobinuria (PNH); this is the leading cause of mortality from BCS[7]. As many as 30% of BCS patients carry a Factor V Leiden mutation (FVLM); such a mutation is present in the majority of pregnancy- or oral contraceptive-related instances of hepatic vein thrombosis[8]. Patients with BCS may show nonspecifically decreased levels of Protein C, Protein S, and antithrombin, attributable to impaired hepatic synthesis, but levels less than 20% of normal suggest the presence of an inherited deficiency[9].

BCS is less common in western countries, but primary membranous obstruction of the inferior vena cava (IVC) is the most common cause of BCS in South Africa and Asia[10]. No underlying etiology can be identified in about 5% of BCS patients. Recent research has suggested that endothelial dysfunction and decreased fibrinolytic activity contribute to idiopathic instances of BCS[1].

The aim of the present epidemiological study was to describe the socio-demographic features, etiology, and risk factors for BCS in Egyptian patients.

MATERIALS AND METHODS

Study design and sampling

The present descriptive study enrolled 94 consecutive Egyptian patients between April 2009 and February 2011. Each patient was confirmed to have primary BCS, were introduced to the Budd-Chiari Study Group (BCSG), and then admitted to the Tropical Medicine Department of Ain Shams University Hospital (Cairo, Egypt). All patients provided written informed consent to participate in the study. Complete histories and clinical examinations were recorded for all patients.

Laboratory investigations included a complete blood count, a liver profile, and a coagulation profile. A thrombophilia workup, performed to determine the underlying etiology of BCS, included measurement of anticardiolipin antibodies, lupus anticoagulant, antinuclear antibodies, protein C, protein S, and antithrombin Ⅲ; and flow cytometry quantitating CD55 and CD59 levels to diagnose PNH. The possible presence of a FVLM was assessed in 64 patients, whereas the statuses of prothrombin and methylene tetrahydrofolate reductase (MTHFR) gene mutations were evaluated in 60 patients. *JAK2* mutational status was assessed, and/or a bone marrow biopsy exploring the possible presence of a myeloproliferative disorder was performed, in 62 patients.

Radiological assessment using abdominal Duplex ultrasonography (US) was performed to assess the patency of all of the hepatic veins (HVs), the portal vein, and the IVC. Abdominal magnetic resonance (MR) imaging, MR venography, or multislice computed tomography, was performed to confirm all diagnoses and to assess vascular anatomy.

Statistical analysis

Analysis of variance was used to compare the mean values of laboratory parameters. Multiple comparisons were performed using the least significant difference post-hoc test and results are presented as means and standard deviations (SDs). Non-parametric data were analyzed using the Kruskal-Wallis test and are presented as medians with interquartile ranges (IQRs). The chi-squared test and Fisher's exact test were used to test for differences among variables; the results are presented as percentages with corresponding *P* values. The unpaired Student's *t* test was used to test for differences in mean values of laboratory parameters between males and females, and the results are presented as means with SDs. Non-parametric data were analyzed using the Mann-Whitney *U* test and data are presented as medians with IQRs. Spearman's correlation coefficient was used to test the strength of associations between variables. All data were analyzed using SPSS version 15. A *P* value less than 0.05 was considered significant (S); a *P* value less than 0.01 was highly significant (HS); and a *P* value less than 0.001 was very highly significant (VHS).

RESULTS

We enrolled 94 Egyptian patients with BCS. There were 58 females (61.7%, mean age: 28.88 ± 9.08 years) and 36 males (38.3%, mean age: 28.64 ± 8.35 years). A total of 34 patients (36.2%) were from Cairo, 39 (41.5%) from the Delta, and 21 (22.3%) from Upper Egypt. A total of 75 patients (79.8%) had chronic BCS, 18 (19.1%) acute or subacute BCS, and 1 (1.1%) fulminant BCS. By the Child-Pugh classification, 30 patients (32%) were class A, 33 (35%) class B, and 31 (33%) class C.

Table 1 summarizes the clinical manifestations of our 94 patients. The most common symptoms were ab-

Table 1 Relevant clinical data on patients with Budd-Chiari syndrome (*n* = 94)

Symptom	*n* (%)
Abdominal enlargement	84 (89.4)
Abdominal pain	78 (83)
History of previous thrombosis	26 (27.7)
Recurrent abortion (females; 58)	14 (24.1)
Gastrointestinal bleeding	15 (15.9)
Recurrent oral and/or genital ulcers	13 (13.8)
Signs	
Ascites	80 (85.1)
Hepatomegaly	78 (83)
Splenomegaly	48 (51.1)
Lower limb edema	46 (48.9)
Dilated abdominal veins	39 (41.5)
Jaundice	36 (38.3)
Abdominal tenderness	34 (36.2)
Encephalopathy	29 (30.9)

Table 2 The primary etiologies of Budd-Chiari syndrome (*n* = 94)

Etiology		*n* (%)
FVLM (64 tested)	Homozygous	10 (15.6)
	Heterozygous	24 (37.5)
MTHFR (60 tested)	Homozygous	8 (13.3)
	Heterozygous	23 (38.3)
PGM (60 tested)	Homozygous	1 (1.7)
	Heterozygous	2 (3.3)
JAK2 (MPD) (62 tested)	+	18 (29)
Primary APA	+	16 (17)
Secondary APA	+	11 (11.7)
Behçet's disease	+	12 (12.8)
Protein C deficiency	+	4 (4.3)
Antithrombin Ⅲ deficiency	+	4 (4.3)
Protein S deficiency	+	1 (1.1)
PNH	+	2 (2.1)
Hormonal therapy (58 females)	+	9 (15.5)
Pregnancy-related (58 females)	+	10 (17.2)

FVLM: Factor V Leiden mutation; MTHFR: Methylene tetrahydrofolate reductase; PGM: Prothrombin gene mutation; *JAK2*: Janus tyrosine kinase-2; MPD: Myeloproliferative disorder; APA: Antiphospholipid antibody syndrome; PNH: Paroxysmal nocturnal hemoglobinuria.

dominal enlargement (89.4%) and abdominal pain (83%), and the most common clinical signs were ascites (85.1%), hepatomegaly (83%), and splenomegaly (51.1%).

Table 2 summarizes the disease etiologies of our 94 patients. The most common etiologies were FVLM mutation (53.1%) and MTHFR mutation (51.6%). A total of 15.5% of female patients were currently, or had recently, received hormonal treatment (oral or injectable) whereas 17.2% had BCS associated with pregnancy. The etiology of BCS was undefined in eight patients (8.5%). Forty-six patients (48.9%) demonstrated a single etiological factor, 29 (30.9%) two such factors, 8 (8.5%) three, and 3 (3.2%) four. There was no statistically significant relationship between disease pattern (acute, subacute, fulminant, or chronic) and etiology.

Table 3 shows the relationship between gender and BCS etiology. Males had significantly higher rates of

Table 3 Relationship between gender and etiology in patients with Budd-Chiari syndrome (*n* = 94)

Etiology		Gender *n* (%)		χ^2	*P* value	Sig
		Male	Female			
PC deficiency	+	1 (2.90)	3 (5.20)		1	NS
PS deficiency	+	1 (2.90)	0 (0.00)		0.38	NS
AT Ⅲ deficiency	+	2 (5.70)	2 (3.40)		0.63	NS
FVLM	Homozygous	5 (20.80)	5 (12.50)	1.77	0.18	NS
	Heterozygous	6 (25.00)	18 (45.00)			
PGM	Homozygous	0 (0.00)	1 (2.60)	1.54	0.21	NS
	Heterozygous	0 (0.00)	2 (5.10)			
MTHFR	Homozygous	6 (28.60)	2 (5.10)	8.41	0.01	HS
	Heterozygous	9 (42.90)	14 (35.90)			
JAK2 (MPD)	+	6 (28.60)	12 (29.30)	0.003	0.95	NS
Primary APA	+	5 (13.90)	11 (19.00)	0.4	0.52	NS
Secondary APA	+	1 (2.80)	10 (17.20)		0.05	S
Behçet's disease	+	11 (30.60)	1 (1.70)		< 0.001	VHS
PNH	+	1 (2.80)	1 (1.70)		1	NS

Sig: Significance; NS: Not significant; S: Significant; HS: Highly significant; VHS: Very highly significant; PC: Protein C; PS: Protein S; AT: Antithrombin; FVLM: Factor V Leiden mutation; PGM: Prothrombin gene mutation; MTHFR: Methylene tetrahydrofolate reductase; *JAK2*: Janus tyrosine kinase-2; MPD: Myeloproliferative disorder; APA: Antiphospholipid antibody syndrome; PNH: Paroxysmal nocturnal hemoglobinuria.

MTHFR gene mutation and Behçet's disease, whereas females had a significantly higher rate of secondary antiphospholipid syndrome (APA).

Table 4 summarizes the Duplex US findings. A total of 74.5% of all patients had HV occlusion, 3.2% IVC occlusion, and 17% both HV and IVC occlusion. PV thrombosis was present in 5.3% of patients. Figure 1 shows a representative color Doppler US of a patient with a dilated congested left HV and significant stenosis at the junction thereof with the IVC. Figure 2 is a representative B-mode sonograph showing occlusion of all hepatic veins, a slit-like IVC, and a markedly enlarged caudate lobe.

Table 5 summarizes the relationship between BCS etiology and radiological data. A highly significant positive relationship was evident between the presence of Behçet's disease and IVC occlusion, either isolated or combined with occlusion of the hepatic veins ($P < 0.0001$). No other significant relationship was evident between any etiological factor and radiological data.

DISCUSSION

Our current epidemiological study of 94 Egyptian patients with confirmed diagnoses of primary BCS describes the socio-demographic features, etiology, and risk factors for BCS. Previous reports found that BCS affects all races, usually during the third or fourth decade of life, and is slightly more common in females[4]. More females than males (61.7% *vs* 38.3%) were present in our population and mean patient age at the time of first visit was 28.64 ± 8.35 years for males and 28.88 ± 9.08 years for females.

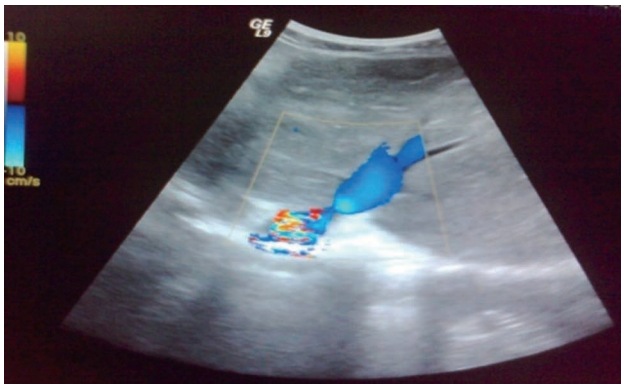

Figure 1 Representative color Doppler ultrasonograph showing a dilated congested left hepatic vein with significant stenosis at the junction thereof with the inferior vena cava.

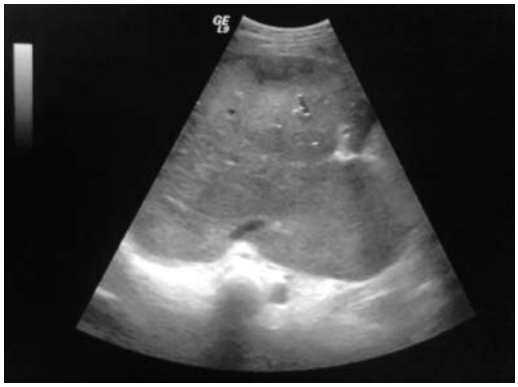

Figure 2 Representative B-mode sonograph showing occlusion of all hepatic veins, a slit-like inferior vena cava and a markedly enlarged caudate lobe.

BCS patients from different geographic regions tend to show distinct disease etiologies. In particular, thromboses are more common in western BCS patients, whereas development of venous webs is more frequent in Eastern and Japanese BCS patients[11]. Recent studies have shown that primary BCS should be regarded as a multifactorial disease, and that co-occurrence of several prothrombotic disorders leads to thrombosis at this uncommon location. A previous study found that several prothrombotic conditions were evident in at least 35% of BCS patients[12]. Thus, in the workup of such patients, identification of a single etiology should not indicate that additional etiologies should not be sought[1]. A previous study by Uskudar *et al*[13] in Turkey identified at least one etiological factor in 72% of patients, two in 18.6%, and three in 1.3%. In our present study of Egyptian patients, we identified at least one etiological factor in 48.9% of patients, two in 30.9%, three in 8.5%, and four in 3.2%.

Table 6 shows the prevalence rates of the major causal factors of primary BCS as reported in the current study and in that of Valla, conducted in 2009[1] and based on the work of Primignani *et al*[12,14], Patel *et al*[15], Colaizzo *et al*[16], and Kiladjian *et al*[17]. In the current study, FVLM was the most common cause of disease (53.1% of patients); 37.5% of patients were FVLM heterozygotes and 15.6% FVLM homozygotes. Similarly, a study in India by Mohanty *et al*[11] found that FVLM was the most common etiology of BCS (26% of patients). Identification of FVLM as a risk factor for venous thrombosis was a major advance in understanding the pathogenesis of BCS[8]; the possible presence of FVLM should be routinely investigated when BCS is diagnosed[11].

Table 4 Duplex ultrasound findings in patients with Budd-Chiari syndrome (n = 94)

Ultrasound finding		Patients n (%)
Hepatomegaly		78 (83)
Splenomegaly		48 (51.1)
No. of instances of occluded HV	0	3 (3.2)
	1	2 (2.1)
	2	12 (12.8)
	3	77 (81.9)
RHV occlusion		85 (90.4)
MHV occlusion		91 (96.8)
LHV occlusion		87 (92.6)
IVC occlusion		22 (23.4)
PV occlusion		5 (5.3)
Anatomical localization of thrombosis at presentation	Isolated HV	70 (74.5)
	Combined HV and IVC	16 (17)
	Isolated IVC	3 (3.2)
	Associated PV thrombosis	5 (5.3)

HV: Hepatic vein; RHV: Right hepatic vein; LHV: Left hepatic vein; MHV: Middle hepatic vein; PV: Portal vein; IVC: Inferior vena cava.

MTHFR gene mutation was the second most common etiology of BCS in the current study, evident in 51.6% of patients, of whom 38.3% were heterozygotes and 13.3% homozygotes. A previous study in China by Li *et al*[18] found that the prevalence of the MTHFR 677/T genotype in BCS patients was 45.12%, a figure similar to ours. Valla[19] reported that the *G20210A* prothrombin gene mutation (PGM), another recently discovered inherited marker, was less common in BCS patients than the other prothrombotic mutations mentioned above. In the present study, very few patients had a PGM (3.3% heterozygous, 1.7% homozygous).

MPD is the leading cause of BCS in western countries, foun d in 20%-53% of patients. Polycythemia vera was reported in 10%-40% of patients, whereas essential thrombocythemia and myelofibrosis were less common[6]. In the current study, we identified MPD in 29% of patients, of whom 28.5% had overt disease and 71.5% were of occult status. We confirmed the presence of such disorders by identification of the *JAK2 V617F* mutation. A recent study reported that clusters of dystrophic megakaryocytes evident in bone marrow biopsy samples were specific for MPD[20]. In our current study, a *JAK2* mutation was present in 29% of patients; this figure was lower than that previously reported by Colaizzo *et al*[21] (34.4%), Spivak[22] (40%), and Patel *et al*[15] (58.5%). These discrepancies may be attributable to the use of different DNA sources (peripheral blood granulocytes in

Table 5 Relationship between etiology and radiological findings [occluded vein(s)] in patients with Budd-Chiari syndrome

Etiology		Anatomical localization of thrombosis at presentation *n* (%)					χ^2	*P* value	Sig
		HV only	HV and PV	HV, PV and IVC	HV and IVC	IVC			
PC deficiency	+	3 (4.3)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	0.001	0.99	NS
PS deficiency	+	1 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.32	0.58	NS
AT Ⅲ Deficiency	+	3 (4.3)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	0.001	0.99	NS
FVLM	Homo	9 (17.0)	0 (0.0)	1 (12.5)	0 (0.0)	9 (17.0)	0.03	0.85	NS
	Hetero	19 (35.8)	2 (100.0)	3 (37.5)	0 (0.0)	19 (35.8)			
PGM	Homo	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0.52	0.47	NS
	Hetero	2 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (4.0)			
MTHFR	Homo	7 (14.0)	0 (0.0)	1 (14.3)	0 (0.0)	7 (14.0)	0.29	0.59	NS
	Hetero	19 (38.0)	0 (0.0)	3 (42.9)	1 (10.0)	19 (38.0)			
JAK2 (MPD)	+	16 (31.4)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	2.08	0.15	NS
Primary APA	+	9 (12.9)	0 (0.0)	1 (33.3)	6 (37.5)	0 (0.0)	2.58	0.11	NS
Secondary APA	+	9 (12.9)	0 (0.0)	0 (0.0)	1 (6.3)	1 (33.3)	0.02	0.88	NS
Behçet's disease	+	2 (2.9)	0 (0.0)	2 (66.7)	7 (43.8)	1 (33.3)	21.25	< 0.0001	HS
PNH	+	2 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.62	0.43	NS

Sig: Significance; NS: Not significant; HS: Highly significant; HV: Hepatic vein; PV: Portal vein; IVC: Inferior vena cava; PC: Protein C; PS: Protein S; AT: Antithrombin; FVLM: Factor V Leiden mutation; PGM: Prothrombin gene mutation; MTHFR: Methylene tetrahydrofolate reductase; *JAK2*: Janus tyrosine kinase-2; MPD: Myeloproliferative disorder; APA: Antiphospholipid antibody syndrome; PNH: Paroxysmal nocturnal hemoglobinuria.

Table 6 Etiologies of Budd-Chiari syndrome described in previous studies and in the current work

Etiology		Reported rate[1]	Rate in current study
PC deficiency		20%	4.30%
PS deficiency		7%	1.10%
AT Ⅲ deficiency		5%	4.30%
FVLM	Homozygous	NA	15.60%
	Heterozygous	20%	37.50%
PGM	Homozygous	NA	1.70%
	Heterozygous	7%	3.30%
MTHFR	Homozygous	NA	13.30%
	Heterozygous	NA	38.30%
JAK2-positive (MPD)		50%	29%
Primary APA		10%	17%
Secondary APA			11.70%
Behçet's disease		5%	12.80%
PNH		2%	2.10%
Hormonal therapy (58 females)		50%	15.50%
Non-identified etiology		5%	8.50%

NA: Not available; PC: Protein C; PS: Protein S; AT: Antithrombin; FVLM: Factor V Leiden mutation; PGM: Prothrombin gene mutation; MTHFR: Methylene tetrahydrofolate reductase; *JAK2*: Janus tyrosine kinase-2; MPD: Myeloproliferative disorder; APA: Antiphospholipid antibody syndrome; PNH: Paroxysmal nocturnal hemoglobinuria. [1]Reported by Valla in *2009*[1] according to Primignani *et al*[12,14], Patel *et al*[15], Colaizzo *et al*[16], and Kiladjian *et al*[17].

our present study and bone marrow films in the work of Patel *et al*[15]) or to differences in patient populations.

APA syndrome was one of the major causes of BCS in the current study, being present in 28.7% of patients; 17% with primary APA and 11.7% with APA secondary to systemic lupus erythematosus (SLE). A previous report found that 3%-17% of patients with venous thrombosis showed measureable levels of antiphospholipid antibodies[23]. In the current study, we were surprised to find Behçet's disease in 12.8% of our patients. This disease is common in certain countries, and Uskudar *et al*[13] reported that the disease was present in 9% of Turkish patients with BCS. Thus, we suggest that patients with BCS should always be screened for Behçet's disease. Such a diagnosis can be difficult, even in male patients from countries where the disease is endemic; use of the diagnostic criteria of the International Study Group of Behçet's Disease may be helpful[24].

The prevalence of primary deficiencies in Protein C, Protein S, and Antithrombin Ⅲ in BCS patients is difficult to determine, for several reasons. Firstly, the liver synthesizes these inhibitors of coagulation, and liver dysfunction related to BCS thus induces non-specific falls in the plasma levels of the inhibitors. Secondly, diagnosis of any primary deficiency is based on measurement of plasma protein level, because most mutations in the relevant genes are unique, rendering diagnosis using molecular biology techniques alone difficult. Finally, complete family screening is recommended to differentiate between inherited and false instances of deficiencies in Proteins C and S, but this is usually impractical. Thus, the question of whether a primary deficiency in the expression of one of the proteins mentioned above exists will remain unanswered in most instances[19]. However, a level below 20% of the normal value suggests the presence of an inherited deficiency[2]. In the present study, we identified deficiencies in Antithrombin Ⅲ, Protein C, or Protein S in 4.3%, 1.1%, and 4.3% of our patients, respectively. Mohanty *et al*[11] studied BCS patients in India, and found deficiencies in Antithrombin Ⅲ, Protein C, and Protein S in 3.8%, 13.2%, and 5.7% of patients, respectively. Uskudar *et al*[13] worked with BCS patients in Turkey, and reported deficiencies in Antithrombin Ⅲ, Protein C, and Protein S in 3%, 9%, and 7% of patients, respectively.

A previous study found that hepatic vein (HV) thrombosis occurred in up to 12% of patients with PNH; this is the leading cause of mortality in BCS patients[7]. In the present study, PNH was documented in 2.1% of patients, similar to what was previously reported by Valla[2] (2%). Membranous obstruction of the IVC is the most

common cause of BCS in South Africa and Asia, but is less common in western countries, where the condition is thought to be a consequence of IVC thrombosis[10]. One of the most striking results of the present study was that development of IVC venous webs was not responsible for any instance of BCS. In contrast, Uskudar *et al*[13] reported that venous webs were one of the most common etiological factors of BCS in Turkey.

Analysis of the relationship between gender and BCS etiology indicated that females were more likely to have secondary APA syndrome than males (17.2% *vs* 2.8%), but that males were more likely to have MTHFR gene mutations (71.5% *vs* 41%) and also had significantly higher rates of Behçet's disease (30.6% *vs* 1.7%). We also found that potential risk factors for BCS included previous thrombosis elsewhere in the body (27.5%), recent pregnancy (17.2% of females), and hormonal therapy (15.5% of females). Each of our BCS patients on hormonal therapy or with pregnancy-related BCS displayed an additional etiological factor. Thus, many women with BCS who are pregnant or users of oral contraceptives appear to have additional thrombophilic conditions[25].

In the present study, we found isolated HV involvement in 74.5% of patients, isolated IVC involvement in 3.2%, and combined HV and IVC occlusion in 17%. In a previous study of 237 patients with BCS, Darwish Murad *et al*[26] reported obstruction of the HV, the IVC, and both veins, in 62%, 7%, and 31% of patients, respectively. The high rate of IVC thrombosis found in the cited study was associated with the high prevalence of Behçet's disease. These findings were similar to those of the Turkish study by Uskudar *et al*[13], who reported HV involvement in 77% of patients and IVC involvement in 53%. Together, the results indicate that the etiology of BCS may be related to the site of obstruction.

Previous studies have indicated that the site of the lesion varies among patients of different countries. In particular, IVC involvement is more common in Nepal, South Africa, China, India, and Japan, whereas HV involvement is more common in the west[4]. In the current study, we assessed the extent of association between BCS etiology and the anatomical site of thrombosis as revealed using different imaging modalities. Our results indicate a significant association between the presence of Behçet's disease and the anatomical site of thrombosis. In particular, Behçet's disease was diagnosed in 12 patients, 9 (75%) with combined IVC and HV thrombosis, 2 (16.7%) with isolated HV thrombosis, and 1 (8.3%) with isolated IVC thrombosis. These results are similar to those reported by Bayraktar *et al*[27], who found that 85.7% of Behçet's disease patients had combined IVC and HV thrombosis and 14.2% had isolated HV thrombosis.

An increasing number of studies support the concept that thrombosis is site-specific, depending on the underlying prothrombotic disorder[28]. For example, MPD is more common in BCS patients than in those with portal vein thrombosis; Factor V Leiden mutation is more strongly associated with BCS than is portal vein thrombosis; and the *G20210A* prothrombin gene mutation is more strongly associated with portal vein thrombosis than is BCS. Further site-specificity may be in play within the hepatic venous outflow tract per se. Indeed, the FVLM appears to be particularly common in patients with IVC obstruction[8] and the use of oral contraceptives, or pregnancy, is specifically associated with hepatic vein involvement[29].

In conclusion, our study of Egyptian BCS patients indicated that FVLM was the most common etiology, MTHFR the second most common, and that BCS commonly occurs during the third decade of life and is more prevalent in females. Comparison of our data with those of previous studies indicates that BCS patients from different geographic regions tend to differ in terms of etiology.

COMMENTS

Background

Budd-Chiari syndrome (BCS) results from hepatic venous outflow obstruction at any level, from the hepatic venules to the right atrium, and is estimated to affect every 1 out of 100 000 subjects in the general population worldwide. BCS affects all races, usually during the third or fourth decade of life, and is slightly more common in females.

Research frontiers

BCS patients from different geographic regions tend to vary in terms of disease etiology. Previous reports have indicated that thromboses are more common in the West and venous webs more frequent in the East and Japan. In the present study of Egyptian patients, the authors demonstrate that Factor V Leiden mutation (FVLM) was the most common etiology, methylene tetrahydrofolate reductase gene mutation the second most common, and that the disease was of multiple etiologies in 42.6% of patients.

Innovations and breakthroughs

This is the first epidemiological study to examine the socio-demographic features, etiology and risk for development of BCS in Egyptian patients.

Applications

In the workup for determining the etiology of BCS, identification of a single cause should not preclude investigation of additional contributing factors. The presence of an underlying hypercoagulable state should be investigated in all patients.

Terminology

FVLM is the most common cause of inherited thrombophilia. The mutated factor Va is resistant to degradation by protein C, a natural anticoagulant. The rise in the level of undegraded factor Va over time increases the risk of uncontrolled thrombin and thrombus formation. Methylene tetrahydrofolate reductase is an enzyme active in re-methylation of homocysteine. Deficiencies in the enzyme cause hyperhomocysteinemia; this condition compromises the functions of the anticoagulant and fibrinolytic systems.

Peer review

The authors investigated the epidemiology of Budd-Chiari syndrome in Egyptian patients. The results indicate that BCS is most common during the third decade of life and is more often diagnosed in females than in males. In Egyptian patients, FVLM was the most common etiology, methylene tetrahydrofolate reductase gene mutation the second most common, and multiple etiologies were present in 42.6% of patients. Thus, in workup for determination of the etiology of BCS, identification of a single cause should not preclude investigation of additional relevant factors. The presence of an underlying hypercoagulable state should be investigated in all BCS patients. The present study provides valuable new information on BCS.

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BRIEF ARTICLE

miR-93 suppresses proliferation and colony formation of human colon cancer stem cells

Xiao-Feng Yu, Jian Zou, Zhi-Jun Bao, Jie Dong

Xiao-Feng Yu, Jian Zou, Zhi-Jun Bao, Jie Dong, Department of Gastroenterology, Huadong Hospital, Fudan University, Shanghai 200040, China
Author contributions: Yu XF and Zou J contributed equally to this work; Zou J designed the research; Zou J, Yu XF, Bao ZJ and Dong J performed the majority of the experiments; Dong J, Bao ZJ and Zou J analyzed data; and Yu XF and Zou J wrote the paper.

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Correspondence to: Dr. Jian Zou, Department of Gastroenterology, Huadong Hospital, Fudan University, 221 Yan an Xi Road, Shanghai 200040, China. apollozou@hotmail.com
Telephone: +86-21-62483180 Fax: +86-21-62484981
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Abstract

AIM: To identify differentially expressed microRNAs (miRNAs) in human colon cancer stem cells (SW1116csc) and study their function in SW1116csc proliferation.

METHODS: SW1116csc were isolated from the human colon cancer cell line, SW1116 and cultured in serum-free medium. A miRNA microarray was used to detect differential expression profiles of miRNAs in SW1116csc and SW1116 cells. Real-time quantitative polymerase chain reaction (PCR) was performed to verify the differential expression of candidate miRNAs obtained from the microarray. Target mRNAs of differentially expressed miRNAs were predicted with target prediction tools. miRNA expression plasmids were transfected into SW1116csc using Lipofectamine 2000 reagent. Cell proliferation curves were generated with trypan blue staining, and the colony formation rate of transfected cells was measured with the soft agar colony formation assay. Expression of target mRNAs and proteins from differentially expressed miRNAs were detected using reverse transcription (RT)-PCR and western blotting.

RESULTS: Compared with expression in SW1116 cells, 35 miRNAs (including hsa-miR-192, hsa-miR-29b, hsa-miR-215, hsa-miR-194, hsa-miR-33a and hsa-miR-32) were upregulated more than 1.5-fold, and 11 miRNAs (including hsa-miR-93, hsa-miR-1231, hsa-miRPlus-F1080, hsa-miR-524-3p, hsa-miR-886-3p and hsa-miR-561) were downregulated in SW1116csc. The miRNA microarray results were further validated with quantitative RT-PCR. miR-93 was downregulated, and its predicted mRNA targets included BAMBI, CCND2, CDKN1A, HDAC8, KIF23, MAP3K9, MAP3K11, MYCN, PPARD, TLE4 and ZDHHC1. Overexpressed miR-93 significantly inhibited cell proliferation and colony formation by SW1116csc. Furthermore, miR-93 negatively regulated the mRNA and protein levels of HDAC8 and TLE4.

CONCLUSION: Some miRNAs were differentially expressed during differentiation of SW1116csc into SW1116 cells. miR-93 may inhibit SW1116csc proliferation and colony formation.

Key words: miR-93; Stem cell; Colon cancer; Expression profile

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INTRODUCTION

Cancer stem cells (CSC) are a sub-population of cancer cells that possess characteristics associated with normal

stem cells, such as self renewal and the ability to differentiate into multiple cell types. CSCs are tumorigenic, in contrast to most cancer cells, which are thought to be non-tumorigenic. The CSC hypothesis infers that if CSCs were eliminated, the tumor would simply regress due to differentiation and cell death. Since the identification and characterization of CSCs in hematological malignancies, an increasing number of studies have described CSCs in solid tumors such as ovarian[1], colon[2], lung[3], breast[4], liver[5], melanoma[6] and pancreatic[7] tumors, raising the possibility that the CSC hypothesis applies to most neoplastic systems. CSCs are the most critical tumor cell type because they are capable of self renewing, differentiating, and maintaining tumor growth and heterogeneity, and thus play an important role in tumorigenesis and therapeutics.

MicroRNAs (miRNAs) are small noncoding RNAs that posttranscriptionally regulate gene expression. Mature miRNAs can specifically bind to 3' untranslated regions of target cellular mRNA, in turn triggering mRNA degradation or inhibition of translation[8]. To date, thousands of miRNAs have been identified in the human genome, where they act as key regulators of a wide variety of biological processes including development, cell differentiation, apoptosis, metabolism, and signal transduction[9]. Consequently, abnormal patterns of miRNAs have been found in various human diseases, most notably cancer[10]. Recent findings indicate that alterations in the expression of several miRNAs are present in human cancers, suggesting potential roles in carcinogenesis[11]. Expression of some miRNAs, such as let-7 in human lung cancers[12], the miR-15a/miR-16-1 cluster in chronic lymphocytic leukemia[13], and neighboring miR-143/miR-145 in colorectal neoplasia and breast cancer[14,15], is reduced, suggesting potential tumor suppressor activity. In contrast, other miRNAs, such as the miR-17-92 cluster in human B-cell lymphomas[16] and miR-155/BIC in Hodgkin lymphoma[17], are overexpressed, suggesting oncogenic potential.

miRNAs are emerging as important regulators of cellular differentiation and proliferation and have been implicated in the etiology of a variety of cancers. However, the role of miRNAs in human colon cancer stem cells remains poorly understood. In this study, we screened and identified differential miRNA expression profiles in colon cancer stem cells using a miRNA microarray and studied the function of differentially expressed miRNAs in the proliferation of colon cancer stem cells.

MATERIALS AND METHODS

Cell culture

The human colon cancer cell line (SW1116) was purchased from Cell Bank, Shanghai Institute of Life Science, Chinese Academy of Sciences and maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Gibco BRL, United States), penicillin G (1×10^5 U/L), and streptomycin (100 mg/L) in a 50 mL/L CO_2 atmosphere at 37 ℃. SW1116csc (Figure 1) were isolated previously[18] and maintained in serum-free DMEM/F12 medium supplemented with human recombinant epidermal growth factor (20 μg/L; Invitrogen), human recombinant basic fibroblast growth factor (20 μg/L; Invitrogen), L-glutamine (2 mmol/L), insulin (4 U/L), penicillin G (1×10^5 U/L), and streptomycin (100 mg/L).

Microarray experiments

Total RNA was extracted from SW1116csc and SW1116 cells using Trizol reagent (Invitrogen). The quantity was measured on a spectrophotometer (Ultraspec 2000, Pharmacia Biotech), and the integrity of the RNA was checked on a 1% agarose gel. Low-molecular-weight RNA (< 200 nt) was separated from the total RNA using mirVana miRNA purification columns (Ambion, Austin, TX, United States) for miRCURY™ array microarray (v.13.0) (Exiqon) analysis according to the manufacturer's protocol, which uses the LNA probe to detect miRNA expression. LNA is a high-affinity RNA analog with a bicyclic furanose unit locked in an RNA-mimicking sugar conformation, which results in unprecedented hybridization affinity toward complementary single-stranded RNA molecules. This makes LNA-modified DNA probes ideally suited for RNA targeting. Microarray images were analyzed using an Axon GenePix 4000B microarray scanner (Molecular Devices), and GenePix pro V6.0 (Molecular Devices) was used to read the raw intensity of the image. Average values of the replicate spots of each miRNA were background subtracted, normalized, and subjected to further analysis. Normalization was performed using the signal of U6 snRNA on the chip, and the cutoff value was set to 1000.

Real-time quantitative reverse transcription polymerase chain reaction

For polymerase chain reaction (PCR) of miRNAs, cDNA synthesis was performed with 100 ng total RNA using a SuperScript Ⅲ Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol. The sequences of primers and probes specific for individual miRNAs and the U6 RNA internal control are shown in Table 1. PCR consisted of denaturation at 95 ℃ followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 3 s. Thermal cycling and fluorescent monitoring were performed on an ABI 7700 sequence analyzer (PE Biosystems). Relative expression (RE) of the sample gene was calculated using the ΔΔCT method using the formula RE = 2ΔΔCT where CT = PCR cycle in which the sample fluorescent intensity exceeds that of background, ΔCT sample = CT sample - CT U6 sample, ΔCT control = CT control - CT U6 control, and ΔΔCT = ΔCT sample - ΔCT control.

miRNA target prediction

A list of potential miRNA targets was created by combining predicted targets from the mirBase, TargetScan, miRanda, and PicTar target prediction algorithms. Po-

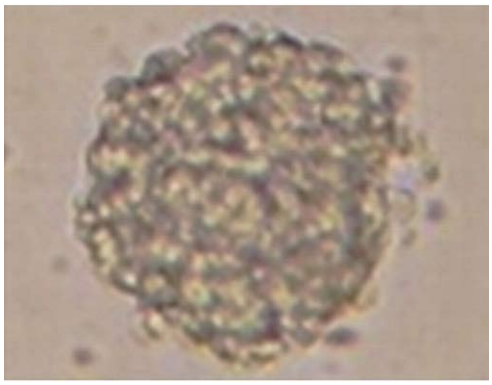

Figure 1 Human colon cancer stem cells grow into clonally derived spheres in serum-free medium.

Table 1 Sequences of primers used in quantitative reverse transcription polymerase chain reaction

Primer	Primer sequence (5'→3')
hsa-miR-93-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGCTACCT
hsa-miR-93-F	ACACTCCAGCTGGGCAAAGTGCTGTTCGTGC
hsa-miR-1231-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGGCAGCT
hsa-miR-1231-F	ACACTCCAGCTGGGGTGTCTGGGCGGAC
hsa-miR-32-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGTGCAAC
hsa-miR-32-F	ACACTCCAGCTGGGTATTGCACATTACTAA
hsa-miR-33a-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGTGCAAT
hsa-miR-33a-F	ACACTCCAGCTGGGGTGCATTGTAGTTGC
hsa-miR-194-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGTCCACA
hsa-miR-194-F	ACACTCCAGCTGGGTGTAACAGCAACTCCA
hsa-miR-215-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGGTCTGT
hsa-miR-215-F	ACACTCCAGCTGGGATGACCTATGAATTG
hsa-miR-29b-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGAACACT
hsa-miR-29b-F	ACACTCCAGCTGGGTAGCACCATTTGAAATC
hsa-miR-192-RT	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGGGCTGT
hsa-miR-192-F	ACACTCCAGCTGGGCTGACCTATGAATTG
URP	TGGTGTCGTGGAGTCG
U6	F-CTCGCTTCGGCAGCACA R-AACGCTTCACGAATTTGCGT

URP: Universal reporter primer; RT: Reverse transcription.

tential targets were chosen based on gene function, the number of predicted target sites, and target prediction by multiple algorithms.

Plasmid constructs and transfection

The human miR-93 precursor (CUGGGGGCUC-CAAAGUGCUGUUCGUGCAGGUAGUGUGAU-UACCCAACCUACUGCUGAGCUAG CACUUCCC-GAGCCCCCGG) was reverse-transcribed and cloned into pSilencer 4.1 (Ambion). The correct plasmid was named pS-miR-93, and the control plasmid (pS-Neg) consisted of a scrambled sequence (Ambion). The sequence of the pS-miR-93 plasmid was confirmed with DNA sequencing. SW1116csc were seeded at 1×10^5 cells/well in a 12-well plate (BD Bioscience). After 48 h, the cells were transfected with 1 μg/well of pS-miR-93 or pS-Neg using 4 μL Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer's protocol. After 24 h, cells were harvested, and total mRNAs and proteins were extracted.

Cell proliferation assay

SW1116csc and pS-miR-93-transfected SW1116csc (SWt) cells were seeded at a density of 1×10^4 in 35-mm Petri dishes. Cultured cells stained with trypan blue were observed and counted in triplicate over 6 wk.

Colony formation in soft agar

Cells were disassociated, suspended in medium containing 0.3% agar and 10% serum, and plated onto a bottom layer containing 0.6% agar. The cells were plated at a density of 3×10^4 cells/6-cm dish, and the number of colonies > 0.5 mm in diameter was counted 14 d later.

RT-PCR

Total RNA was extracted, and cDNA was synthesized as above. PCR was performed in a 50-μL volume containing 1 μL Taq DNA polymerase (Hua Mei Co.), 5 μL 10 × buffer, 1 μL dNTPs (10 mmol/L), 2 μL primers (10 μmol/L), and 1 μL cDNA (0.1 μg/μL). Amplification was performed in a thermal cycler (Perkin-Elmer Co., United States). The PCR products were analyzed and photographed with a gel documentation system (FR-200, Shanghai Fu Ri Bio Co.). For HDAC8, the forward primer used was F: 5'-TGGGCAGTCGCT-GGT-3', and the reverse primer was R: 5'-GTGGCT-GGGCAGTCATAA-3' (product size: 285 bp). For TLE4, F: 5'-ACTCCCACTGCTGTGCAAG-3', R: 5'-GTTTCTGGCACAATGCACAG-3' (194 bp). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F: 5'-TTGGTATCGTGGAAGGACTCA-3', R: 5'-TGTCATCATATTTGGCAGGTT-3' (270 bp). RT-PCR primers were synthesized by Shanghai Sangon Co.

Western blotting

Cell extracts were prepared in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin) and centrifuged at 12 000 × *g* at 4 ℃. The total protein concentration was measured using a BCA assay. Cellular extracts containing 50 μg total proteins were subjected to 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen). Blots were probed at 4 ℃ overnight with primary antibodies in 5% milk/TBST. The antibodies for western blotting were HDAC8, TLE4 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Table 2 Differentially expressed miRNAs in colon cancer stem cells

miRNA name	SW1116csc	SW1116	Fold
	image intensity	image intensity	change
hsa-miR-93	0.46	8.13	0.06
hsa-miR-1231	0.02	0.08	0.22
hsa-miRPlus-F1080	0.01	0.05	0.23
hsa-miR-524-3p	0.05	0.16	0.34
hsa-miR-886-3p	3.34	8.37	0.40
hsa-miR-561	0.02	0.04	0.45
hsa-miR-497	0.01	0.03	0.51
hsa-miR-23a	11.92	22.51	0.53
hsa-miR-886-5p	2.89	5.18	0.56
hsa-miRPlus-A1087	0.47	0.79	0.59
hsa-miRPlus-E1170	30.71	49.93	0.61
hsa-miRPlus-E1102	0.42	0.28	1.50
hsa-miR-138-2	0.39	0.26	1.50
hsa-miR-31	0.45	0.30	1.52
hsa-miR-17	2.50	1.64	1.52
hsa-miR-374a	5.40	3.55	1.52
hsa-miR-424	3.38	2.21	1.53
hsa-miRPlus-F1181	0.64	0.42	1.53
hsa-miRPlus-E1238	0.28	0.18	1.54
hsa-miR-542-3p	0.38	0.24	1.56
hsa-miR-582-3p	0.27	0.17	1.57
hsa-miR-584	0.28	0.17	1.61
hsa-miR-522	0.29	0.18	1.64
hsa-miR-590-5p	0.41	0.25	1.67
hsa-miR-487b	2.88	1.72	1.68
hsa-miR-29c	1.53	0.89	1.72
hsa-miR-96	2.39	1.34	1.78
hsa-miR-193a-3p	22.24	12.50	1.78
hsa-miR-20a	0.74	0.41	1.79
hsa-miR-301a	2.28	1.27	1.80
hsa-miRPlus-E1106	0.56	0.31	1.82
hsa-miR-30e	1.21	0.65	1.86
hsa-miR-874	0.20	0.10	1.99
hsa-miR-19a	17.38	8.71	2.00
hsa-miR-519a	1.73	0.77	2.24
hsa-miRPlus-A1065	0.30	0.13	2.32
hsa-miR-521	0.94	0.40	2.36
hsa-miR-876-5p	0.23	0.10	2.37
hsa-miR-493	0.28	0.11	2.53
hsa-miR-101	4.92	1.28	3.85
hsa-miR-32	1.89	0.35	5.48
hsa-miR-33a	3.97	0.64	6.16
hsa-miR-194	0.79	0.11	7.11
hsa-miR-215	0.97	0.11	9.09
hsa-miR-29b	3.00	0.33	9.20
hsa-miR-192	1.47	0.14	10.67

Statistical analysis

All data are presented as the mean ± SE. SPSS for Windows (version 15.0, SPSS Inc., United States) was used for statistical analysis. P values < 0.05 were considered statistically significant.

RESULTS

miRNA expression profiling with miRNA microarray

We used an miRNA microarray to evaluate miRNA expression profiles of colon cancer stem cells (SW1116csc) and differentiated colon cancer cells (SW1116). Analysis of data derived from the miRCURY™ array microarray indicated that compared with SW1116 cells, there were 46 differentially expressed miRNAs in SW1116csc. Among them, 35 miRNAs (including hsa-miR-192, hsa-miR-29b, hsa-miR-215, hsa-miR-194, hsa-miR-33a and hsa-miR-32) were overexpressed more than 1.5-fold and 11 miRNAs (including hsa-miR-93, hsa-miR-1231, hsa-miRPlus-F1080, hsa-miR-524-3p, hsa-miR-886-3p and hsa-miR-561) were downregulated (Table 2). Expression of miR-93 in SW1116csc was decreased by 16.7 times, and we chose this miRNA for further study.

Validation of microarray results using quantitative PCR

To validate our microarray results, we performed quantitative PCR with some of the miRNAs that were differentially expressed in SW1116csc according to the microarray data. We selected miR-93, miR-1231, miR-32, miR-33a, miR-194, miR-215, miR-29b and miR-192 for quantitative PCR. The quantitative PCR results indicated that the expression of miR-93 and miR-1231 was decreased, whereas the expression of miR-32, miR-33a, miR-194, miR-215, miR-29b and miR-192 was significantly increased in SW1116csc cells (Figure 2). The quantitative PCR result was in agreement with that of the microarray.

Prediction of miR-93 target gene

By combining predicted targets generated with the mirBase, TargetScan, miRanda and PicTar target prediction algorithms, potential targets of miR-93 were chosen. The stem relevant mRNA targets included BAMBI, CCND2, CDKN1A, HDAC8, KIF23, MAP3K9, MAP3K11, MYCN, PPARD, TLE4, and ZDHHC1.

Inhibition of cell proliferation and colony formation of SW1116csc by pS-miR-93 transfection

We tested for differences in the proliferation rate between SW1116csc, pS-Neg-transfected SW1116csc (SWcon), and pS-miR-93-transfected SW1116csc (SWt). The cells were examined from week 1 to week 7 after seeding. As shown in Figure 3, there was a difference in the growth rate between SWcon and SWt cells. SWt cells grew slowly and showed growth inhibition after week 3. The self-renewing capacity of SWt cells was also examined with the colony formation assay. When plated at a density of 100 cells/well, SW1116csc and SWcon cells generated a greater mean number of tumor spheres (72.3 ± 4.2 and 64.5 ± 3.6, respectively) than did SWt cells (19.6 ± 2.1) (Figure 4).

Overexpression of miR-93 reduces the mRNA and protein levels of HDAC8 and TLE4

To provide additional evidence for the role of miR-93 in inhibition of SW1116csc proliferation and colony formation, we examined the effect of miR-93 on the expression of HDAC8 and TLE4. As shown in Figure 5, miR-93 mimics significantly attenuated the mRNA and protein levels of HDAC8 and TLE4.

DISCUSSION

Mature functional miRNAs of approximately 22 nucleo-

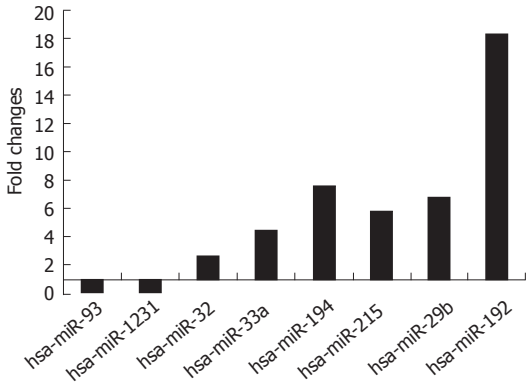

Figure 2 Expression levels of select miRNAs in SW1116csc as measured with quantitative reverse transcription polymerase chain reaction.

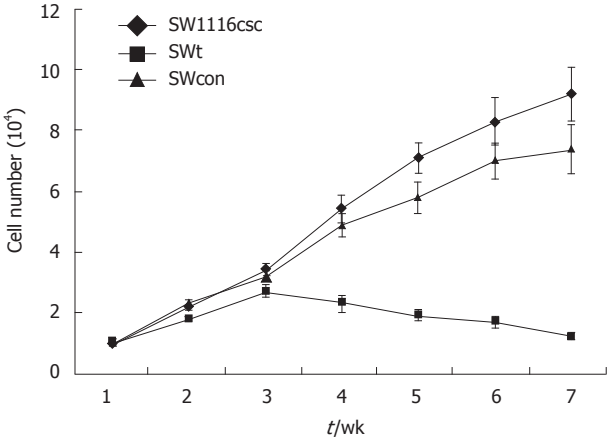

Figure 3 Growth curves of SW1116csc, SWcon cells, and SWt cells. mean ± SD are shown.

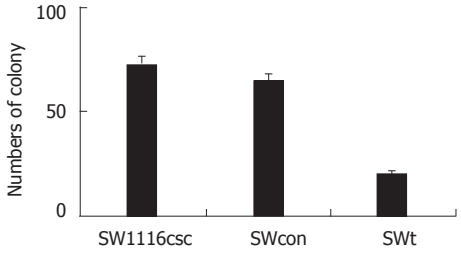

Figure 4 Colony formation after incubation of 100 separate cells for 14 d. mean ± SD are shown.

tides that are generated from long primary miRNA transcripts control gene expression at the posttranscriptional level by degrading or repressing target mRNAs. Some miRNAs aberrantly expressed in cancer have been well documented[19,20]. These miRNAs regulate the expression of signaling molecules such as cytokines, growth factors, transcription factors, and proapoptotic and antiapoptotic molecules. Recently, miRNAs were found to play a role in the differentiation of stem cells. Proper regulation of differentiation of stem cells is crucial to normal development and the avoidance of cancer[21]. However, the differential expression of miRNAs in human colon cancer

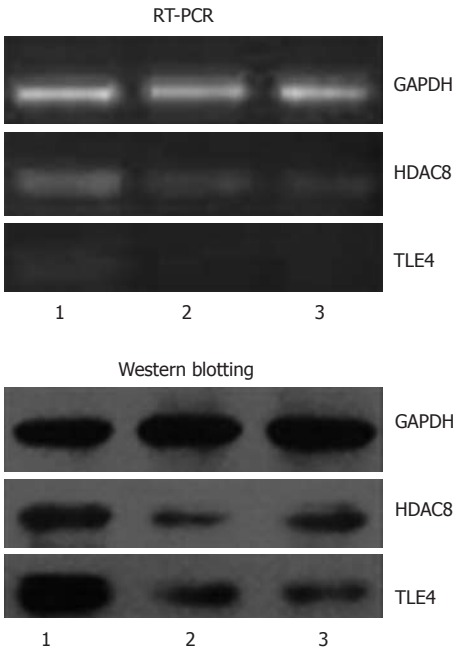

Figure 5 Expression of HDAC8 and TLE4 mRNA and protein in SW1116csc, SW1116 cells and SWt cells. 1: SW1116csc; 2: SW1116 cells; 3: SWt cells. Glyceraldehyde-3-phosphate dehydrogenase was evaluated as an internal control. RT-PCR: Reverse transcription polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

stem cells has not been addressed.

Here we analyzed the differential expression of miRNAs in human colon cancer stem cells and differentiated colon cancer cells. We identified 46 differentially expressed miRNAs in SW1116csc. We observed miRNAs that were upregulated and others that were downregulated. The miR-93 expression level was significantly lower in SW1116csc than in SW1116 cells, indicating that miR-93 may be involved in the development or replication of colon CSCs.

One of the key characteristics of stem cells is their ability to divide for long periods of time when most other cells are quiescent[22]. Because the function of miR-93 in colon CSCs was unknown, we identified the biological effects of miR-93 on colon CSCs when its expression in these cells was upregulated by examining the role of miR-93 on cellular growth and proliferation of transfected SW1116csc. Overexpression of miR-93 consistently inhibited cell proliferation and colony formation of SW1116csc. To the best of our knowledge, this study is the first to demonstrate the differential expression of miRNAs in colon CSCs and the effect of miR-93 on colon CSCs. However, the underlying mechanisms of these effects are not completely understood. Using miRNA target prediction tools and RT-PCR, we found that inhibition of overexpressed miR-93 probably affects cell proliferation and colony formation of colon cancer stem cells by targeting HDAC8 and TLE4.

Histone deacetylase (HDAC) enzymes are a family of proteins with complex, multifunctional roles *in vivo*, including transcriptional regulation, regulation of tubulin

and cytoskeletal function, control of cardiac cell growth, regulation of thymocyte development, and facilitation of DNA repair[23]. HDAC enzymes function in part to control the acetylation state of nucleosomal histones, thereby regulating transcription. More recently, however, it has been shown that HDAC enzymes have many non-histone acetylation targets as well, including tubulin, heat-shock proteins, and a variety of transcription factors such as p53 and NF-kB subunit p65[24,25].

HDAC8 is a newly identified HDAC that was cloned and characterized in 2000. It is a novel marker of smooth muscle differentiation and is expressed at low levels in normal white blood cells but overexpressed in some malignant hematological cell lines[26]. cDNA microarray analysis suggests that there is differential expression of HDAC8 between mammary tumors and normal lactating mammary glands and that it may play a key regulatory role in mammary gland tumorigenesis[27]. Here we found that the expression level of HDAC8 in human colon cancer cells was high. Thus, accumulating data increase our understanding of the role of abnormally elevated HDAC8 activity in the pathogenesis of tumors.

Groucho (Gro) or Transducin-like Enhancer of Split (TLE) proteins constitute a family of highly conserved cofactors for transcription. They act as non-DNA-binding corepressors and are recruited to promoters *via* interaction with a DNA binding partner. Gro corepressor proteins interact with multiple transcription factors and thus affect different signaling pathways[28]. They contact histones and recruit HDACs, thereby altering local chromatin structure. Gro corepressor proteins confer repressing functions on binding partners with an activating potential[29].

The Gro/TLE family of corepressors interacts with at least five families of transcription factors and plays critical roles in Drosophila and vertebrate development. During B-lymphocyte differentiation, TLEs mediate the repressive effect of Pax5 *via* recruitment by Pu.1, limiting alternative cell fates[30]. However, the ability of Gro/TLEs to interact with other signaling pathways suggests a potentially broader role for Gro/TLEs in both normal and malignant hematopoiesis. Members of the Gro/TLE family of corepressors bind to all known Tcf/LEF complexes and act as inhibitors of Wnt/β-catenin signaling[31,32], a pathway implicated in expansion and self-renewal of the hematopoietic stem cell compartment[33]. Similarly, Gro/TLEs inhibit NF-κB signaling[34], a pathway constitutively activated in acute myeloblastic leukemia and thought to play an important role in hematopoietic cell proliferation, survival, and chemoresistance. *Gro/TLE* gene family members are also key effectors of Notch signaling, a pathway implicated in HSC fate determination and self-renewal[35]. The high expression of TLE4 in human colon CSCs indicates interactions with Wnt, NF-κB, or Notch signaling and suggests that TLE4 may be involved in the proliferation and differentiation of colon CSCs.

In summary, our study suggests that during the course of colon CSC differentiation towards colon cancer cells, some miRNAs are differentially expressed. miR-93 is one of the miRNAs that was downregulated, and overexpressed miR-93 significantly inhibited cell proliferation and colony formation by colon CSCs. Furthermore, overexpression of miR-93 negatively regulated mRNA and protein expression of HDAC8 and TLE4. The inhibition of cell proliferation by miR-93 in colon CSCs may occur by targeting HDAC8 and TLE4.

COMMENTS

Background

The cancer stem cell (CSC) hypothesis is currently at the center of a rapidly evolving field, involving a change of perspective on the development and treatment of cancers. However, research has been hampered by the lack of distinct molecular markers of CSCs.

Research frontiers

Since the identification and characterization of CSCs in hematological malignancies, an increasing number of studies have described CSCs in solid tumors such as ovarian, colon, lung, breast, liver, melanoma and pancreatic tumors, suggesting that the CSC hypothesis applies to most neoplastic systems.

Innovations and breakthroughs

Previously isolated SW1116csc were compared with SW1116 cells. The authors identified 35 miRNAs that were upregulated and 11 miRNAs that were downregulated in SW1116csc. Overexpressed miR-93 significantly inhibited cell proliferation and colony formation of SW1116csc. Furthermore, miR-93 negatively regulated the mRNA and protein expression levels of HDAC8 and TLE4.

Applications

The study of CSCs has important implications for future cancer treatment and therapies. The CSC hypothesis states that if the CSCs were eliminated, the tumor would simply regress due to differentiation and cell death. By selectively targeting CSCs, it may be possible to treat patients with aggressive, non-resectable tumors and prevent the tumor from metastasizing.

Terminology

CSCs are a sub-population of cancer cells that possess characteristics associated with normal stem cells, such as self renewal and the ability to differentiate into multiple cell types. CSCs are tumorigenic, in contrast to most cancer cells, which are thought to be non-tumorigenic. CSCs persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors.

Peer review

The authors presented an original work about colon CSCs and identified a potentially new pathway that could be targeted for colon cancer management.

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BRIEF ARTICLE

CpG island methylator phenotype in plasma is associated with hepatocellular carcinoma prognosis

Ji-Bin Liu, Yi-Xin Zhang, Shu-Hui Zhou, Min-Xin Shi, Jin Cai, Yan Liu, Ke-Ping Chen, Fu-Lin Qiang

Ji-Bin Liu, Yi-Xin Zhang, Ke-Ping Chen, Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, Jiangsu Province, China
Ji-Bin Liu, Min-Xin Shi, Jin Cai, Yan Liu, Fu-Lin Qiang, Institute of Tumor, Nantong Tumor Hospital, Nantong 226361, Jiangsu Province, China
Shu-Hui Zhou, Department of Radiology, School of Radiology and Public Health, Soochow University, Suzhou 215006, Jiangsu Province, China
Author contributions: Liu JB and Chen KP designed the study and analyzed and interpreted data; Zhang YX and Cai J revised critically for important intellectual content; Zhou SH did statistical analysis of data; Shi MX and Qiang FL were responsible for acquisition of data.
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Correspondence to: Ke-Ping Chen, Professor, Institute of Life Sciences, Jiangsu University, 301 Xuefu Rd., Zhenjiang 212013, Jiangsu Province, China. kpchen@ujs.edu.cn
Telephone: +86-511-88791923 Fax: +86-511-88791923
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Abstract

AIM: To evaluate the clinical significance of CpG island methylator phenotype (CIMP) in plasma and its association with hepatocellular carcinoma (HCC) progress.

METHODS: CIMP status of 108 HCC patients was analyzed using a methylation marker panel in tumor tissues and plasma with methylation-specific polymerase chain reaction. Fifteen samples of non-neoplastic liver tissues and 60 of plasma from healthy persons were examined simultaneously. Examined genes included *APC, WIF-1, RUNX-3, DLC-1, SFRP-1, DKK* and *E-cad*.

RESULTS: The frequencies of high-level methylation in HCC tissue and plasma were at least 15% for the seven genes: *APC*, 48/108, 44.44% in tissue and 26/108, 24.07% in plasma; *WIF-1*, 53/108, 49.07% in tissue and 35/108, 32.41% in plasma; *RUNX-3*, 52/108, 48.14% in tissue and 42/108, 38.89% in plasma; *DLC-1*, 38/108, 35.18% in tissue and 23/108, 21.30% in plasma; *SFRP-1*, 40/108, 37.04% in tissue and 31/108, 28.7% in plasma; *DKK*, 39/108, 36.1% in tissue and 25/108, 23.14% in plasma; and *E-cad*, 37/108, 34.3% in tissue and 18/108, 16.67% in plasma. CIMP+ ($\geqslant$ 3 methylated genes) was detected in 68 (60.2%) tumor tissue samples and 62 (57.4%) plasma samples. CIMP was not detected in non-neoplastic liver tissues or plasma of healthy persons. CIMP status in tumor tissues differed significantly in gender, hepatitis B surface antigen, alpha-fetoprotein, and tumor-node-metastasis stage ($P < 0.05$). Similar results were obtained with plasma samples ($P < 0.05$). There was no difference in CIMP status in age, presence of hepatitis C virus antibody, cirrhosis, number of nodes, number of tumors, tumor size, or Edmondson-Steiner stage. A one-year follow-up found that the metastatic rate and recurrence rate in the CIMP+ group were significantly higher than in the CIMP- group as assessed with plasma samples ($P < 0.05$).

CONCLUSION: Plasma DNA can be a reliable sample source for CIMP analysis. CIMP in plasma may serve as a molecular marker of late-stage and poor-prognosis HCC.

Key words: CpG island methylator phenotype; Methylation; Plasma; Prognosis; Hepatocellular carcinoma

Peer reviewer: Takashi Kobayashi, MD, PhD, Department of Surgery, Showa General Hospital, 2-450 Tenjincho, Kodaira, Tokyo 187-8510, Japan

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world[1] and one of the leading causes of cancer-related death[2] because of late-stage diagnosis, lack of effective therapy, and easy recurrence[3]. Despite much progress in diagnostic and treatment strategies for HCC, recurrence and metastasis remain the main factors affecting patient survival. The 5-year survival rate is between 35% and 41% after resection[4,5] and between 47% and 61% after liver transplantation[6]. In general, prognosis remains poor, thus identification of useful molecular prognostic markers is necessary.

DNA methylation is an enzyme-mediated chemical modification that usually occurs in cytosine-guanine dinucleotide-rich areas (CpG islands) in the promoter region of genes. Aberrant promoter hypermethylation is an important mechanism leading to loss of gene function in tumors[7] including HCC[8,9]. The methylation pattern of multiple genes can provide useful information on global epigenetic alterations[10]. The hypermethylated subtype in tumors, called the CpG island methylator phenotype (CIMP) in which multiple genes are concurrently methylated, is a novel marker of tumor progression[11,12]. Methylation of CpG islands in the promoters of many tumor suppressor genes effectively silences those genes[13]. These epigenetic alterations may be important early events in carcinogenesis and may also be potential biomarkers for early detection[14]. CIMP is an important mechanism in HCC development and may serve as a molecular marker of late-stage HCC with poor prognosis[15]. Tumor tissue is the current gold standard to detect methylation in HCC[16]. However, not all patients' tissue samples can be used, but blood plasma samples are readily available from all patients.

Because determining the methylation status of single genes has limited prognostic value[17], CIMP has been reported as a promising predictive biomarker in many human cancers[17-19]. Many studies have investigated the relationship between CIMP and HCC prognosis[15,20], but these studies used tumor tissue as a research tool. These studies only examined the patients who underwent hepatic resection or liver puncture. Promoter methylation in breast cancer can be detected in the circulating plasma DNA[21]. The usefulness of future DNA methylation studies will be greatly enhanced if the efficacy is equivalent by using plasma and tumor tissues (Iyer *et al*[22], 2010). In this study, we analyzed the CIMP status of 108 HCC patients based on a methylation marker panel. Examined genes included *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK* and *E-cad*. These genes were selected because they have been found to be frequently methylated in HCC and other malignancies. The aim of our current study was to evaluate the clinical significance of CIMP in plasma DNA and its association with prognosis in HCC.

MATERIALS AND METHODS

Patients and specimens

Between 2008 and 2010, 108 paired samples of primary HCC and corresponding non-neoplastic liver tissues and plasma from Chinese patients were collected during surgical resection at the Nantong Tumor Hospital. Samples from 60 healthy persons were used as matched controls. The controls had no self-reported history of cancer and were frequently matched to age (± 5 years), gender, and residential area. After resection, tissue specimens were immediately frozen in liquid nitrogen and stored at -70 ℃. Patients consisted of 85 men and 23 women, ranging in age from 32 to 74 (52.48 ± 7.56) years. Questionnaire data and blood samples were also collected from all patients and controls. After centrifugation, separated plasma samples were stored at -70 ℃. The diagnosis of HCC was confirmed by pathological examination or elevation of alpha-fetoprotein (AFP, > 400 g/L) combined with imaging examinations including magnetic resonance imaging (MRI) and/or computerized tomography (CT). Tumor-node-metastasis (TNM) stage was classified according to the 6th edition TNM classification of the American Joint Committee on Cancer (Greene *et al*, 2002). Fifteen samples of noncancerous liver tissues were obtained from patients with liver hemangioma. Eighty-six patients had cirrhosis, 84 patients were positive for hepatitis B surface antigen (HBsAg), and 19 patients were positive for hepatitis C virus antibody (anti-HCV). Written informed consent was obtained from each patient, and the study protocol was approved by the local ethics committee.

DNA extraction and sodium bisulfite

DNA from both plasma (200 μL per column) and tissue samples was treated with proteinase K and then extracted with phenol-chloroform according to the manufacturer's instructions (Shanghai ShineGene Molecular Biotech, Inc., Shanghai, China) and stored at -20 ℃. A final elution volume of 50 μL was collected. The extracted DNA was treated with sodium bisulfite using an EZ DNA-methylation™ kit (Zymo Research, Orange, CA, United States) to convert all unmethylated cytosines to uracils. Bisulfite-modified DNA was resuspended in 10 μL elution buffer and stored at -20 ℃ until methylation-specific polymerase chain reaction (MSP).

MSP

MSP was used to determine the methylation status of CpG islands in genes after bisulfate treatment[23,24]. The methylation status of the promoters of *APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK* and *E-cad* was determined with a two-step MSP protocol that consists of amplification and detection. Polymerase chain reaction (PCR) products were analyzed on 2% agarose gels, stained with ethidium bromide, and visualized with ultraviolet illumina-

Table 1 Primer sets for nested methylation-specific polymerase chain reaction

Genes			Primer sequence (5'-3')
APC	U	F	GTGTTTTATTGTGGAGTGTGGGTT
		R	CCAATCAACAAACTCCCAACAA
	M	F	TATTGCGGAGTGCGGGTC
		R	TCGACGAACTCCCGACGA
WIF-1	U	F	GGGTGTTTTATTGGGTGTATTGT
		R	AAAAAAACTAACACAAACAAAATACAAAC
	M	F	CGTTTTATTGGGCGTATCGT
		R	ACTAACGCGAACGAAATACGA
RUNX3	U	F	TTATGAGGGGTGGTTGTATGTGGG
		R	AAAACAACCAACACAAACACCTCC
	M	F	TTACGAGGGGCGGTCGTACGCGGG
		R	AAAACGACCGACGCGAACGCCTCC
DLC-1	U	F	AAACCCAACAAAAAAACCCAACTAACA
		R	TTTTTTAAAGATTGAAATGAGGGAGTG
	M	F	CCCAACGAAAAAACCCGACTAACG
		R	TTTAAAGATCGAAACGAGCGAGCG
SFRP-1	U	F	GAGTTAGTGTTGTGTGTTTGTTGTTTTGT
		R	CCCAACATTACCCAACTCCACAACCA
	M	F	GTGTCGCGCGTTCGTCGTTTCGC
		R	AACGTTACCCGACTCCGCGACCG
DKK	U	F	TTAGGGGTGGGTGGTGGGGT
		R	CTACATCTCCACTCTACACCCA
	M	F	GGGGCGGGCGGCGGGGC
		R	ACATCTCCGCTCTACGCCCG
E-cad	U	F	TAATTTTAGGTTAGAGGGTTATTGT
		R	CCACCCCAATACTAAATCACAACA
	M	F	TTAGGTTAGAGGGTTATCGCGT
		R	TAACTAAAAATTCACCTACCGAC

M: Methylated sequence; U: Unmethylated sequence; F: Forward sequence; R: Reverse sequence.

tion. All experiments were performed in duplicate. Table 1 lists the primer sequences.

Follow-up

Patients were followed up for one year. The last follow-up was on September 8, 2010. None of the patients received chemotherapy prior to surgery. The median follow-up time was 5 mo (range, 3-12 mo). Patients were given a physical examination, abdominal ultrasonography, and chest X-ray, and serum was collected and tested for AFP. During the first year, local recurrence and distant metastasis were monitored every 3 mo with CT and/or MRI. Only 98 of the 108 patients were completely followed up. Twenty-one patients (19.44%) were found to have HCC recurrence; these patients all had AFP levels >20 μg/L, and CT examination revealed a clear image of the liver cancer. These 21 patients appeared to have different degrees of multicentric new lesions of the liver, including multiple nodules, apparent mass, the portal vein, hepatic vein thrombosis and sub-lesions. Twenty-four (22.2%) patients had HCC metastasis. According to their clinicopathological characteristics, formation of portal vein tumor thromboses, and dissemination into lymph nodes, 18 patients showed liver metastasis, four patients had bone metastasis, and two patients had brain metastasis. Forty-four (40.74%) patients died of cancer-related causes. Fifty-four patients were still alive at the time of the last follow-up.

Table 2 Frequencies of gene methylation in hepatocellular carcinomas, corresponding non-neoplastic liver tissues and normal liver tissues

Genes	No. of methylated samples					
	Tumor (n = 108)	Plasma (n = 108)	Non-neoplastic tissues (n = 102)	Normal tissues (n = 15)	Healthy plasma (n = 60)	χ^2
APC	48[1]	26	5	0	0	43.47
WIF- 1	53[1]	35	6	0	0	48.44
DLC-1	38[1]	23	4	0	0	32.05
DKK	39[1]	25	5	0	0	30.85
SFRP-1	48[1]	26	5	0	0	43.47
E-cad-1	37[1]	18	3	0	0	33.37
RUNX3	52[1]	42	4	0	0	52.47

[1]χ^2 test comparing the frequencies of gene methylation between tumor and non-neoplastic samples, $P < 0.05$.

Statistical analysis

The SPSS 13.0 software package (SPSS, Inc., Chicago, IL, United States) was used for all statistical analyses. Values for the clinical and biological characteristics of patients were expressed as means ± SD. Comparison was done with Student's t test. A χ^2 test or Fisher's exact test was used to compare the incidence of methylation. All P values presented are two-sided, and a P value of less than 0.05 was considered statistically significant.

RESULTS

Clinical data

Median tumor size was 6.0 cm (range, 1.2-19.4 cm). According to the Edmondson-Steiner classification system, 7 cases were classified as grade Ⅰ, 55 cases were grade Ⅱ, 43 cases were grade Ⅲ, and 3 cases were grade Ⅳ. According to the 6th edition TNM classification of the American Joint Committee on Cancer (Greene *et al*, 2002), 51 cases were classified as grade Ⅰ, 20 cases were grade Ⅱ and 37 cases were grade Ⅲ.

Methylation of tumor-associated genes in HCC

We examined the methylation status of seven tumor-associated genes (*APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK*, *E-cad*) in tissue and plasma samples from HCC patients and controls. The results are summarized in Table 2. No methylation was detected in the promoters of these genes in normal tissues or control plasma. Table 2 shows that methylation was more frequent in HCC than in adjacent non-neoplastic liver tissue and normal liver tissues for all seven genes. The same results were observed for plasma (Table 2). Methylation at any level was detected in one or more of the genes in 99 (91.67%) of 108 cases. The frequencies of high-level methylation in HCC tissue and plasma were at least 15% for the seven genes, including 44.44% for *APC*, 49.07% for *WIF-1*, 48.14% for *RUNX3*, 35.18% for *DLC-1*, 37.04% for *SFRP-1*, 36.1% for *DKK*, and 34.3% for *E-cad* in tissue. In plas-

Table 3 Relationship between CpG island methylator phenotype and clinicopathological characteristics in hepatocellular carcinomas

Characteristics	CIMP+		CIMP-		χ^{21}	χ^{22}
	Tumor	Plasma	Tumor	Plasma		
Age (yr)						
< 52	27	25	21	23	0.5584	1.002
⩾ 52	38	37	22	23		
Gender						
Female	9[a]	8[a]	13	15	4.28	6.115
Male	56	54	30	31		
HBsAg						
Negative	9[a]	7[a]	15	17	6.625	10.06
Positive	56	55	28	29		
Anti-HCV						
Negative	52	52	37	37	0.655	0.2151
Positive	13	10	6	9		
AFP (μg/L)						
< 20	2[a]	3[a]	40	41	88.1	77.72
20-400	29	30	3	5		
> 400	34	29	0	0		
Cirrhosis						
Yes	53	50	32	36	0.7827	0.0925
No	12	12	11	10		
Node number						
Single	44	37	31	32	0.2362	1.119
Multiple	21	25	12	14		
Tumor number						
Single	41	37	28	31	0.0467	0.6738
Multiple	24	25	15	15		
Tumor size						
< 6	30	27	24	22	0.966	0.195
⩾ 6	35	35	19	24		
Edmondson-Steiner						
Ⅰ-Ⅱ	38	35	24	21	0.0742	1.234
Ⅲ-Ⅳ	27	27	19	25		
TNM stage						
Ⅰ	31[a]	34[a]	20	17	5.325	4.872
Ⅱ	16	12	4	8		
Ⅲ	18	16	19	21		

[1]Indicates χ^2 test comparing the clinicopathological characteristics between CIMP+ and CIMP- in tumor tissues; [2]Indicates the comparison in the plasma. [a]$P < 0.05$. CIMP: CpG island methylator phenotype; HBsAg: Hepatitis B surface antigen; Anti-HCV: Hepatitis C virus antibody; AFP: Alpha-fetoprotein; TNM: Tumor-node-metastasis.

ma, the frequencies were 24.07% for *APC*, 32.41% for *WIF-1*, 38.89% for *RUNX3*, 21.30% for *DLC-1*, 28.7% for *SFRP-1*, 23.14% for *DKK* and 16.67% for *E-cad*.

Concordance of data obtained from plasma and tissue samples

The frequencies of promoter methylation in tissue and plasma samples for the seven tumor-associated genes were as follows: *APC*, 48/108, 44.44% in tissue and 26/108, 24.07% in plasma; *WIF-1*, 53/108, 49.07% in tissue and 35/108, 32.41% in plasma; *RUNX3*, 52/108, 48.14% in tissue and 42/108, 38.89% in plasma; *DLC-1*, 38/108, 35.18% in tissue and 23/108, 21.30% in plasma; *SFRP-1*, 40/108, 37.04% in tissue and 31/108, 28.7% in plasma; *DKK*, 39/108, 36.1% in tissue and 25/108, 23.14% in plasma; and *E-cad*, 37/108, 34.3% in tissue and 18/108, 16.67% in plasma. We observed significant concordance of promoter methylation between plasma and tissue samples for all seven genes.

CIMP in HCC

According to the criteria in a related study[25], the CIMP status of each of our 108 HCC samples was classified as CIMP+ (with ⩾ 3 methylated genes) or CIMP- (with two or fewer methylated genes). In this study, because of a cutoff value of 3, the average number of methylated genes was 2.8 in tumor tissue and 2.0 in plasma. Regarding tumor tissues, 65 cases of HCC (60.2%) were classified as CIMP+, and 43 (39.8%) cases were classified as CIMP-. When plasma was examined, 62 cases of HCC (57.4%) were classified as CIMP+, and 46 cases (42.6%) were classified as CIMP-. No CIMP+ samples were from non-neoplastic tissues or healthy controls.

Relationship between CIMP and clinicopathological characteristics

We analyzed the relationship between CIMP and clinicopathological characteristics including age, gender, HBsAg, anti-HCV, serum AFP level, liver cirrhosis, number of nodes, tumor size, number of tumors, Edmondson-Steiner grading, and TNM stage. In tumor tissues, we found significant differences between CIMP and gender, HBsAg, AFP, and TNM stage ($P < 0.05$ for all; Table 3). The same result was obtained with plasma samples, including differences between CIMP and gender, HBsAg, AFP, and TNM stage ($P < 0.05$ for all; Table 3). There were no differences between CIMP status and age, anti-HCV, cirrhosis, number of nodes, number of tumors, tumor size, or Edmondson-Steiner stage.

Prognostic significance of CIMP in HCC

Follow-up for up to one year found that the rates of metastasis differed significantly between the CIMP+ and CIMP- groups when tumor tissue ($P < 0.05$) and plasma were examined ($P < 0.05$). CIMP+ tumors appeared to frequently undergo metastasis. The recurrence rates were significantly higher in the CIMP+ group compared with the CIMP- group when both tumor tissue and plasma were examined ($P < 0.05$ for both). Survival rates differed significantly between the CIMP+ and CIMP- groups ($P < 0.05$ for both; Table 4).

DISCUSSION

Little is known about the many risk factors that are likely to affect the metastasis and recurrence of HCC. The development and progression of HCC is a multistep process, and the basic molecular pathway of HCC development remains largely unknown. Abnormal gene expression may be an early event in tumorigenesis and a potential biomarker for early detection[14]. Recurrence, which is frequent after surgical resection, and metastasis are the main factors affecting the long-term prognosis of HCC patients. Currently, predicting the development and guiding the treatment for HCC are generally based on clinical characteristics and/or the stage of HCC[26].

Table 4 Relationship between CpG island methylator phenotype and prognosis of hepatocellular carcinomas after a one-year follow-up ($n = 98$)

Prognosis	CIMP+		CIMP-		χ^{21}	χ^{22}
	Tumor	Plasma	Tumor	Plasma		
Metastasis						
Yes	22[a]	19[a]	2	5	11.032	5.762
No	40	38	34	36		
Recurrence						
Yes	18[a]	17[a]	3	4	5.796	4.257
No	44	40	33	37		
Survival						
Yes	26[a]	23[a]	24	27	5.574	4.621
No	36	32	12	16		

[1]Indicates χ^2 test comparing the prognosis between CIMP+ and CIMP- in tumor tissues; [2]Indicates the comparison in the plasma. [a]$P < 0.05$. CIMP: CpG island methylator phenotype.

However, genetic and epigenetic changes can also be used as indicators of cancer progression and/or markers. Epigenetic processes, in particular, abnormal methylation of CpG islands in HCC, may play a crucial role in cancer development[27,28]. In our study, we examined the methylation status of seven tumor-associated genes (*APC*, *WIF-1*, *RUNX-3*, *DLC-1*, *SFRP-1*, *DKK* and *E-cad*) on the basis of their biological significance in tissue and plasma in HCC and controls. We observed no methylation in the promoters of these genes in normal tissues and control plasma. The frequencies of high-level methylation in HCC tissue and plasma were at least 15% for these seven genes. The results showed that examining the methylation status of multiple genes may aid the diagnosis of early HCC. In this study, we also found that promoter methylation in plasma DNA was highly specific for certain cancer-related genes and that results for plasma were similar to those for tissue samples. There was good concordance in DNA methylation between plasma and tumor tissues in HCC.

CIMP+ tumors tend to occur in older patients with colorectal cancer, and this phenotype is overrepresented in proximal colon cancer tumors[29]. In esophageal adenocarcinoma, CIMP is associated with poor prognosis[30]. CIMP is also associated with environmental factors and serum AFP levels in HCC[31,32]. Many studies have investigated the relationship between CIMP and HCC prognosis[15,20]. However, these studies used tumor tissue samples, thereby limiting the applicability of the research. In our study, we found that CIMP in tumor tissues as well as plasma was associated with metastasis and recurrence of HCC. The presence of CIMP+ tumors indicates the likelihood of metastasis and recurrence in HCC. Thus, CIMP may play an important role in the pathogenesis, metastasis, and recurrence of HCC. Methylation may silence the genes associated with suppression of metastasis and recurrence. We also observed that there were no CIMP+ samples from corresponding non-neoplastic tissues and healthy controls, consistent with the known importance of methylation in cancer development. Our study also shows that promoter methylation in plasma DNA was highly specific and was seen in both plasma and tissue samples. Therefore, CIMP detection in plasma rather than tumor tissues may be used as a reliable source for predicting the prognosis of patients with HCC.

It has been shown that methylation of genes associated with tumors and CIMP is intimately involved in the early process of carcinogenesis and tumor progression[13]. We found that CIMP is a common phenomenon in HCC. Thus, CIMP may ultimately offer a new tool for predicting patients' clinical outcomes.

CIMP that is associated with tumors seems to have distinct epidemiology, histology, and molecular features[20].

The functional significance of methylation of tumor-associated genes in HCC may be that this process initiates progressive inactivation of these genes[15]. CIMP may be useful for stratifying the prognosis of patients with TNM stage I HCC and for identifying patients who are at higher risk for recurrence[20]. In our study, the presence of CIMP+ tumors indicates a poor prognosis in HCC. Thus, CIMP detection may determine the prognosis of patients with HCC. Furthermore, our study shows that promoter methylation in plasma DNA was highly specific and plasma and tissue samples yielded similar results. Therefore, CIMP detection in plasma, rather than tumor tissues, may be used as a reliable index for predicting the course of patients with HCC. Limitations of this study are that the results were based on a relatively small sample of patients, and the number of cancer-related genes we examined was small. Furthermore, the length of follow-up was only one year. Thus, in the future, a larger-scale multi-gene study that includes an extended follow-up period is needed to confirm our results.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the the fifth most common malignant cancer in the world, for later diagnosis, lack of effient therapy and easy recurrence. Despite a lot of progress in diagnostic and treatment strategies for HCC, recurrence and metastasis are still the main factors affecting the long-term prognosis for patients. In general, prognosis is still poor, and identification of useful molecular prognostic markers is required.

Research frontiers

DNA methylation is an enzyme-induced chemical modificationthat usually occurs in cytosine-guanine dinucleotide-rich areas (CpG islands) in the gene promoter regions. Aberrant promoter hypermethylation is an important mechanism for loss of gene function in tumors including HCC. The methylation pattern of multiple genes can provide useful information and an overall picture of epigenetic alterations. The hypermethylated subtype in tumors, called the CpG island methylator phenotype (CIMP), where multiplegenes are concurrently methylated, is a novel marker for tumor for progression. CIMP is an important mechanism in hepatocellular carcinoma development and could serve as a molecular marker of late stage and poorly prognostic HCC development.

Innovations and breakthroughs

Many studies have investigated the relationship between CIMP and prognosis associated with HCC, and also made many admirable achievements. However, these studies based on tumor tissue as a research object. These patients were only confined to patients with hepatic resection or liver puncture and part of the patients would be missed. Plasma DNA can be reliable for testing methylation profile in hepatocellular carcinoma patients. The productivity level of future DNA methylation studies will be greatly enhanced if the efficacy of using plasma is

equivalent to tumor tissue.In this study, the authors analysed the CIMP status of HCC patients based on a methylation marker panel. Associated genes include the *APC*, *WIF-1*, *RUNX3*, *DLC-1*, *SFRP-1*, *DAPK* and *E-cad*. These genes were selected because they have been found to be methylated frequently in HCC and other malignancies. The aim of the present study was to evaluate the clinical significance of CIMP in plasma associated with prognosis in HCC.

Applications

In this study, the authors found that plasma DNA could be used as a reliable resource and replace tumor tissue for CIMP research. This results also suggested that CIMP in plasma could serve as a molecular marker of late stage and poorly prognostic HCC.

Terminology

Epigenetic changes: Heritable changes in gene structure that without changing the gene sequence. CpG islands: CpG rich areas located in the promoter regions of many genes. CpG island methylation: The addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides. CIMP: The hypermethylated subtype in tumors, called the CIMP, where multiplegenes are concurrently methylated.

Peer review

This article is new and clinically informative in that it has firstly reported that the CIMP in plasma is associated with the presence of HCC.

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BRIEF ARTICLE

Comparison of laparoscopic *vs* open liver lobectomy (segmentectomy) for hepatocellular carcinoma

Ben-Shun Hu, Ke Chen, Hua-Min Tan, Xiang-Ming Ding, Jing-Wang Tan

Ben-Shun Hu, Ke Chen, Xiang-Ming Ding, Jing-Wang Tan, Hepato-bilio-pancreatic Surgery Department, Northern Jiangsu People's Hospital, Yangzhou 225001, Jiangsu Province, China
Hua-Min Tan, General Surgery Department, Fuzhou General Hospital of Nanjing Military Command, Fuzhou 350000, Fujian Province, China
Author contributions: Tan JW and Hu BS proposed the study; Hu BS and Tan JW wrote the first draft of the manuscript; Chen K, Tan HM and Ding XM analyzed the data; All authors contributed to the design and interpretation of the study and further drafts; Tan JW acquired the funding for these studies.
Correspondence to: Jing-Wang Tan, Chief Physician, Hepato-bilio-pancreatic Surgery Department, Northern Jiangsu People's Hospital, No. 98, Nantong West Rd, Yangzhou 225001, Jiangsu Province, China. tanjingwang02@yahoo.com.cn
Telephone: +86-514-8737004 Fax: +86-514-8737004
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Abstract

AIM: To investigate the effects of laparoscopic hepatectomy for the treatment of hepatocellular carcinoma (HCC).

METHODS: From 2006 to January 2011, laparoscopic hepatectomies were performed on 30 cases of HCC at Northern Jiangsu People's Hospital. During this same time period, 30 patients elected to undergo conventional open hepatectomy over laparoscopic hepatectomy at the time of informed consent. The degree of invasiveness and outcomes of laparoscopic hepatectomy compared to open hepatectomy for HCC were evaluated.

RESULTS: Both groups presented with similar blood loss amounts, operating times and complications. Patients in the laparoscopic hepatectomy group started walking and eating significantly earlier than those in the open hepatectomy group, and these more rapid recoveries allowed for shorter hospitalizations. There were no significant differences between procedures in survival rate.

CONCLUSION: Laparoscopic hepatectomy is beneficial for patient quality of life if the indications are appropriately based on preoperative liver function and the location and size of the HCC.

Key words: Laparoscopy; Hepatocellular carcinoma; Liver resection; Liver lobectomy

Peer reviewers: Ioannis Kanellos, Professor, 4th Surgical Department, Aristotle University of Thessaloniki, Antheon 1, Panorama, Thessaloniki 55236, Greece; Venkatesh Shanmugam, MBBS, MS (Gen Surg), Dip NB (Gen Surg), FRCS (Glasg), MD, Specialist Registrar (Trent Deanery), Royal Derby Hospital, Uttoxeter Road, Derby, DE22 3NE, United Kingdom

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INTRODUCTION

Laparoscopic resection of liver tumors was first reported in 1993[1]. It was initially adopted to treat peripheral, benign tumors that were amenable to non-anatomical wedge resection. The relatively slow development of laparoscopic liver resection compared to other subspecialties of laparoscopic surgery can be attributed to several reasons. First, some technical maneuvers that are frequently applied in open liver resection, such as organ mobilization, control of vascular inflow, and hanging

maneuvers, are difficult to reproduce in the laparoscopic setting. Difficulty in controlling hemorrhage, especially when profuse bleeding arises from the inferior vena cava or occurs deep in the liver parenchyma, is another major concern. Risk of gas embolism secondary to pneumoperitoneum can lead to dire consequences. In addition, increased risks of postoperative bile leakage and bleeding and uncertain long-term oncological outcomes concomitant with the resection of malignant tumors are obstacles that have undermined the development of laparoscopic hepatectomy[2].

With advancements in surgical instruments and increasing experience in laparoscopic treatment for benign liver diseases, there has been a growing interest in its application for hepatocellular carcinoma (HCC). We have actively performed laparoscopic hepatectomies for hepatic tumors, particularly for HCCs, since 2000. Recent experience has persuaded us that there are great potential benefits of laparoscopic hepatectomy; however, few reports exist regarding the usefulness, morbidity, and mortality of laparoscopic hepatectomy for HCC[3]. In this study, we addressed the indications, evaluated the degree of invasiveness, and analyzed the outcomes of laparoscopic hepatectomy for HCC based on our 5 years of experience.

MATERIALS AND METHODS

Patients

From 2006 to January 2011, laparoscopic hepatectomies were performed on 30 cases of HCC: 29 Child class A and one Child class B. Hepatitis B and hepatitis C viruses were positively noted in 24 and three cases, respectively. Twenty-three patients had chronic hepatitis, and 25 had liver cirrhosis. The localization of HCC by Couinaud's classification was as follows: segment Ⅰ, eight patients; segments Ⅱ and Ⅲ, ten patients; segment Ⅳ, four patients; segment Ⅴ, six patients; and segment Ⅵ, two patients.

During this same time period, 30 patients elected to undergo conventional open hepatectomy over laparoscopic hepatectomy at the time of informed consent. Twenty-eight cases (left lateral segmentectomy, 10; partial hepatectomy, 20) were retrospectively selected by the same criteria: the tumor was solid, was located in the left lateral or lower segment, and was < 10 cm in size. All operations were performed by the authors. The patients undergoing laparoscopic hepatectomy were compared to patients who underwent open hepatectomy with respect to operation time, blood loss, and blood chemistry. For statistical analysis, Student's *t* test, Kaplan-Meier method (for survival rates), and log-rank test were used. $P < 0.05$ was considered statistically significant.

Surgical procedure

The technique of laparoscopic hepatectomy has been described elsewhere[4]. Briefly, patients received general anesthesia according to the same protocol. Each patient's position and trocar placement were determined based on the location of the tumor. Laparoscopic hepatectomies were performed with the four- or five-trocar technique. Exploration of the extent of the tumor and its relationship with the vascular anatomy and other tumors in the liver was performed using intraoperative ultrasonography. In laparoscopic hepatectomy, there is the possibility of a CO_2 embolism by pneumoperitoneum during transection of the liver parenchyma and vessels[5]. Therefore, the pneumoperitoneum was shifted using abdominal wall lifting, particularly during liver parenchymal transection. The line of the intended liver parenchymal transection was marked on the liver surface using diathermy or microwaves. After the liver was punctured using a microwave scalpel along the line of the transection, it was irradiated with microwaves (average power 75 W) for a 30-s interval. Ultrasonic dissection of the liver was performed using an ultrasonic surgical system. The branched vessels were clipped and transected. In recent cases, laparoscopic coagulating shears were actively employed, and an endoscopic linear stapler was applied for liver transection if the tumor was pedunculated. In addition, the use of an endolinear stapler allowed surgeons to achieve rapid dissection and transection of Glisson's sheath and the hepatic veins in a left lateral segmentectomy. The resected liver was maneuvered into a plastic bag. Extraction of the undivided specimen was performed in all patients through the slightly enlarged trocar incision, which enabled histological review.

Table 1 Clinicopathological features

	L-Hr	O-Hr	*P* value
Age (yr)	46 ± 12	48 ± 15	NS
Sex (male:female)	20:10	19:11	NS
Tumor size (cm)	6.7 ± 3.1	8.7 ± 2.3	NS
Tumor site			
Left lobe of liver	26	16	NS
Right lobe of liver	4	14	
Child			
A	29	24	NS
B	1	6	
T-Bil (mmol/L)	13.4 ± 6.3	14.8 ± 6.8	NS
ALT (IU/L)	37.6 ± 4.3	39.3 ± 7.2	NS

L-Hr: Laparoscopic hepatectomy; O-Hr: Open hepatectomy; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; NS: Not significant.

RESULTS

Demographic data of the clinicopathological features in the open and laparoscopic hepatectomy groups are shown in Table 1. There were no differences in any of the preoperative background variables between the two groups, including tumor size.

All laparoscopic hepatectomies were successful, and there were no notable differences in operating times compared with open hepatectomy (Table 2). Postoperatively, the peak values of total bilirubin and alanine aminotransferase did not differ significantly between

Table 2 Clinicopathological features

	L-Hr	O-Hr	*P* value
Operation time (min)	180 ± 45	170 ± 32	> 0.05
Blood loss (g)	520 ± 30	480 ± 46	> 0.05
Tumor size (cm)	6.7 ± 3.1	8.7 ± 2.3	> 0.05
POD1 T-Bil (mmol/L)	27.8 ± 5.3	29.4 ± 4.6	> 0.05
POD1 ALT (IU/L)	359 ± 70	461 ± 68	> 0.05
Drain off (POD)	5.4 ± 1.2	6.6 ± 1.7	< 0.05
Ambulation (POD)	2.3 ± 2.5	5.7 ± 3.2	< 0.01
Oral intake (POD)	2.2 ± 1.4	5.5 ± 1.6	< 0.05
Hospital stay	13 ± 2.1	20 ± 3.2	< 0.01
Postoperative complication	4 (13.3%)	3 (10%)	> 0.05
Incision infection	0	1	
Subphrenic dropsy	1	2	
Bile leakage	3	0	
Liver failure	0	0	
Postoperative bleeding	0	0	

L-Hr: Laparoscopic hepatectomy; O-Hr: Open hepatectomy; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; POD: Postoperative day.

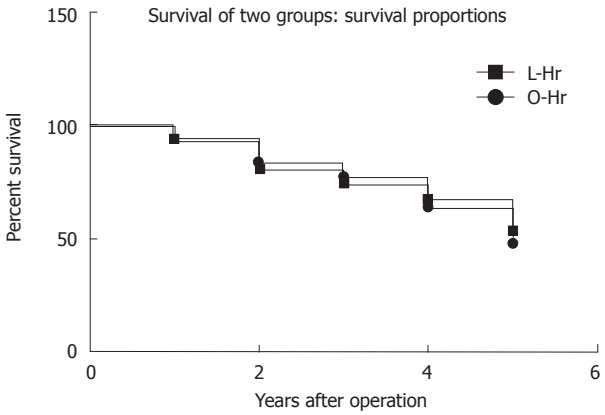

Figure 1 Prognosis (survival rate). L-Hr: Laparoscopic hepatectomy; O-Hr: Open hepatectomy. L-Hr *vs* O-Hr, $P > 0.05$.

the two groups. The patients started walking and eating significantly earlier in the laparoscopic hepatectomy group, and recovered more rapidly, which consequently allowed for shorter hospitalization. The laparoscopic hepatectomy group had a 13.3% complication rate, and the open hepatectomy group had a complication rate of 10%. However, this difference did not reach statistical significance (Table 2). Three patients in the laparoscopic hepatectomy group suffered bile leakage from the transected liver surface and recovered after drainage for 3 d. There were no hospital deaths in either group.

No recurrences related to laparoscopy, such as peritoneal dissemination and port-site recurrences, were observed. There were no significant differences in the survival rates between procedures, although more clinical cases and longer follow-up periods are needed to reach definitive conclusions. The 5-year survival rates for open and laparoscopic hepatectomy groups were 53.3% and 50%, respectively (Figure 1, $P > 0.05$).

DISCUSSION

Laparoscopic surgery for liver resection is a highly specialized field because the liver has unique anatomical features that present technical obstacles for surgery, such as difficulties in the control of bleeding and the potential for bile leakage from the intrahepatic vessels. However, important technological developments and improved endoscopic procedures have been established. Equipment modifications, such as intraoperative ultrasonography, ultrasonic dissection, microwave coagulators, and argon beam coagulators, and the introduction of endoscopic linear staplers and laparoscopic coagulation shears have been recognized for their efficacy in liver surgery. These advances have led to a higher frequency of laparoscopic partial hepatectomies performed in recent years. There have been reports of laparoscopic right and left lobectomy[6-9]. The future direction of laparoscopic liver resection is the use of surgical robotic systems[1,10,11]. Liver resection with the use of the Da Vinci robotic system has been reported[5]. The potential advantages of using robots include the visualization of high-quality 3D images and tremor filtering. The EndoWrist robotic instrument is even able to perform a 360-degree range of motion. However, the advantages of employing robotic systems must be balanced with the high cost of surgery, lack of training schemes, and difficulty of conversion to open surgery. Nonetheless, the use of surgical robots is expected to become more common in the treatment of both benign and malignant liver tumors in the foreseeable future.

The treatment of HCC and, more specifically, the indications for hepatectomy, are very limited. Nonsurgical ablation therapy, an alternative method to surgery, has been advocated by some because it yields improved quality of life, which has subsequently provoked controversy over curability[12]. However, the Liver Cancer Study Group of Japan has reported that patients who underwent hepatic resection had a higher survival rate than a nonsurgical treatment group, even for small-sized HCCs[13]. Under these circumstances, laparoscopic hepatectomy represents an intermediate option between ablation therapy and conventional hepatectomy; ablation therapy is less invasive than surgical resection, but laparoscopic hepatectomy is superior in its ability to resect the tumor completely and in allowing optimal pathological evaluation from the resected specimen. Although laparoscopic surgery is less invasive than standard hepatectomy, laparoscopic hepatectomy is inferior to open hepatectomy in terms of anatomical resection. Laparoscopic systematic resection is currently contraindicated because of its technical difficulties, except in the case of left lateral segmentectomy. A recent report has demonstrated the safety associated with more limited blood loss for laparoscopic left lateral segmentectomies in a case–control study[14].

Laparoscopic liver resection is a safe and feasible treatment option for HCC, even in cirrhotic liver[15]. However, converting to a new surgical method does not mean that the old surgical method was a failure. With advances in surgical techniques and instruments, laparoscopic liver

resection has been performed more frequently, even for tumors in difficult anatomical locations. Nonetheless, oncological clearance should not be jeopardized in exchange for the postoperative benefits of laparoscopic surgery. Laparoscopic liver resection offers advantages over open resection in terms of ambulation, oral intake and hospital stay. These more rapid recoveries allow for shorter hospitalization, which is reflected in reduced cost and better patient quality of life. The equivalent 5-year survival rates that are observed with laparoscopy promote this approach as a promising avenue of research for the resection of HCCs; however, further evaluation is required.

COMMENTS

Background

With the development of laparoscopic instruments and parenchymal transection devices and an improved understanding of the vascular anatomy of the liver, it is now widely accepted that laparoscopy will be used increasingly in liver surgery.

Research frontiers

The underlying intent of laparoscopic surgery for hepatocellular carcinoma is to provide curative resection while minimizing complications. In this study, the authors demonstrated that laparoscopic hepatectomy avoids some of the disadvantages of open hepatectomy and is beneficial for quality of life, as a minimally invasive procedure. There were no significant differences in the survival rates between procedures.

Innovations and breakthroughs

This paper describes a retrospective evaluation of a cohort of patients who underwent laparoscopic hepatectomy at a single center. A good clinical outcome depended on the reasonable choice of indication, suitable parenchymal transection devices, and sufficient experience in hepatic resection and laparoscopy.

Applications

Laparoscopic hepatectomy can be performed in selected patients.

Peer review

This is a comparative observational study on laparoscopic liver resection for cancer. Its publication seems important in a time of intense and controversial discussion about the use of laparoscopy for liver cancer.

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CASE REPORT

Neuroendocrine and squamous colonic composite carcinoma: Case report with molecular analysis

Sabrina C Wentz, Cindy Vnencak-Jones, William V Chopp

Sabrina C Wentz, Department of Pathology, Vanderbilt University Medical Center, Nashville, TN 37232-2561, United States
Cindy Vnencak-Jones, Molecular Genetics Laboratory, Vanderbilt University Medical Center, Nashville, TN 37232-2561, United States
William V Chopp, Department of Pathology, Veterans Affairs Medical Center, Nashville, TN 37232-2561, United States
Author contributions: Wentz SC is the manuscript author and contributed 90% of the manuscript text and all photomicrographs; Vnencak-Jones C and Chopp WV contributed equally to the paper with histopathologic and molecular data interpretation and manuscript supervision.
Supported by Intradepartmental funds through Vanderbilt University Medical Center Department of Pathology for resident career development
Correspondence to: Sabrina C Wentz, MD, Department of Pathology, Vanderbilt University Medical Center, 1161 21st Ave S., CC3322 Nashville, TN 37232-0011, United States. sabrina.c.wentz@vanderbilt.edu
Telephone: +1-615-3434882 Fax: +1-615-3437023
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Abstract

Composite colorectal carcinomas are rare. There are a modest number of cases in the medical literature, with even fewer cases describing composite carcinoma with neuroendocrine and squamous components. There are to our knowledge no reports of composite carcinoma molecular alterations. We present a case of composite carcinoma of the splenic flexure in a 33 year-old Caucasian male to investigate the presence and prognostic significance of molecular alterations in rare colonic carcinoma subtypes. Formalin-fixed paraffin-embedded (FFPE) tissue was hematoxylin and eosin- and mucicarmine-stained according to protocol, and immuno-stained with cytokeratin (CK)7, CK20, CDX2, AE1/AE3, chromogranin-A and synaptophysin. DNA was extracted from FFPE tissues and molecular analyses were performed according to lab-developed methods, followed by capillary electrophoresis. Hematoxylin and eosin staining showed admixed neuroendocrine and keratinized squamous cells. Positive nuclear CDX2 expression confirmed intestinal derivation. CK7 and CK20 were negative. Neuroendocrine cells stained positively for synaptophysin and AE1/AE3 and negatively for chromogranin and mucicarmine. Hepatic metastases showed a similar immunohistochemical profile. Molecular analysis revealed a G13D *KRAS* mutation. *BRAF* mutational testing was negative and microsatellite instability was not detected. The patient had rapid disease progression on chemotherapy and died 60 d after presentation. Although the G13D *KRAS* mutation normally predicts an intermediate outcome, the aggressive tumor behavior suggests other modifying factors in rare types of colonic carcinomas.

Key words: Composite carcinoma; Neuroendocrine; Squamous; *KRAS*; *BRAF*; Microsatellite instability

Peer reviewer: Dr. Ashok Kumar, MD, Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow 226014, India

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INTRODUCTION

Composite carcinomas are composed of at least two different intermingled malignant cell types. Such tumors have been documented in numerous gastrointestinal organs[1-4]. The usual composite carcinoma contains

adenocarcinoma and neuroendocrine components; however, spindle cell, osteoid metaplasia, squamous, and pleomorphic patterns have been identified[5]. Although up to 20% of colonic adenocarcinomas contain neuroendocrine cells, these have not been shown to influence prognosis[5]. When rendering a diagnosis of composite carcinoma, it is recommended that tumors contain a neuroendocrine component comprising at least one-third to one-half of the tumor[5].

The behavior of composite carcinomas is variable. In some cases, tumor behavior is similar to adenocarcinoma[5]; however, some case reports show progression to death within months[6]. The follow-up interval is not known in all of these cases. In some composite carcinomas, metastatic disease is solely from the neuroendocrine component[7], while others have shown composite metastases[8].

Primary squamous carcinoma of the colon occurs in one out of every 2000-4000 colorectal carcinomas[9]. Diagnostic criteria include the exclusion of metastases from other organs, the absence of a squamous fistula tract, the absence of concurrent anal squamous carcinoma, and the presence of squamous features on histologic examination[10]. The most frequent sites of primary squamous carcinoma of the colon are the proximal colon and rectum. Patients usually present during the fifth decade of life and more than 15% have distant metastases at presentation[11]. The average survival after diagnosis is 30 mo and the 5-year survival is approximately 30%[11]. Surgical resection remains the mainstay of therapy, and cisplatin and 5-fluorouracil are commonly used chemotherapeutic agents[12]. Squamous carcinomas with small cell or undifferentiated features have a very poor prognosis[13].

Tumor microsatellite instability (MSI) and *KRAS* and *BRAF* mutations have prognostic and predictive significance in colorectal adenocarcinomas, but little is known regarding these findings in the rarer subtypes of colorectal carcinomas such as high grade neuroendocrine carcinoma or squamous cell carcinoma. Microsatellite instability is found in 10-15% of sporadic colorectal carcinomas. MSI-H patients typically have proximal tumors with an overall more favorable outcome compared to tumors with microsatellite-instability. *BRAF* mutations, of which greater than 90% are V600E, occur in 70% of sporadic MSI cases and in 10%-15% of microsatellite-stable cases[14]. *KRAS* mutations are found in 30%-50% of colorectal carcinomas. The G12D, G12V and G13D mutations are the most common *KRAS* mutations, in order of decreasing frequency[15]. In particular, the G12V mutation is an independent risk factor for a 30% increase in relapse or death[16], while the G13D mutation predicts an intermediate outcome between two broad groups of cases; those with *BRAF*, G12D and G12V mutations and those with MSI-H with concurrent G13D mutations[15]. To our knowledge molecular alterations have not been characterized in composite carcinomas of the colon with neuroendocrine and squamous components. This case report describes an aggressive and fatal tumor in an unusual gastrointestinal location in an unusual age demographic, with a G13D *KRAS* mutation.

CASE REPORT

A 33 year-old Caucasian male veteran presented to the Nashville Veterans Affairs Medical Center with a 3-wk history of anorexia, dry heaves, bloating, mid-epigastric pain, and night sweats. The patient's past medical history is unremarkable except for an esophageal stricture treated with endoscopic dilatation, and gastroesophageal reflux disease treated with proton pump inhibitors. On examination, the abdomen was mildly distended with no palpable mass. Laboratory studies revealed a leukocytosis (white blood cell 18 600/μL) and a creatinine of 1.19 mg/dL (to convert to millimoles per liter, multiply by 0.0555), indicating renal impairment due to spontaneous tumor lysis. Alkaline phosphatase was 929 U/L, and aspartate aminotransferase and alanine aminotransferase were 221 and 93 U/L. A carcinoembryonic antigen level was not obtained. Right upper quadrant ultrasound revealed a multinodular liver, and non-contrast computed tomography of the chest, abdomen and pelvis showed descending colon wall thickening with enlarged mesenteric lymph nodes, and an enlarged liver with innumerable coalescing lesions measuring 2-4 cm. No other masses were identified.

The patient underwent percutaneous liver biopsy and endoscopic biopsy of the circumferential and nearly obstructing colonic lesion. Initial Hematoxylin and Eosin (HE) sections of the colonic and hepatic biopsies showed a metastatic, predominantly neuroendocrine tumor which positively stained for CDX2, AE1/AE3 and synaptophysin and negatively stained for cytokeratin (CK)7, CK20 and chromogranin-A. There was extensive necrosis and mitotic activity. The patient began cisplatin and etoposide chemotherapy; however interval computed tomography showed enlarging hepatic metastases. The patient underwent emergent sleeve resection with colonic diversion for obstruction. Final histopathologic staging showed a ypT3ypN2ypM1 composite carcinoma, which was grossly white-tan with slight lobulations. HE sections showed submucosal keratinizing squamous cells admixed with neuroendocrine cells (Figure 1). There was extensive necrosis and mitotic activity. The immunohistochemical profile was similar to the initial biopsies (Figures 2 and 3). Mucicarmine staining in several sections confirmed the absence of mucin production. Lymphovascular invasion was composed of neuroendocrine cells, and four of seven lymph nodes were involved by both tumor components. There was no associated squamous metaplasia or fistula. The patient's functional and clinical status declined, and therapy was shifted to palliative. The patient died at home, 60 d after initial presentation. An autopsy was not performed. Post-mortem molecular analyses on the surgical resection specimen demonstrated the tumor was microsatellite-stable, was negative for the V600E *BRAF* mutation, and harbored a G13D *KRAS* mutation (Figure 4).

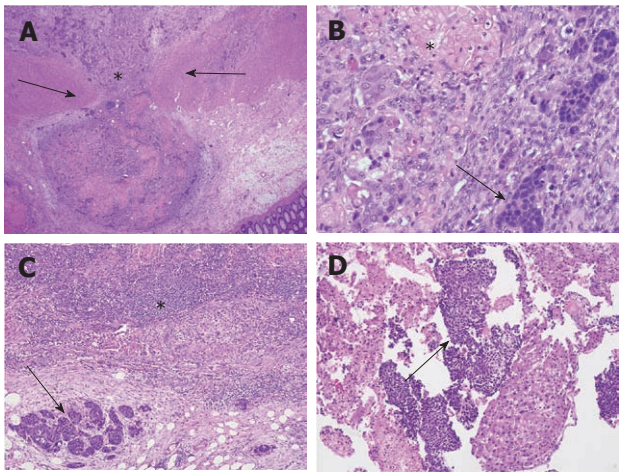

Figure 1 Hematoxylin and eosin-stained sections of tumor. A: The tumor (asterisk) is submucosal with extension through inner and outer muscularis propria (arrows), 100 ×; B: Intimate mixing of squamous (asterisk) and neuroendocrine (arrow) components, 400 ×; C: Lymph node metastasis of both components (squamous, asterisk; neuroendocrine, arrow), 100 ×; D: Liver fine needle aspirate showing predominantly neuroendocrine cells (arrow) with background bland hepatocytes, 200 ×.

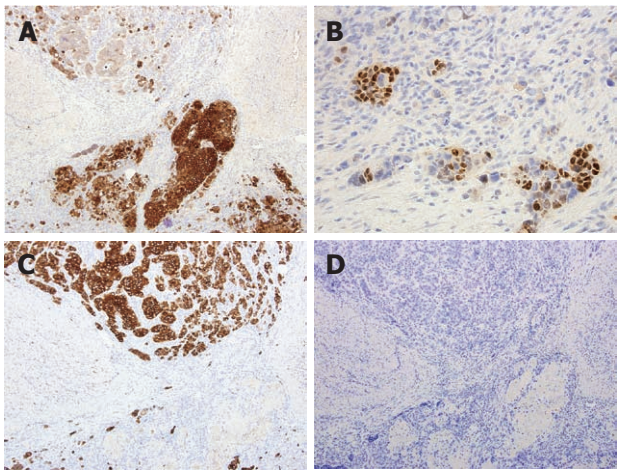

Figure 2 Immunohistochemical profile of the surgical resection specimen. A: Cytokeratin AE1/AE3; B: Synaptophysin; C: CDX2; D: CK20. A, B and D at 100 ×, C at 400 ×.

MATERIALS AND METHODS

Biopsy and surgical sections were formalin-fixed, paraffin-embedded, and HE stained according to standard protocol. CK7, CK20, CDX2, AE1/AE3, chromogranin-A and synaptophysin immunostains were run on Leica BondMax instruments (Bannockburn, IL) as follows: primary antibody incubation for 15 min, post-primary (proprietary, Leica) for 8 min, polymer (proprietary, Leica) for 8 min, peroxidase block for 5 min, DAB chromogen for 10 min, and hematoxylin counterstain for 10 min. CK7 additionally uses Epitope Retrieval Solution 1 (proprietary, Leica) for 10 min. Mucicarmine staining was performed according to standard protocol. For molecular analyses, DNA was extracted from three 10-micron thick tissue sections representing tumor or normal tissue, respectively, using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) following prior tissue lysis and treatment with proteinase K.

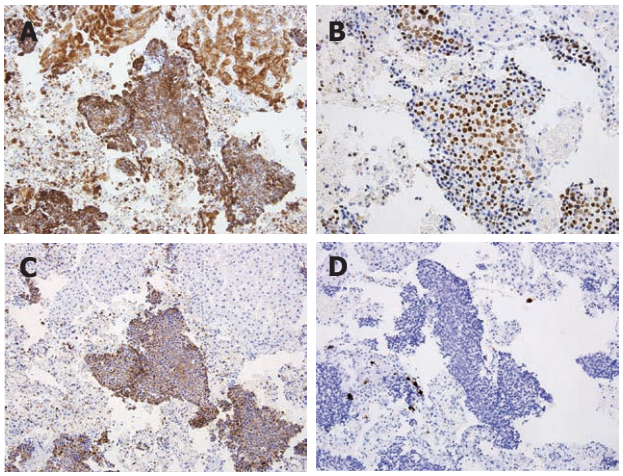

Figure 3 Immunohistochemical profile of the liver biopsy specimen. A: Cytokeratin AE1/AE3; B: Synaptophysin; C: CDX2; D: CK20. A, B and D at 200 ×, C at 400 ×.

MSI analysis

Five ng of normal and tumor DNA were amplified using multiplex polymerase chain reaction (PCR) containing fluorescently labeled primers for 5 mononucleotide markers corresponding to NR-21, BAT-26, BAT-25, NR-24, MONO-27 and 2 pentanucleotide markers for identity confirmation (Promega, Madison WI). The assay was performed according the instructions listed by the manufacturer, amplicons were subjected to capillary electrophoresis using an ABI 3130xl, and the results were analyzed using GeneMapper v3.7 software.

BRAF analysis

Five ng of tumor DNA was analyzed for the common *BRAF* mutation V600E using a lab developed allele-specific (AS) PCR assay for this mutation. The AS PCR assay uses a common forward primer (5'-TGTTTTCCTTTACTTACTACACCTCAGA-3') and two reverse primers (5'-CAGTGGAAAAATAGCCTCAATTCT-3' and 5'-ACCCACTCCATCGAGATTTCT-3'), each tagged with a different fluorochrome to produce an internal control amplicon and, if present, a V600E (c.1799T > A) mutation-specific amplicon. Amplicons were separated using capillary electrophoresis on an ABI 3130xl and the results were analyzed using GeneMapper v3.7 software.

KRAS analysis

Ten ng of tumor DNA was analyzed for common *KRAS* mutations using allele specific PCR and previously described primers for the detection of mutations G12A, G12C, G12D, G12R, G12S, G12V, G13C and G13D[17]. In addition to the fluorescent tagged mutation specific primer, each of the eight PCR reactions also contained a previously described common wild type reverse primer to produce a mutation-specific amplicon of 176, 179 or

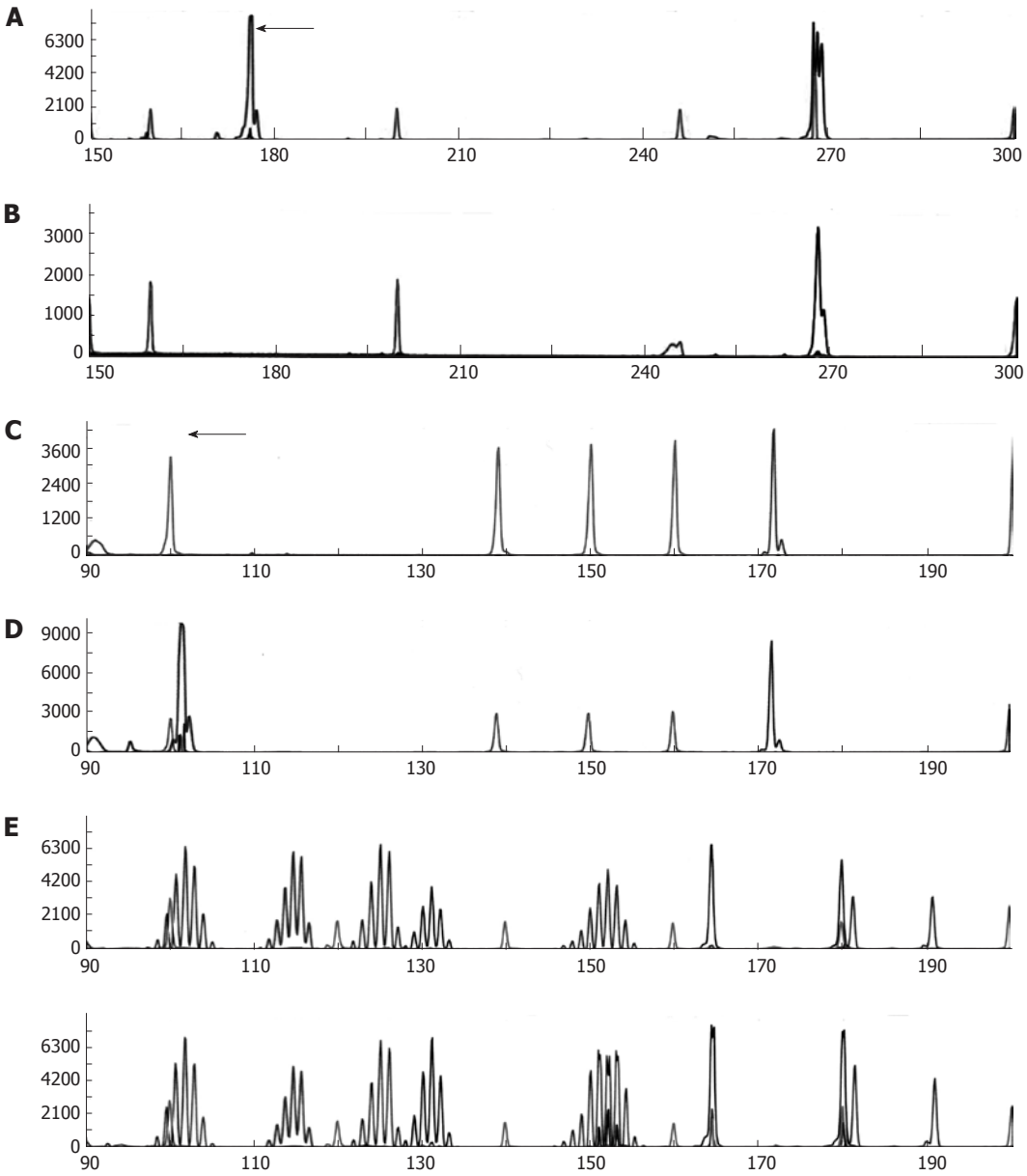

Figure 4 Molecular analysis. A, B: *KRAS* mutation analysis; A: The peak at 176 bp (arrow) corresponds to the G13D mutation; B: Absent peak in the G12D mutation analysis (internal size standards at 160, 200, 248 and 300 bp; internal control amplicon peak at 267 bp for A and B); C, D: *BRAF* V600E mutation analysis; C: The tumor is negative for the *BRAF* V600E mutation as shown by the absence of a peak at 101 bp (arrow); D: *BRAF* V400E positive control with peak at 101 bp (internal size standards at 100, 140, 150, 160 and 200 bp; internal control amplicon peak at 171 bp for C and D); E: Microsatellite instability testing demonstrates a microsatellite-stable tumor, top; normal tissue, bottom (internal size standards at 120, 140, 160 and 200 bp).

180 base pairs in length depending on the location and mutation present[18]. To confirm the amplification efficiency of the DNA, a third, lab-developed wild-type forward primer (5'-GTGTATTAACCTTATGTGTGACATG-3') was included to yield a wild type amplicon of 267 base pairs in length. Amplification conditions included a 5 min denaturation at 95 ℃ followed by 40 cycles of 95 ℃ for 30 s, 60 ℃ for 2 min and 72 ℃ for 2 min. The PCR assay concluded with a final extension phase of 10 min at 72 ℃. PCR products were separated using capillary electrophoresis on an ABI 3130xl, and the results were analyzed using GeneMapper v3.7 software.

DISCUSSION

This case report highlights a rare subtype of colorectal carcinoma with unusual features, including early age of onset and location at the splenic flexure. Additionally, this case documents molecular alterations which have not previously been documented in composite carcinomas with squamous and neuroendocrine features.

Most composite carcinomas are neuroendocrine and adenomatous, while this case showed squamous, rather than adenomatous, differentiation. While squamous carcinomas typically arise in the proximal colon and rectum when patients are in the fifth decade of life[11], this case

describes a 33 year-old with a lesion at the splenic flexure. Furthermore, the tumor was entirely submucosal and intramural, with no mucosal involvement, raising the possibility of a distant metastasis from an unidentified primary tumor. A computerized tomography scan of the chest, abdomen, and pelvis failed to demonstrate any other mass which could have potentially metastasized. The tumor was CDX2 positive, confirming lower gastrointestinal origin.

Due to the young age of the patient, aggressive nature of the tumor, and unusual histology, we performed molecular analyses of well-known genetic alterations in colorectal carcinoma which have prognostic and predictive significance. We anticipated that the tumor was microsatellite-stable, as it did not have mucinous or medullary histology or prominent tumor-infiltrating lymphocytes. As *BRAF* mutations are the least common of the alterations analyzed, we also anticipated this would be negative. Due to the patient's rapid progression, it was possible that the tumor harbored a *KRAS* mutation. The G13D mutation we identified would have excluded the patient from treatment with cetuximab or panitumumab. Current literature suggests this mutation results in an intermediate prognosis; however, this patient's clinical course was representative of a mutation with a very poor outcome. Given that the majority of colorectal carcinomas are adenocarcinomas, it is possible that rarer colorectal carcinoma histologic subtypes with similar genetic alterations may not have similar clinical courses as adenocarcinomas. The presence of other genetic alterations or modifier genes in these histologic subtypes remains to be determined. This study demonstrates prognostic and predictive value of molecular alterations is not uniform across tumor subtypes and further information is needed before such results drive therapeutic decision-making in cases of rare tumors.

In summary, we present a rare case of metastatic composite colorectal carcinoma containing squamous and neuroendocrine components. The young age of presentation and location within the colon are unusual. The tumor harbored a G13D *KRAS* mutation, with more aggressive behavior than expected from such a mutation.

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CASE REPORT

Primary malignant melanoma of the esophagus: A case report

Joana Machado, Paula Ministro, Ricardo Araújo, Eugénia Cancela, António Castanheira, Américo Silva

Joana Machado, Paula Ministro, Ricardo Araújo, Eugénia Cancela, António Castanheira, Américo Silva, Department of Gastroenterology, S Teotónio Hospital, 3504-509 Viseu, Portugal

Author contributions: Machado J contributed to literature review and wrote the manuscript; Ministro P contributed to the endoscopic diagnosis and revised the manuscript; Cancela E and Castanheira A contributed to the follow up of the patient; Araújo R and Silva A contributed to literature research.

Correspondence to: Joana Machado, MD, Department of Gastroenterology, S Teotónio Hospital, Av Rei D Duarte, 3504-509 Viseu, Portugal. jlvmachado@hotmail.com
Telephone: +351-232-420500 Fax: +351-232-282190

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Abstract

The authors present the clinical case of an 87-year-old Caucasian male admitted to the emergency room with hematemesis. He had a history of intermittent dysphagia during the previous month. Endoscopic evaluation revealed an eccentric, soft esophageal lesion located 25-35 cm from the incisors, which appeared as a protrusion of the esophagus wall, with active bleeding. Biopsies were acquired. Tissue evaluation was compatible with a melanoma. After excluding other sites of primary neoplasm, the definitive diagnosis of Primary Malignant Melanoma of the Esophagus (PMME) was made. The patient developed a hospital-acquired respiratory infection and died before tumor-directed treatment could begin. Primary malignant melanoma represents only 0.1% to 0.2% of all esophageal malignant tumors. Risk factors for PMME are not defined. A higher incidence of PMME has been described in Japan. Dysphagia, predominantly for solids, is the most frequent symptom at presentation. Retrosternal or epigastric discomfort or pain, melena or hematemesis have also been described. The characteristic endoscopic finding of PMME is as a polypoid lesion, with variable size, usually pigmented. The neoplasm occurs in the lower two-thirds of the esophagus in 86% of cases. PMME metastasizes *via* hematogenic and lymphatic pathways. At diagnosis, 50% of the patients present with distant metastases to the liver, the mediastinum, the lungs and the brain. When possible, surgery (curative or palliative), is the preferential method of treatment. There are some reports in the literature where chemotherapy, chemohormonotherapy, radiotherapy and immunotherapy, with or without surgery, were used with variable efficacy. The prognosis is poor; the mean survival after surgery is less than 15 mo.

Key words: Esophagus; Melanoma; Esophagoscopy; Upper gastrointestinal tract; Neoplasms

Peer reviewer: John K Marshall, MD, Associate Professor of Medicine, Division of Gastroenterology (4W8), McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

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INTRODUCTION

Primary malignant melanoma of the esophagus (PMME) is a rare neoplasm. Only 0.1% to 0.2% of all esophageal malignant tumors are attributed to PMME and only 0.5% of all noncutaneous melanomas are found in the esophagus[1].

Although PMME was first reported in 1906[2] and confirmed with histological evidence in 1952[3], the diagnosis remained controversial until 1963 when the

presence of benign melanocytes within the esophageal mucosa was demonstrated[4]. This observation was confirmed later in two other studies[5,6].

Risk factors are not yet defined but esophageal melanosis has been indicated as predisposing factor[6-8].

PMME is more common in men between de 6th and 7th decades[1,9,10]. Cases have been described at young ages[11,12]. A higher incidence has been described in Japan[1,9].

Symptoms are usually present for less than 3 mo. Dysphagia is the most frequent complain at presentation but retrosternal or epigastric discomfort or pain are also common. Hematemesis or melena are less frequent[1].

A polypoid, pigmented lesion in the lower two-thirds of the esophagus is the typical endoscopic finding[10]. Less frequently they are non-pigmented, but melanin can be identified in histological examination in a large proportion of those cases[1,9,13], and the true incidence of amelanotic melanoma of the esophagus is less than 2%[14].

Thoracic and abdominal computed tomography (CT), barium swallow examination and endoscopic ultrasonography are useful as staging methods[15-18].

Most PMME are diagnosed at advances stages of the disease[10]. Although there is not a formal recommendation, surgery is the preferential method of treatment but the prognosis remains poor.

The authors present a case of PMME and review the literature.

CASE REPORT

An 87-year-old Caucasian male, without relevant or previously known health problems, was brought to our emergency room with hematemesis. He had a history of intermittent dysphagia during the previous month. After hemodynamic stabilization, an upper gastrointestinal endoscopy was performed revealing an eccentric, soft lesion located 25-35 cm from the incisors, which appeared as a protrusion of the esophagus wall, with active bleeding and numerous clots. A CT scan was performed to characterize the nature of the lesion. The CT identified a solid enhancing mass with soft tissue attenuation, extending above the aortic arch and without any imaging signs of aortic invasion (Figure 1). No apparent pulmonary, hepatic or mediastinal nodes or adenopathies were identified. After excluding the possibilities of an aortic aneurysm or aorto-esophageal fistula, an upper gastrointestinal endoscopy was repeated with endoscopic hemostasis, administering 10 mL of adrenaline (1:10 000). The next day an endoscopic review was performed (Figure 2) and biopsies were acquired. Histological examination revealed "tissue infiltrated with crowded cells, most of them without visible cytoplasm (when visible, it contained a brown pigment that stained black with Fontana Masson technique) and hyperchromatic, rounded nuclei" (Figure 3A). Immunohistochemical findings included positive vimentin, protein S-100 and HMB-45 staining and negative keratins (MNF, LP 34, CAM 5.2) and LCA (Figure 3B). The patient was observed by the dermatology and ophthalmology departments and no suspicious skin, eye or anal lesions were found; the diagnosis of primary esophageal melanoma was made. During his hospital stay, the patient developed a hospital-acquired respiratory infection. The patient died after 35 d, before tumor-directed treatment could begin.

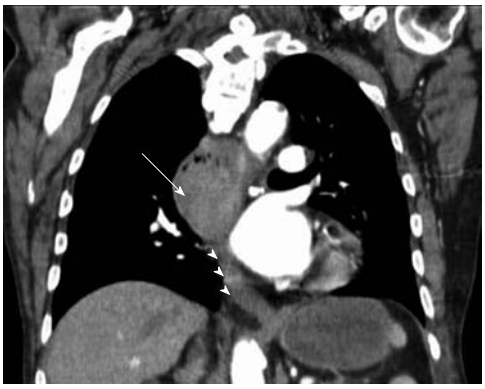

Figure 1 Coronal reformat of a contrast-enhanced chest computed tomography demonstrates a solid enhancing mass with soft tissue attenuation in the middle third of the esophagus (arrow) causing obstruction and proximal dilatation. The distal third of the esophagus (arrowheads) is unremarkable.

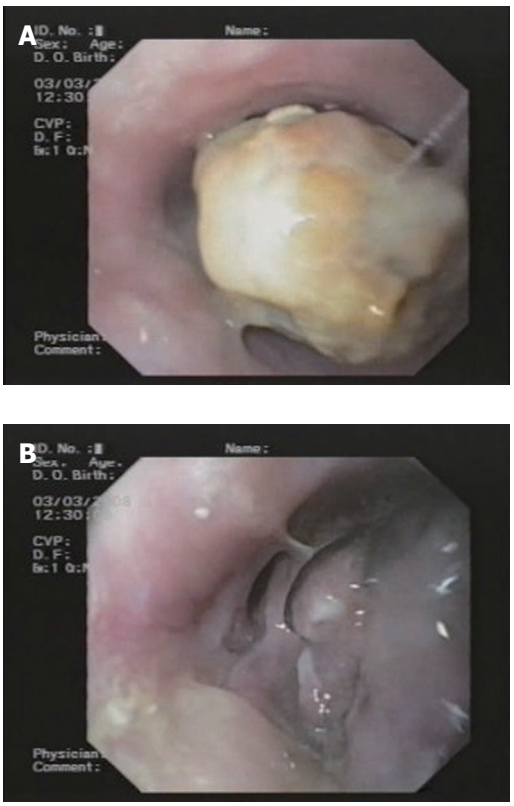

Figure 2 Upper gastrointestinal endoscopy of the patient. A: Soft polypoid tumor protruding to the esophageal lumen, located 25cm to 35cm from the incisors; B: A closer view of the lesion shows that tumor surface is darker than esophageal mucosa due to its melanin content.

DISCUSSION

Primary malignant melanoma of the esophagus is a rare

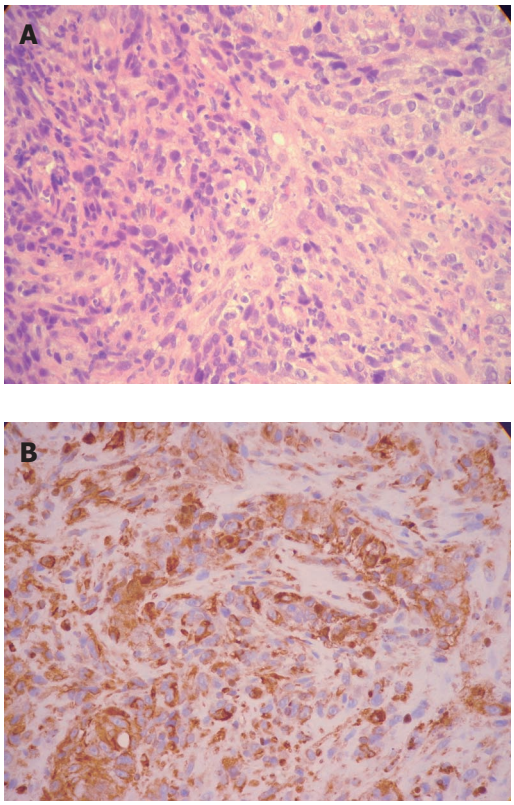

Figure 3 Histopathological findings. A: Hematoxylin and eosin staining discloses crowded rounded or elongated cells within distinct cytoplasm and hyperchromatic nuclei (magnification, x 100); B: Immunohistochemical study for HMB45 (anti-melanoma protein mAb). The positive reaction (brown cytoplasmic reaction) supports the diagnosis of malignant melanoma (magnification, x 100).

neoplasm and represents 0.1% to 0.2% of all esophageal malignant tumors and 0.5% of all noncutaneous melanomas[1].

The first to report a case of PMME was Bauer in 1906[2]. In 1952, Garfinkle and Cahan were the first to confirm the diagnosis with histological evidence[3]. The diagnosis remained controversial until 1963 when la Pava *et al*[4] demonstrated the presence of benign melanocytes within the esophageal mucosa in 4 of 100 autopsy specimens. Later, Tateishi *et al*[5] (1974) and Ohashi *et al*[6] (1990) confirmed this observation and demonstrated that 2.5 % to 8% of the general population has typical melanocytes within the esophageal mucosa[4-6].

The origin of melanocytes of the esophagus remains unclear. Two hypotheses are proposed: (1) migration of melanoblasts from the neural crest; and (2) pluripotent immature cells migrate to the esophagus and then differentiate into melanocytes[1,5].

Risk factors are not yet defined. No association between tobacco or alcohol consumption appears to exist. Melanosis, a benign condition defined as an increased number of melanocytes within the basal layer and an increased quantity of melanin in these melanocytes, has been indicated as a predisposing factor and it has been described in association with or preceding PMME[6,7]. In 2007, Oshiro *et al*[8] described a case of an esophageal melanotic lesion found incidentally during upper gastrointestinal endoscopy that after 19 mo of follow-up underwent malignant transformation.

PMME occurs predominantly in males between the ages of 50-70 years old[1,9,10]. One pediatric case has been reported[11] as well as another in a young adult[12]. A higher incidence of PMME has been described in Japan[1,9] and a greater number of esophageal melanocytes have been observed in this population[6].

Dysphagia, predominantly for solids, is the most frequent symptom at presentation (79.8% of all cases[1]). Retrosternal or epigastric discomfort or pain appears in 33.1% of the cases[1]. Occasionally, melena or hematemesis are reported, as occurred in our case. Symptoms are typically present for less than 3 mo[1]. The reported size of the tumors at diagnosis and the short duration of symptoms demonstrate that this is a rapidly growing neoplasm.

The characteristic endoscopic finding of PMME is as a polypoid lesion, with variable size, usually pigmented. The neoplasm occurs in the lower two-thirds of the esophagus in 86% of cases[10]. They are non-pigmented in 10%-25% of cases but melanin can be identified at histological examination in some of them[1,9,13]. The true incidence of amelanotic melanoma is less than 2%[14]. PMME should be included in the differential diagnosis list of all polypoid tumors found during endoscopic evaluation of the esophagus. Other possible diagnoses are leiomyoma, lipoma, fibroma, neurofibroma, some epidermoid carcinomas, sarcoma, small cell carcinoma, carcinosarcoma and metastatic melanoma.

Thoracic and abdominal CT allows lesion visualization and staging. Barium swallow examination can show a bulky, polypoid intraluminal filling defect[15]. Endoscopic ultrasonography as a staging method has been used in a few cases but the results were coincident with those obtained in histological examination of the resected specimens[16-18].

Diagnostic criteria have been developed by Allen and Spitz[19] and include a typical histologic pattern of melanoma and the presence of melanin granules within the tumor cells; origin from squamous epithelium with junctional activity; junctional activity with melanotic cells in the adjacent epithelium. Histological examination of specimens obtained by endoscopic biopsies are often misdiagnosed and described as poorly differentiated carcinoma, especially when the melanoma cells contain few or no melanin granules (in the series of Sabanathan[1] only 54% of cases were diagnosed preoperatively as malignant melanoma). More recently, immunohistochemical staining with S-100 or anti-melanoma protein mAb (HBM-45) allows accurate identification of this kind of tumor.

PMME metastasizes *via* hematogenic and lymphatic pathways. At diagnosis, about 50% of patients present distant metastases to the liver (31%), the mediastinum (29%), the lungs (18%) and the brain (13%)[10].

Treatment should be individualized for each patient. The choice should be based on tumor size and location, presence or absence of metastases, age and comorbidities of the patient.

When possible, surgery (curative or palliative), is the

preferential method of treatment. Radical resection with great margins is recommended as this tumor has a widespread intramucosal component. Survival after radical resection is 14.18 mo and after limited local excision is only 9 mo[1].

Adjuvant or neoadjuvant chemotherapy or chemohormonotherapy are not globally beneficial. However, some authors have described sporadic successful cases using these therapies. A frequently utilized course, similarly to that used in cutaneous melanoma, includes dacarbazine, nimustin and vincristine. Two cases with at least 7 years survival have been described[20,21]. In 2004, Uetsuka *et al*[20] described a case using dacarbazine, nimustine, cisplatin and tamoxifen before and after surgery and interferon-β monthly only after surgery. In 2007, Kawada *et al*[21] described a case using chemotherapy (dacarbazine, nimustine and vincristine) and local endoscopic injection of interferon-β before and after surgery.

Radiotherapy can be effective but should be reserved for PMME with metastatic illness, patients with high surgical risk or for those who refuse a surgical approach[9]. When used as neoadjuvant or adjuvant therapy of radical surgery, a 16.72 mo survival was achieved[1].

Khoury-Helou *et al*[22] described a 9-year survival in the case of a patient who underwent surgery followed by chemotherapy (5-fluorouracile and cisplatin) and radiotherapy (40Gy) after identification of a ganglionar metastasis.

Immunotherapy using autologous monocyte-derived dendritic cells pulsed with the epitope peptides of melanoma-associated antigens (MAGE-1, MAGE-3), in association with passive immunotherapy with lymphokine-activated killer cells, is an alternative proposed for adjuvant treatment[23].

Intraluminal brachytherapy in association with photoablation with Nd: YAG laser has also been used as an alternative treatment for a patient with surgical contraindications[24]. Another option for palliative treatment is esophageal stent implantation[25].

The prognosis is poor; the mean survival after surgery is less than 15 mo[9,10]. At least 7 patients have survived 5 or more years; they were all submitted to total esophagectomy but only three of them were submitted to some kind of adjuvant treatment[20,22,26-30].

Eighty-five percent of PMME patients die of disseminated disease[1].

Our patient died of a fatal hospital-acquired infection and therefore was not submitted to any tumor-directed therapy. PMME represents a rare condition, especially in western countries. Clinicians should be aware of PMME when considering the differential diagnosis of an esophageal polypoid lesion. The prognosis is poor and treatment should be promptly initiated, yet there is no formal recommendation on this subject.

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Uses of probe-based confocal laser endomicroscopy: Responses to a question to practitioners

Navin Paul, Kevin A Ghassemi

Navin Paul, Kevin A Ghassemi, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, United States
Author contributions: Paul N and Ghassemi KA designed, performed and analyzed data from the research; Paul N wrote the article and Ghassemi KA reviewed the final article.
Correspondence to: Navin Paul, MD, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave, Los Angeles, CA 90095, Unite States. npaul@mednet.ucla.edu
Telephone: +1-424-6032122 Fax: +1-310- 2684510
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Abstract

Confocal laser endomicroscopy is a novel imaging technology, which allows real-time visualization and interpretation of microscopic details in live tissues. Although several potential uses have been identified for this technology, no data are available regarding its real-world uses. We report the results of an email-based survey of experts in North America regarding their use of the technology.

Key words: Confocal laser endomicroscopy; Expert opinion

Peer reviewers: Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, University of Naples Federico Ⅱ, School of Medicine, Naples 80131, Italy; Jonas Mudter, MD, Medical Department 1, University of Erlangen, Ulmenweg 18, Erlangen 91054, Germany

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TO THE EDITOR

Confocal laser endomicroscopy (CLE) is a novel tool which allows real-time *in vivo* visualization of histological details in the setting of endoscopy[1]. Since the first description of use of an endoscope-based system in humans in 2004, the technology has been studied for several indications. These include use to identify Barrett esophagus, *in vivo* gastric cancer, gastric intestinal metaplasia, celiac disease, colorectal polyps, ulcerative colitis surveillance, graft-*vs*-host disease, biliary tract strictures, pancreatic cysts, and use in association with endoscopic mucosal resection[2]. In a recent issue of this journal, Gheonea *et al*[3] reviewed data regarding its use in studying vascularization patterns of gastrointestinal malignancies. Despite this expanding list of potential applications, no data are available regarding the indications for which the technology is currently used in real-world settings.

While preparing a departmental presentation on CLE, we contacted gastroenterologists who were listed as using probe-based CLE (pCLE) in North America in an effort to identify expert opinion regarding current and potential clinical applications. Users were identified from the website of Mauna Kea Technologies[4]. All users were contacted on September 4, 2010 by email and were posed a single open-ended question requesting their personal views on current applications and real-world utility of pCLE. No questionnaire was used. Data were abstracted from responses and were analyzed.

Fifty-one gastroenterologists across 31 institutions were listed as using pCLE. Email addresses were gleaned for 43 physicians from 23 institutions by searching hospital or university websites or accessing publications where the practitioner was the corresponding author. Of 43 emails sent, 4 were returned as undeliverable, leaving 39/51 (76.5%) who were assumed to be contacted suc-

cessfully. Among 39, responses were obtained from 30 (76.9%). An analysis of the email response times of contacted experts has been published separately[5].

The most frequently cited uses for pCLE among current practitioners of the technology were as follows: Barrett esophagus (21/30; 70%); indeterminate biliary strictures (14; 46.7%); use in conjunction with endoscopic mucosal resection (13; 43.3%); colon polyps (12; 40%); ulcerative colitis surveillance (9; 30%); gastric intestinal metaplasia (4; 13.3%); molecular imaging (2; 6.7%); and neuroendocrine hyperplasia (1; 3.3%). Needle-based CLE was cited as a potential application by four (13.3%). Six responses (20%) listed no potential applications. Twelve respondents (40%) described the technology as "experimental" or as a "research tool", and an additional seven (23.3%) described its use as currently "not ready for practice" or "undetermined".

Our study sheds light on physician practices and opinions regarding the current clinical utility of pCLE. Our data show that while many of the respondents believed pCLE had several potential applications, a majority (63%) felt that the technology was not yet ready for primetime. Cost of the equipment, need to learn a new technology, a learning curve associated with the technology, uncertain reimbursement, and potentially increased time spent for certain applications are some reasons which may slow the adoption of this new technology in community gastroenterology practices. In the face of an increasing array of uses, we believe there is a need for an analysis of the cost-effectiveness of the technology, and for an expert consensus on indications for which its use would be best-suited.

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Asmaa Gaber Abdou, Assistant Professor, Department of Pathology, Faculty of Medicine, Menofiya University, Shebein Elkom 002-048, Egypt

Adam S Cheifetz, MD, Assistant Professor, Clinical Director, Center for IBD, Beth Israel Deaconess Medical Center, 330 Brookline Ave, Boston, MA 02215, United States

Dr. Pankaj Garg, Consultant, Department of General Surgery, Fortis Super Speciality Hospital, Mohali, Punjab, Panchkula 134112, India

Ralph Graeser, PhD, Group Leader, Molecular and Cellular Biology, ProQinase GmbH, Breisacher Str. 117, Freiburg 79106, Germany

Wei Jia, PhD, Professor, Department of Nutrition, University of North Carolina at Greensboro, 500 Laureate Way, Suite 4226, Kannapolis, NC 28081, United States

Jin-Hong Kim, Professor, Department of Gastroenterology, Ajou University Hospital, San 5, Wonchon-dong, Yeongtong-gu, Suwon 442-721, South Korea

Michael Keese, Associate Professor, Clinic For Vascular and Endovascular Surgery, Johann Wolfgang Goethe Universität Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

Atsushi Masamune, MD, PhD, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Alan C Moss, MD, FACG, Assistant Professor of Medicine, Director of Translational Research, Center for Inflammatory Bowel Disease, Beth Israel Deaconess Medical Center, Harvard Medical School, Rose 1 / East, 330 Brookline Ave, Boston, MA 02215, United States

Kotaro Miyake, MD, PhD, Department of Surgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Pradyumna Kumar Mishra, MS, PhD, Professor, Division of Translational Research, Tata Memorial Centre, ACTREC, Navi Mumbai 410 210, India

Ricardo Marcos, PhD, Lab Histology and Embryology, Institute of Biomedical Sciences Abel Salazar, ICBAS, Lg Prof Abel Salazar, 2, Porto 4099-003, Portugal

Satoshi Osawa, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan

Dr. Stephan Johannes Ott, PhD, MD, Clinic for Internal Medicine I, University-Hospital Schleswig-Holstein (UK S-H), Campus Kiel, Arnold-Heller-Str. 3, Hs. 6, 24105 Kiel, Germany

Dr. Nagarajan Perumal, Compliance veterinarian, Center for Life Science, IACUC Office, National University of Singapore, 117456, Singapore

Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Andi Utama, PhD, Molecular Epidemiology Division, Mochtar Riady Institute For Nanotechnology, Jl. Boulevard Jend. Sudirman 1688, Lippo Karawaci-Tangerang, Banten 15810, Indonesia

Pietro Valdastri, MScEE, PhD, Assistant Professor of Industrial BioEngineering, The BioRobotics Institute , Scuola Superiore Sant'Anna, Polo Sant'Anna Valdera, Viale Rinaldo Piaggio, 34, 56025 Pontedera, Italy

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Events Calendar 2011

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AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

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Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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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]

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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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Books

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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

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

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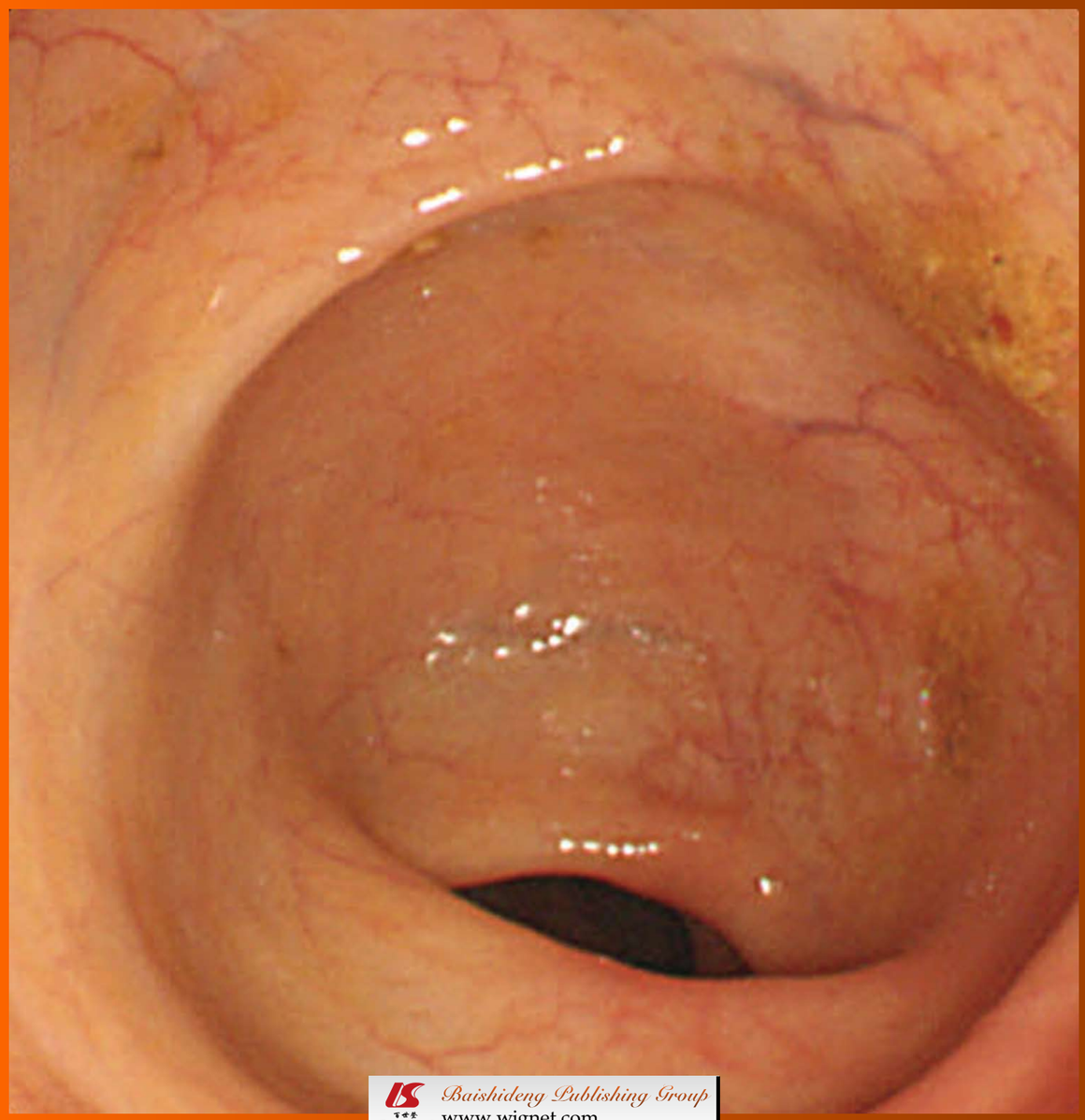

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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Beijing Baishideng BioMed Scientific Co., Ltd.
Room 903, Building D, Ocean International Center,
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Beijing 100025, China
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EDITORIAL

Liver transplantation for hepatocellular carcinoma on cirrhosis: Strategies to avoid tumor recurrence

Marco Vivarelli, Andrea Risaliti

Marco Vivarelli, Department of Surgery and Transplantation, University of Bologna, S Orsola Hospital, 40138 Bologna, Italy
Andrea Risaliti, Department of Hepatobiliopancreatic and Transplantation Surgery, Polytechnic University of Marche, Ospedali Riuniti, 60100 Ancona, Italy
Author contributions: Vivarelli M wrote the paper; Risaliti A provided critical expertise and helped with focusing the topics.
Correspondence to: Dr. Marco Vivarelli, Department of Surgery and Transplantation, University of Bologna, S Orsola Hospital, Via Massarenti 9, 40138 Bologna, Italy. vivarelli63@libero.it
Telephone: +39-051-6363721 Fax: +39-051-397661
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Abstract

Hepatocellular carcinoma (HCC) is one of the most frequent neoplasms worldwide and in most cases it is associated with chronic liver disease. Liver transplantation (LT) is potentially the optimal treatment for those patients with HCC who have a poor functional hepatic reserve due to their underlying chronic liver disease. However, due to the limited availability of donors, only those patients whose oncologic profile is favorable can be considered for LT. Despite the careful selection of candidates based on strict rules, 10 to 20% of liver transplant recipients who have HCC in the native cirrhotic liver develop tumor recurrence after transplantation. The selection criteria presently employed to minimize the risk of recurrence are based on gross tumor characteristics defined by imaging techniques; unfortunately, the accuracy of imaging is far from being optimal. Furthermore, microscopic tumor features that are strictly linked with prognosis can not be assessed prior to transplantation. Pre-transplantation tumor downstaging may allow transplantation in patients initially outside the selection criteria and seems to improve the prognosis; it also provides information on tumor biology. The main peculiarity of the transplantation setting, when this is compared with other modalities of treatment, is the need for pharmacological immunosuppression: this is based on drugs that have been demonstrated to increase the risk of tumor development. As HCC is an aggressive malignancy, immunosuppression has to be handled carefully in patients who have HCC at the time of transplantation and new categories of immunosuppressive agents should be considered. Adjuvant chemotherapy following transplantation has failed to show any significant advantage. The aim of the present study is to review the possible strategies to avoid recurrence of HCC after liver transplantation based on the current clinical evidence and the more recent developments and to discuss possible future directions.

Key words: Chemotherapy; Hepatocellular carcinoma; Immunosuppression; Liver transplantation; Tumor recurrence

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent neoplasms worldwide with more than 500 000 new cases diagnosed each year. In more than 90% of cases, HCC is associated with chronic liver disease, par-

ticularly disease caused by hepatitis B (HBV) or hepatitis C (HCV) viral infection[1].

Resective surgery of HCC with curative intent has two main limitations: the impaired functional reserve of the liver in the presence of cirrhosis often contraindicates surgery, while the persistence of the underlying chronic liver disease leads to recurrence of the tumor within the liver in more than 50% of cases in the long-term[2]. Furthermore, liver cirrhosis is by itself a progressive condition that substantially limits life expectancy.

For these reasons, liver transplantation (LT) theoretically represents the ideal treatment of HCC on cirrhosis as it removes both the tumor and the underlying condition that results in HCC.

However, the initial experience with LT for HCC in the eighties was discouraging, as recurrence rates of more than 50% were reported in many series; the indication for LT in the presence of HCC was therefore questioned[3-5].

In 1995, Mazzaferro *et al*[6] published the results of a prospective study where he demonstrated that, by adopting strict selection criteria with regard to the size and number of tumor nodules, it was possible to achieve a satisfactory long-term recurrence-free survival after LT. The criteria established in that study, known as the "Milan criteria" have from then on been the basis for the selection of patients with HCC on cirrhosis for transplantation. Currently, HCC on cirrhosis represents one of the main indications for LT: according to the report of the United Network for Organ Sharing (UNOS), in 2008, 17.5% of 6069 recipients of a liver graft from a deceased donor were HCC carriers, while 10 616 LTs have been performed up to 2009 in Europe on HCC patients according to the data of the European Liver Transplant Registry (ELTR).

Although in absolute terms the present results of LT for HCC are outstanding, considering that we are dealing with a highly aggressive tumor, 10% to 20% of recipients still develop tumor recurrence. This failure rate is of particular relevance in the transplantation field where great effort is made not to waste precious and rare resources such as liver grafts[7,8].

The purpose of the present review is to analyze possible strategies to avoid HCC recurrence following LT.

PATIENT SELECTION

Although they provide excellent results, the selection criteria introduced by Mazzaferro result in the exclusion of a great number of possible LT candidates whose tumor slightly exceeds the limits. In addition, in the original article the Milan criteria were verified by pathology of the explanted liver, while in the clinical setting HCC is staged by imaging techniques. The accuracy of imaging in preoperative staging is far from optimal and ranges between 14% and 80%[9-12] (Table 1).

Since 1996, attention has been focused on the possibility of recruiting a larger number of LT candidates whose tumor is beyond the Milan criteria but still within a safety zone in terms of likelihood of recurrence. Several proposals for revision of the Milan criteria have been made over the years, the most popular of which are summarized in Table 2[6,9,13,14]. The latest of these proposals is from Mazzaferro *et al*[14] and is known as the "up-to-seven" rule: the sum of the number of tumor nodule(s) and the maximum diameter of the nodule(s) must not exceed the value of seven.

A system has also been developed to predict the survival probability of a LT candidate with HCC as a function of size and number of tumor nodules; using freely accessible software it is possible to calculate the probability of survival linked to given tumor features. This software, called the Metroticket Calculator takes into account another parameter, the presence of microvascular tumor invasion (MVI) that has been shown to be intimately related to the biology of the tumor[15]. The importance of MVI as a determinant of recurrence has been known for years; histological grading has also been associated with tumor recurrence in several reports[16-17]. Unfortunately, MVI and histological grading can not be precisely assessed prior to transplantation; percutaneous biopsy of the tumor nodule can miss the area of the tumor where MVI is present or may fall in an area of the nodule were the tumor is more differentiated. In fact, grading varies within the same nodule and is eventually defined by the area were the highest degree of undifferentiation is found[18].

The main bias of all the selection systems currently in use is that they are based on the gross pathological features of the tumor. Apart from the difficulty of cor-

Table 1 Accuracy of imaging techniques in the pre-transplant staging of hepatocellular carcinoma

Author, yr, center	No. of pts	Accuracy in detecting No. of nodules (%)	Accuracy in detecting diameter of nodules (%)	Accuracy in assessing Milan criteria (%)
Yao FY, 2001, San Francisco	70	75	62-80	nr
Sotiropoulus GC, 2005, Essen	70	34	14	60
Shah SA, 2006, Toronto	118	75	78	57
Grasso A, 2006, London	74	80	80	nr

pts: Patients; nr: Not reported.

Table 2 Proposed criteria for liver transplantation in patients with hepatocellular carcinoma

Author, yr, center	No. of pts	Selection criteria	Survival
Mazzaferro V, 1996, Milan	48	1 nodule ≤ 5 cm or ≤ 3 nodules ≤ 3 cm	83% (4-yr RF)
Yao FY, 2001, San Francisco	70	1 HCC ≤ 6.5 cm or ≤ 3 HCC ≤ 4.5 cm with cumulative diameter ≤ 8 cm	75.2% (5-yr overall)
Toso C, 2008, Edmonton	288	Total HCC volume ≤ 115 cm^3	80% (5-yr overall)
Mazzaferro V, 2009, multicenter study	1556	No. of HCC nodules + maximum diameter (cm) ≤ 7	71% (5-yr overall) if MVI absent

pts: Patients; RF: Recurrence-free; HCC: Hepatocellular carcinoma; MVI: Microvascular tumor invasion.

rectly staging the tumor at imaging, size and number of nodules are not always related to the effective biology of the tumor. Esnaola showed that MVI is certainly more frequent in multiple and large tumors; however, 53% of patients with multiple nodules and 50% of patients with nodules larger than 4 cm in diameter did not display MVI at histology[19]. Jonas *et al*[20] has recently proposed an index based on DNA cytometry in tumor tissue that can establish the relative risk of recurrence; unfortunately, this index can not be evaluated prior to transplantation. Fiorentino *et al*[21] a few years ago demonstrated that biological markers such as membrane expression of E-cadherin and β-catenin, and nuclear expression of β-catenin were predictors of prognosis. These markers could be assessed on preoperative biopsy; however, the value of these markers has never been tested prospectively.

Serum alpha fetoprotein level at the time of LT is also linked to prognosis[22-24]; however, it is not considered in the more widely used selection systems for LT candidates.

PRE-TRANSPLANT DOWNSTAGING

Neo-adjuvant therapy prior to LT has been proposed mainly to slow the progression of HCC while the patient is on the waiting list, to avoid drop-out[25]. Other authors have proposed downstaging, within the Milan criteria, of tumors that were initially beyond the criteria, thus allowing transplantation[26,27]. Among the possible techniques used to downstage HCC, the most widely used is transarterial chemoembolization (TACE), followed by radiofrequency ablation (RFA)[28]. In 2008, Yao *et al*[29] reported the results of a prospective trial of downstaging that included 61 patients originally outside the Milan criteria; in the majority of cases, TACE was used for downstaging however, some patients were also treated with RFA or surgical resection. No tumor recurrences were observed at a median follow-up of 25 mo in the 35 patients who underwent LT following downstaging. There is some evidence that response to downstaging can be used as a prognostic factor to select patients with best prognosis after LT; in particular, it has been observed that the degree of tumor necrosis after TACE is linked to prognosis[30,31].

ADJUVANT CHEMOTHERAPY

Adjuvant chemotherapy has historically failed to show any advantage after resection of HCC[32]. In the setting of liver transplantation, the first relevant report on adjuvant chemotherapy was published by Olthoff and colleagues in 1995; a series of 25 LT recipients with HCC received intravenous fluorouracil, doxorubicin, and cisplatin for 6 mo after LT. Many of these patients had tumors well beyond the Milan criteria, 6 patients did not complete the treatment because of severe side-effects and a 3-year tumor-free survival of 46% was reported; the authors concluded that this result was satisfactory when compared to that achieved in an historical series of 17 patients transplanted between 1984 and 1988 whose tumor-free survival was worse[33].

In 2002, Roayaie published the results of a prospective study carried out on 43 patients who had tumors larger than 5 cm in diameter at the time of LT and were administered 6 cycles of systemic doxorubicin after surgery. In 11 of these 42 patients, adjuvant treatment was discontinued, mainly due to side-effects; recurrence-free survival was 48%[34].

In more recent years, two different prospective randomized trials showed no significant advantage of adjuvant therapy with systemic doxorubicin administered after LT[35,36].

Another recent report on adjuvant therapy with cisplatinum and adriamycin on a series of 12 LT recipients with poor prognostic factors again failed to show any advantage[37].

Although promising preliminary results have been reported with the use of targeted molecular drugs such as sorafenib in advanced HCC, the use of these agents in the transplantation field has not yet been assessed[38].

PHARMACOLOGICAL IMMUNOSUPPRESSION

Some 20 years ago, Yokoyama *et al*[39] from the Pittsburgh group noticed that the doubling time of HCCs that recurred after LT was significantly shorter than that observed in HCCs that arose in non-transplanted patients. The authors concluded that the accelerated growth rate of HCC in LT recipients was due to pharmacological immunosuppression.

Indirect evidence of a favoring effect of immunosuppression on tumor genesis comes from the observation that the incidence of malignancies is significantly higher in organ recipients than in the general population[40].

The cornerstone of pharmacological immunosuppression in organ transplantation is represented by the calcineurin inhibitors (CNIs), namely cyclosporine and tacrolimus. These agents affect T-cell recognition of alloantigen and signal transduction *via* the calcium-dependent calcineurin pathway. Besides inhibiting interleukin (IL)-2 expression, they increase transforming growth factor (TGF)-β 1, a potent inhibitor of IL-2-stimulated T-cell proliferation; unfortunately, TGF-β 1 depresses the natural killer cell-mediated anti-tumor immune response, and is implicated in the development of the metastatic process[41-43].

A study published in the Lancet by Dantal *et al*[44] in 1998, showed that there was a significant relationship between the dosage of cyclosporine administered to kidney transplant recipients and the development of posttransplant *de novo* malignancies, while Freise *et al*[45] demonstrated in an animal model that cyclosporine has an adverse effect on recurrence of hepatoma after liver transplantation.

In 2002, the group of Bologna first demonstrated that there was a direct relationship between the cumulative dosage of cyclosporine administered in the first year

after LT and HCC recurrence-free survival: the higher the dosage of cyclosporine given, the lower the recurrence-free survival[46]. This observation was confirmed by a subsequent study from the same group, where exposure to cyclosporine was shown to be an independent determinant of recurrence-free survival[47]. A later report published in 2008, showed that both tacrolimus and cyclosporine exposure were independently associated with HCC recurrence together with MVI and tumor histological grading[48].

A newer category of immunosuppressant drugs called m-TOR (mammalian target of rapamycin) inhibitors have raised a high degree of interest. These drugs are associated with strong immunosuppressant activity, due to the blocking of IL-2 stimulation of lymphocyte proliferation, and have a potential anti-cancer effect which has been demonstrated in the experimental setting. The anti-cancer effect is mainly related to the impairment of vascular endothelial growth factor (VEGF) production and the blockage of VEGF-induced vascular endothelial cell stimulation[41]. Several reports are available in the literature that seem to indicate that m-TOR inhibitors have some efficacy in reducing the incidence of *de novo* malignancies, or even arresting the progression of aggressive neoplasms after organ transplantation[49-56].

In the clinical setting, m-TOR inhibitors, namely sirolimus and everolimus, have been initially tested in association with CNIs to reduce renal damage associated with the use of these latter agents.

However, a report by Knetemann *et al*[57] published in 2004, showed that by minimizing the use of CNIs through the use of m-TOR inhibitors it was possible to achieve a satisfactory disease-free survival when transplanting patients whose HCC was beyond the Milan criteria.

Since then several centers have employed m-TOR inhibitors in patients with HCC; unfortunately, this has been done outside controlled multicentric trials.

Few reports have compared the results of LT for HCC in patients under CNIs-based immunosuppression to those observed under m-Tor inhibitor-based immunosuppression. The first of these reports comes from Bologna: in a matched case-control study where the clinical and pathological risk factors were identical in the two groups, the 3-year recurrence-free survival was 30% higher in patients who received sirolimus as part of their immunosuppressive schedule than in those who remained on standard treatment based on tacrolimus[58].

Some confirmation of this finding has been provided by Toso *et al*[59], who analyzed the Scientific Registry of Transplant Recipients in the United States to ascertain whether survival after LT in HCC patients was influenced by type of immunosuppression: the author concluded that sirolimus-based maintenance therapy was associated with improved survival.

CONCLUSION

Of the possible strategies to avoid tumor recurrence after LT in HCC patients, the careful selection of candidates based on macroscopic tumor findings is a cornerstone that seems to have been exploited to the maximum. Thus, better characterization of tumor biology is necessary; the main problem in this field is outlining markers assessable on tumor biopsy.

While there is no evidence of the efficacy of adjuvant chemotherapy, more research on immunosuppressive agents is warranted. It is reasonable to suggest that CNIs should be administered at their effective minimum in HCC LT recipients. Although definitive evidence is not yet available, there are enough data to support the use of m-TOR inhibitors in association with CNIs for immunosuppression, or when the risk of tumor recurrence is high, due to the presence of poor prognostic factors, as the main immunosuppressants in a CNI-sparing regimen.

Further prospective studies on large numbers of patients are warranted to confirm the efficacy of this strategy and to quantify the extent of the possible gain in terms of recurrence-free survival.

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EDITORIAL

Pancreatic metastases from renal cell carcinoma: The state of the art

Roberto Ballarin, Mario Spaggiari, Nicola Cautero, Nicola De Ruvo, Roberto Montalti, Cristina Longo, Anna Pecchi, Patrizia Giacobazzi, Giuseppina De Marco, Giuseppe D'Amico, Giorgio Enrico Gerunda, Fabrizio Di Benedetto

Roberto Ballarin, Mario Spaggiari, Nicola Cautero, Nicola De Ruvo, Roberto Montalti, Cristina Longo, Giuseppe D'Amico, Giorgio Enrico Gerunda, Fabrizio Di Benedetto, Liver and Multivisceral Transplant Center, University of Modena and Reggio Emilia, 41100 Modena, Italy
Anna Pecchi, Department of Radiology, University of Modena and Reggio Emilia, 41100 Modena, Italy
Patrizia Giacobazzi, Giuseppina De Marco, Unit of Oncological Radiotherapy, University of Modena and Reggio Emilia, 41100 Modena, Italy
Author contributions: Ballarin R was involved in the conception of the editorial, was primary writer of the manuscript, performed manuscript editing and manuscript review; Spaggiari M, Cautero N, De Ruvo N, Montalti R, Longo C, Pecchi A, Giacobazzi P, De Marco G, D'Amico G, Gerunda GE and Di Benedetto F reviewed the manuscript and contributed to manuscript preparation.
Correspondence to: Roberto Ballarin, MD, Liver and Multivisceral Transplant Center, University of Modena and Reggio Emilia, No. 71, Via del Pozzo, 41100 Modena, Italy. ballarinroberto@hotmail.com
Telephone: +39-059-4223683 Fax: +39-059-4223765
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Abstract

Pancreatic metastases are rare, with a reported incidence varying from 1.6% to 11% in autopsy studies of patients with advanced malignancy. In clinical series, the frequency of pancreatic metastases ranges from 2% to 5% of all pancreatic malignant tumors. However, the pancreas is an elective site for metastases from carcinoma of the kidney and this peculiarity has been reported by several studies. The epidemiology, clinical presentation, and treatment of pancreatic metastases from renal cell carcinoma are known from single-institution case reports and literature reviews. There is currently very limited experience with the surgical resection of isolated pancreatic metastasis, and the role of surgery in the management of these patients has not been clearly defined. In fact, for many years pancreatic resections were associated with high rates of morbidity and mortality, and metastatic disease to the pancreas was considered to be a terminal-stage condition. More recently, a significant reduction in the operative risk following major pancreatic surgery has been demonstrated, thus extending the indication for these operations to patients with metastatic disease.

Key words: Pancreatic metastases; Renal cell carcinoma; Pancreatic surgery; Prognostic factors; Therapeutic approach; Radiological findings

Peer reviewer: Anna S Gukovskaya, Professor, VA Greater Los Angeles Health Care System, University of California, Los Angeles, 11301 Wilshire Blvd, Los Angeles, CA 91301, United States

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INTRODUCTION

Renal cell carcinoma (RCC) remains an important cause of cancer death in the United States, with an estimated 58 240 new cases and approximately 13 040 deaths in 2010[1].

The natural history of RCC is characterized by a high 5-year survival (up to 95%) when the tumor is limited to the kidney (stage 1)[2-4]. Among patients with RCC, 20% to 30% have metastases at presentation, and up to 40%-50% will develop widespread metastatic disease after nephrectomy. The 5-year survival rate is < 10%-15% once metastases have spread[5,6].

Pancreatic metastases are rare, with a reported incidence varying from 1.6% to 11% in autopsy studies of patients with advanced malignancy[7,8]. In clinical series, the frequency of pancreatic metastases ranges from 2% to 5% of all pancreatic malignant tumors[9-12].

The tumors that metastasize most commonly to the pancreas are RCC, lung cancer (adenocarcinoma and non-small cell lung carcinoma), lobular breast carcinoma, and colorectal adenocarcinoma, followed by melanoma, soft-tissue sarcoma (leiomyosarcoma), and a large number of other neoplasms[7,8,13-19].

However, the pancreas is an elective site for metastases from carcinoma of the kidney and this peculiarity has been reported by several studies[11,19-27]. Pancreatic metastases from RCC are frequently the only metastatic site and they typically occur a long time after nephrectomy[20,23,26].

MECHANISM OF DISEASE

According to Sellner *et al*[23], local lymphogenous or local venous spread through abnormal lymphatic or venous communications between the RCC and the pancreas[11,21,28] cannot play an important role, because this would fail to explain the lack of relationship between the site of isolated pancreatic metastases and the side affected by the primary tumor: i.e., the left or right kidney. The localization anywhere in the pancreas irrespective of the site of the RCC argues, instead, in favor of a hematogenous systemic spread. However, a systemic spread would not explain the discrepancy between the relative frequency of multiple pancreatic metastases and the absence of metastases to other organs. The most likely explanation for this unique behavior of isolated pancreatic metastases would seem to lie in the special biology of the tumor. Tumor cells apparently have a high affinity for the parenchyma of the pancreas and only there do they find the conditions they need to mature to manifest metastases. The high affinity of some renal cancer cells for the parenchyma of the pancreas is supported by some reports[23] of metachronous late metastases which again occurred solely in the residual pancreas[11,22]. The results of basic research, which is slowly unraveling the local biochemical mechanisms that underlie the development of metastases, are promising, so it is hoped that the biochemical causes of the unique spreading pattern of some RCC will perhaps be understood one day[29].

CLINICAL CHARACTERISTICS

As with primary pancreatic cancer, the early signs and symptoms of isolated pancreatic metastases are often non-specific and subtle. Isolated pancreatic metastases are often found with routine surveillance imaging for primary lesions or as an incidental finding on imaging done for an unrelated indication.

Pancreatic metastasis of RCC generally occurs in the seventh decade of life and is usually asymptomatic[14,15,30], although cases of abdominal pain, gastrointestinal bleeding caused by duodenal involvement, weight loss, jaundice, and pancreatitis due to pancreatic duct obstruction have been described[14,15,26,31].

Most of the studies reviewed report mean time intervals greater than 10 years, and a period as long as 32.7 years has been described[12,19,21-24,26,32]. In fact, renal carcinoma is the primary tumor that metastasizes to the pancreas following the greatest disease-free interval. This singular feature of RCC makes long-term follow-up essential in these patients.

Multiple lesions throughout the pancreatic gland have been more frequently detected in patients with RCC than in those with other primary tumors. Other single-center series reported similar frequencies of multifocality with respect to pancreatic RCC metastases, ranging from 20% to 45%[12,22,23,26,27,33,34]. In the review by Sellner *et al*[23], multiple lesions occurred in 39% of the 187 patients. This issue has important clinical implications: surgical treatment of patients with suspected pancreatic metastases from RCC must take into account the high probability that the patient will have more than one pancreatic lesion.

In a recent review by Masetti *et al*[35], univariate survival analyses conducted in a subgroup of patients with RCC metastases (n = 157), with a median follow-up of 24 mo (range 1 to 134 mo), showed that the factors associated with worse survival were symptoms at diagnosis, and a disease-free interval less than 2 years in patients with metachronous lesions.

RADIOLOGICAL FINDINGS

Computed tomography and magnetic resonance imaging

The diagnosis of pancreatic metastasis is usually made on radiological or endoscopic criteria, since most patients do not present related symptoms.

The disparity in prognosis and management of patients with primary and secondary pancreatic tumors, as well as the fact that in very selected cases a radical surgical resection can be considered as treatment of pancreatic metastases and achieve prolonged survival, underline the importance of detection and characterization of these lesions by computed tomography (CT) and magnetic resonance imaging (MRI)[9,24,36].

Identifying the sites and extent of the metastatic lesions within the pancreas helps determine the feasibility and extent of pancreatic surgery.

There is comparatively little difficulty in identifying large lesions within the pancreas when using a standard CT technique because they typically deform the contour of the pancreas. Small lesions, however, may be easily missed. The CT evaluation should be performed with

a multidetector CT, a high rate of intravenous contrast media injection (3-5 mL/s) and scanning during the arterial (20 s delay) and portal (50-60 s delay) phases. The MR evaluation should be performed with a 1.5 or 3 T scanner with T1 and T2 weighted sequences, without and with contrast media injection at dynamic acquisition in arterial, portal and venous phases.

The growing use of imaging techniques, in particular of CT in the periodic follow-up of oncological patients, allows earlier detection of small pancreatic metastases, and in most cases the oncological background and existence of previous follow-up permit a correct diagnosis. Moreover, in controversial cases, CT can also be considered as an important tool in providing guidance to biopsy in order to obtain a definitive diagnosis[9,10,36,37].

Imaging features of metastatic pancreatic tumors point to their primary origin and the enhancement pattern reflects the vascular perfusion of the lesions. RCC metastases are usually hypervascular and consequently show intense homogeneous contrast enhancement in the arterial phase, greater than normal pancreatic parenchyma, and a tendency to pass undetected in more delayed post-contrast phases, since the difference in density between the mass and the normal pancreatic gland decreases.

In lesions larger than 1.5 cm, rim enhancement with hypodense central areas of necrosis may be seen.

Pancreatic metastases do not appear to show a predilection for a particular part of the pancreas[37,38]. Three types of metastatic involvement of the pancreas have been described in the literature. The most common type of all metastases and in particular of RCC metastases, reported in 50%-73% of cases, is that of a solitary, localized, well-defined mass. A second pattern of multiple pancreatic lesions has been reported in 5%-10% of cases, and a third pattern of diffuse metastatic infiltration causing generalized enlargement of the organ in 15%-44% of cases[9,30,39,40].

Other features described in this type of lesion are calcifications, ductal and biliary obstruction, vascular extension, and cystic degeneration, although these findings are quite non-specific.

On MRI, pancreatic lesions typically appear hypointense, compared with normal gland tissue on unenhanced T1 weighted images, both with and without fat saturation. Following intravenous contrast media injection, homogeneous enhancement is typically demonstrated in smaller lesions and rim enhancement in larger ones. On T2 weighted images, the lesions are slightly heterogeneous and moderately hyperintense. Hypointense nodules are sometimes visible on T2 weighted images, especially in the diffusely enlarged type. Diffusion weighted imaging was recently included in the standard MRI protocol; metastatic lesions typically also show a hyperintensity signal in sequences with high b-values (700-1000).

When hypervascular pancreatic lesions are depicted on contrast-enhanced CT and MRI, differentiation from a primary pancreatic endocrine tumor might be difficult. The other differential diagnoses include metastasis of hypervascular neoplasm, intrapancreatic accessory spleen and vascular lesions, such as arteriovenous fistulas or aneurysms of the splenic artery[10,19,22,32,41]. In most cases, the oncological background and existence of previous follow-up of the neoplastic disease allows a correct diagnosis, and in controversial cases a CT-guided biopsy can be performed.

Endoscopic ultrasonography

The development of imaging technology has improved the detection and differentiation of small lesions but difficulties remain. Pancreatic lesions are commonly undetected at an early stage since the symptoms of small pancreatic lesions are frequently vague and non-specific.

Endoscopic ultrasonography (EUS) is a highly sensitive diagnostic method for detection of pancreatic lesions, especially small lesions.

Pancreatic metastases appear as solid intraparenchymal space-occupying lesions with an internal structure that is much more hypoechoic than the normal pancreatic tissue, or isoechoic. These lesions are homogeneous, round, well circumscribed and associated with enhancement through transmission of the ultrasonic beam[36,42,43].

At Power Doppler or Color Doppler evaluation and with ultrasound contrast agent (CE-EUS), metastatic pancreatic tumors from renal cell carcinomas are hypervascular (hypervascular enhancement compared with the surrounding pancreatic tissue).

F-18 fluorodeoxyglucose positron emission tomography

There is no firm consensus regarding the role of this technique in pancreatic cancer and especially in metastases from RCC.

Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) has not been extensively studied for the evaluation of distant metastases from RCC. Reported studies of FDG-PET in metastatic RCC have involved few patients, and most series have compared the results of FDG-PET with clinical outcome determined by follow-up with conventional anatomic radiological techniques; moreover, pathologic confirmation of metastatic disease, if performed, was usually combined with radiological follow-up for reporting results.

In a study reported by Ramdave *et al*[44], FDG-PET identified distant metastases from RCC in six out of six patients evaluated for possible metastatic disease. Presence of metastatic disease was confirmed by pathology (fine-needle aspiration cytology) in only one of these six patients. In the same study, FDG-PET detected unsuspected metastatic disease not seen on CT in two of 17 patients evaluated for primary RCC. Metastatic disease was confirmed by biopsy at laparotomy in only one of these cases. In another study by Brouwers *et al*[45], among 20 RCC patients with 112 distant metastatic lesions evaluated by FDG scintigraphy and followed clinically, FDG-PET detected 69% (77 of 112) of the metastatic lesions. Of these, 32 lesions had not been detected by routine imaging modalities.

Table 1 Studies of renal cell cancer that include more than five patients

Ref.	Yr	*n*	5-yr survival	Median survival
Butturini *et al*[56]	1998	5	NR	24.5 mo
La Borgne *et al*[15]	2000	5	0%	35.5 mo
Kassabian *et al*[11]	2000	5	67%	Not reached
Ghavamian *et al*[59]	2000	11	81%	120 mo
Hioits *et al*[14]	2001	10	NR	4-8 yr
Faure *et al*[21]	2001	9	88%	Not reached
Law *et al*[22]	2003	14	75%	Not reached
Wente *et al*[27]	2005	15	NR	Not reached
Kohler *et al*[61]	2006	5	100%	Not reached
Crippa *et al*[10]	2006	5	80%	Not reached
Eidit *et al*[57]	2007	7	88%	Not reached
Varker *et al*[60]	2007	5	NR	Not reached
Bahra *et al*[55]	2007	9	100%	Not reached
Zerbi *et al*[12]	2008	23	88%	Not reached
Reddy *et al*[54]	2008	21	45%	58 mo
Tanis *et al*[63]	2009	10	NR	Not reached
Masetti *et al*[35]	2010	6	NR	Not reached
Konstantinidis *et al*[62]	2010	20	61%	8.7 yr

NR: Data not reported. Survival data is from the time of resection of the pancreatic metastasis, not from diagnosis of the primary tumor.

Using FDG-PET for restaging 36 patients with advanced RCC, Safaei *et al*[46] demonstrated a sensitivity and specificity of 87% and 100%, respectively.

Majhail *et al*[47] calculated the sensitivity, specificity, positive predictive value, and negative predictive value of FDG-PET for detection of distant metastases from RCC. They observed the sensitivity of FDG-PET to be 63.6%; however, in the same work the authors noted that FDG-PET was more sensitive for imaging larger lesions (sensitivity was 83.3% for lesions > 1.5 cm and 92.9% for lesions > 2.0 cm in size). Again, true-positive lesions were larger in size (mean size, 2.2 cm) compared with false-negative lesions (mean size, 1.0 cm).

Overall, FDG-PET scintigraphy is not a very sensitive imaging modality for the evaluation of metastatic RCC and may not adequately characterize small metastatic lesions. However, positive FDG-PET is predictive for the presence of RCC in imaged lesions, may complement anatomic radiological imaging modalities, and may alleviate the need for a biopsy in selected situations. A negative FDG-PET, however, does not rule out active malignancy.

PET could be used in early assessment of the response to chemotherapy, radiotherapy or both modalities. Indeed, the ability to predict therapeutic response early during a course of treatment is important since prognosis is poor, lifespan is limited and toxicity in non-responding patients is not acceptable.

OUTCOME OF SURGERY

Resection of liver, lung, and brain metastases has proven to be effective in the treatment of several types of tumors. The strongest evidence in favor of this practice exists for colorectal cancer, in which the resection of liver metastases has been shown to prolong patient survival and to improve the quality of life[16]. Moreover, study results published in the literature suggest that the resection of metastases from other types of tumors can improve patient outcome[48-50].

Although pancreatic surgery is considered one of the most technically demanding and challenging surgical disciplines, steady improvements in surgical techniques and advances in perioperative supportive care, based on a modern interdisciplinary approach that includes anesthesiology, oncology, radiology, nutritional science, and nursing, has reduced mortality to less than 5% in high-volume centers.

Standardized pancreatic resection adapted to the location of the tumor, in terms of partial pancreaticoduodenectomy, distal pancreatectomy, and total pancreatectomy, is generally recommended for the management of isolated pancreatic metastases. Because of the high recurrence rate[20], atypical local resection is confined to some exceptional cases.

Treatment recommendations for multiple pancreatic metastases vary. Whereas some advise total pancreatectomy, others[51-53] critically reject surgery on the assumption that multiple pancreatic metastases signal incipient fatal disseminated metastatic disease.

A few isolated articles describing small series of patients who underwent surgery for pancreatic metastatic disease have recently been published, reporting encouraging results[10,14,54].

We have identified 18 studies addressing pancreatic metastasectomy for RCC with five or more patients[10-12,14,15,22,27,35,54-63]. Survival results from these studies are listed in Table 1. Of the 12 studies that included long-term survival data, seven (58.3%) reported 5-year survival rates from the time of the pancreatic metastasectomy higher than 80%.

OUTCOME WITHOUT RADICAL RESECTION

In the case of unresectable disease, surgical or endoscopic palliation in association with chemotherapy can improve the quality of life but not survival, even if, in rare cases of renal cell metastases, an improved survival has been reported after palliative surgery[34,64].

In the series described by Zerbi *et al*[12], 13 patients who did not undergo resection, because of locally advanced disease or due to the presence of extrapancreatic disease, all received immunotherapy with interferon (IFN) α as first-line treatment; 6 patients underwent other therapies (interleukin-2 in 3 cases, thalidomide in 1 case, radiotherapy in 1 case, bone marrow transplantation in 1 case). The median survival was 27 mo (range 17.5 to 50.2 mo).

In this group of patients who did not undergo surgical resection, 2- and 5-year survival rates were 59% and 47%, respectively.

Table 2 Prognostic factors for risk group stratification model[70]

Factors for risk group	Poor prognostic category and factors
Factor	Category
Time from diagnosis to study entry	< 12 mo
Hemoglobin	Below lower limit of reference range
Lactate dehydrogenase	> 1.5 x upper limit of reference range
Corrected serum calcium	> 10.0 mg/dL
Previous radiotherapy	Yes
No. of metastatic sites	⩾ 2
Risk Group	No. of factors
Favorable	Zero or one
Intermediate	Two
Poor	Three or more

Patients are stratified into three groups depending on the number of poor prognostic factors found, as follows: favorable risk group, patients with zero or one poor prognostic factor; intermediate risk group, presence of two poor prognostic factors; poor risk group, presence of three or more poor prognostic factors.

PROGNOSTIC FACTORS

No prospective randomized or case-controlled studies have been performed to evaluate the role of surgical resection. In addition, many of the existing retrospective studies are limited because of the small number of patients who were treated for prolonged periods of time.

The pooled data from the 18 studies in Table 1 do not contain adequate detail to assess features for the prediction of outcome in the context of pancreatic metastasectomy for RCC. However, analyzing the three largest series reported in the literature it can be seen firstly that in their series of 21 patients who underwent pancreatic metastasectomy for RCC, Reddy *et al*[54] reported that tumor size greater than 4 cm ($P = 0.13$) and perineural invasion ($P = 0.26$) were not associated with significant differences in outcome, whereas lymph node involvement (hazard ratio 24.1, $P = 0.01$) and vascular invasion (hazard ratio 20.4, $P = 0.007$) were each associated with worse overall survival. The median survival was 4.8 years (range 0.35 to 18.3 years). Metachronous RCC had a similar survival to synchronous lesions ($P = 0.98$).

Zerbi *et al*[12] addressed the question of whether or not pancreatic metastasectomy changes the progression of RCC. They assessed a non-matched control group of patients with non-operatively managed pancreatic RCC metastasis. In this study, 13 out of 36 patients with pancreatic RCC were not offered surgery on the basis of functional status or extent of disease. These 13 patients were the control group, and their outcome was compared with outcomes for patients undergoing pancreatic metastasectomy. For the entire cohort, the median survival was 27 mo (range 0.6 to 222 mo) and 5-year survival was 47%. Patients who had tumors resected had a 5-year survival of 88% compared with 47% for the non-operative group ($P = 0.026$), suggesting a benefit of pancreatic metastasectomy.

On the other hand, in their series of 20 patients with pancreatic metastases from RCC, Konstantinidis *et al*[62] reported that, on univariate analysis, the number of metastatic nodules (solitary *vs* multiple, $P = 0.87$), the size of metastases (greater or smaller than median of 3 cm, $P = 0.78$), the location of the metastases ($P = 0.72$), or an R1 resection ($P = 0.62$) had no prognostic significance for overall survival. Hemoglobin values below the lower level of the reference range also did not predict worse outcome ($P = 0.99$). Patients had median and mean follow-up of 36.8 and 38.1 mo, respectively (range 0.5 to 143 mo). Their median survival from the time of metastasectomy was 8.7 years (range 1.3 to 12 years) and 5-year actuarial survival was 61%.

However, the treatment of pancreatic metastases from RCC remains controversial because of the relatively low frequency of this localization and the complex natural history of RCC, ranging from cases with a long disease-free interval to others with early development of widespread metastases[65].

To predict the behavior of disease in patients with metastatic RCC, several prognostic models have recently been developed[66-68]. In particular, Motzer *et al*[68,69] from Memorial Sloan-Kettering Cancer Center (MSKCC) proposed categorizing patients with metastatic RCC into one of three classes by using a model with five prognostic factors. This prognostic score was recently validated and modified by a study from the Cleveland Clinic Foundation group[70](Table 2).

CHEMOTHERAPY

Since RCC is highly resistant to chemotherapy, interleukin-2 or IFN α have been widely used as first-line treatment of metastatic disease. These agents have limited efficacy and are associated with considerable toxic effects. Response rates with these cytokines were low (5% to 20%), the median overall survival was approximately 12 mo, and the overall survival rate at 5 years was less than 10%[71-75].

Several molecularly targeted therapies have recently shown substantial clinical efficacy in patients with advanced RCC, in particular the multitargeted tyrosine kinase inhibitors (such as sunitinib and sorafenib), anti-vascular endothelial growth factor (VEGF) antibody (such as bevacizumab), and mammalian target of rapamycin (mTOR) pathway inhibitors (such as everolimus and temsirolimus). The treatment regimen is based on the MSKCC risk stratification, on the type of the tumor (clear cell renal carcinoma or not clear cell renal carcinoma), and on the previous treatment (cytokines or multitargeted therapy). Sunitinib is chosen as first-line therapy in patients with favorable or intermediate prognosis. Two phase 2 trials on sunitinib in patients treated with cytokines as first-line therapy showed that the objective response rates (as assessed by investigators and by an independent review) were 34%-40%, better than with INF α[76,77].

The subsequent randomized phase 3 trial enrolled 750 patients. There were 375 patients in the sunitinib group and 372 in the IFN group. The median progression-free survival (PFS) was 11 mo for patients in the sunitinib group and 5 mo for patients in the IFN group

Table 3 Therapeutic algorithm

Patients with clear cell renal carcinoma		First-line therapy	Second-line therapy
Without previous treatment	Prognostic grade: favorable or intermediate prognosis	Sunitinib or Bevacizumab + interferon α	IL-2 high dose or clinical trial
	Prognostic grade: poor	Temsirolimus	Sunitinib or clinical trial
With previous treatment	With cytokines	Sorafenib	Sunitinib
	With multitargeted therapy	Everolimus	Tyrosine kinase inhibitor or clinical trial
Patients with not clear cell renal carcinoma		Temsirolimus	Sunitinib or sorafenib

($P < 0.001$), with a 58% reduction in the risk of progression. Sunitinib also improved the objective response rates (47% *vs* 12%)[78]. Patients with this prognostic grade can also be treated with bevacizumab plus IFN. In the first phase 2 trials, bevacizumab improved PFS with respect to placebo (4.8 mo *vs* 2.5 mo)[79].

The AVOREN study[80], a randomized phase 3 trial, enrolled 649 patients with 327 patients on IFN plus bevacizumab and 322 patients on IFN plus placebo. Bevacizumab plus IFN showed better PFS (median 10.2 mo *vs* 5.4 mo; $P = 0.0001$) and response rate (31% *vs* 13%). The mortality risk fell by about 14%. An American phase 3 trial (CALGB 90206)[81] confirmed the good results of bevacizumab.

In patients with poor prognosis, temsirolimus is the first option. A phase 3 trial[82] compared 3 therapeutic regimens, temsirolimus *vs* IFN *vs* the combination of these 2 agents. Temsirolimus alone improved both PFS and overall survival (OS), with a reduction of the mortality risk (14%). It is also the first choice in patients with not clear cell renal carcinoma.

Sorafenib showed superiority as a second-line therapy over placebo in terms of PFS and OS in patients treated with cytokines. The TARGET study[83], a phase 3 trial, randomized 903 patients; there were 451 patients in the sorafenib group and 452 in the placebo group. The median overall survival was 19.3 mo for patients in the sorafenib group and 15.9 mo for patients in the placebo group ($P = 0.02$). PFS was also significantly prolonged with sorafenib treatment (5.5 mo *vs* 2.8 mo; $P < 0.001$), with a 49% reduction in the risk of progression. Significantly more patients in the sorafenib group than in the placebo group had partial responses or stable disease ($P < 0.001$).

In patients treated previously with multitargeted therapy, everolimus can be the first choice. A phase 3 trial compared everolimus *vs* placebo in patients treated with sorafenib and/or sunitinib. Everolimus doubled the PFS compared with placebo (4 mo *vs* 1.9 mo) and decreased the risk of recurrence.

Based on all the clinical trials, we can summarize all the evidence in a therapeutic algorithm (Table 3). In the future, personalized treatments will be prescribed according not only to clinical criteria, but also to biological and molecular factors such as for breast and colorectal cancer.

RADIOTHERAPY

Pancreatic metastases are commonly treated with surgical resection. However, such a resection cannot always be performed safely, especially in elderly patients or those with diabetes mellitus. Pancreatic metastases of RCC may be treated successfully with radiation therapy (RT).

In the literature there is only one case report of four patients[84]. These patients presented with multiple pancreatic metastases of RCC and were treated with RT combined with IFN α. Radiotherapy was delivered at 2 Gy/fraction, up to 50 Gy in 25 fractions, with three-dimensional conformal radiation therapy technique and 10 MV photon beams from linear accelerators.

In three patients, stable local disease persisted for an average time of 33.6 mo (range11 to 69 mo) after RT. In one patient, a partial response was obtained, lasting 25 mo after RT.

The European Association of Urology guidelines recommend RT for the treatment of brain and bone lesions to induce symptomatic relief of metastases from RCC. To our knowledge, there are no other previous reports of radiotherapy for pancreatic metastases of RCC.

In patients with pancreatic metastases of RCC, surgical resection with or without systemic treatment, such as cytokine therapy or antiangiogenic therapy, should be considered if complete resection is possible.

On the other hand, systemic treatment with or without RT to induce symptomatic relief and to prevent disease progression can be considered for high-risk patients, such as those with a poor performance status, diabetes mellitus, and older age.

ALTERNATIVE APPROACHES

Treatment options for unresectable pancreatic metastases of RCC are limited and new therapeutic measures should be advocated. Radiofrequency ablation (RFA) is a local ablative method that can destroy the tumor by thermal coagulation and protein denaturation. RFA has been used successfully in the treatment of unresectable solid tumors in the liver, lung, kidney, brain, breast, prostate, bone, adrenal glands and spleen[85-92]. Application of RFA to the pancreas presents potential problems related to anatomical considerations and particular properties of the pancreatic parenchyma. The risk of inadvertent thermal injury to the distal common bile duct, duodenum, transverse colon and portal vein is considerable with RFA. In addition, thermal injury to normal pancreatic tissue may cause acute necrotizing pancreatitis, pancreatic fistula or pancreatic ascites[93-101].

Elias *et al*[94] reported two cases of multiple pancreatic metastases from RCC treated by RFA. Both patients manifested acute necrotizing pancreatitis with massive destruction of normal pancreatic parenchyma.

Hadjicostas *et al*[96], based on results obtained in four patients, concluded that RFA seems to be a feasible, potentially safe and promising option in patients with advanced and non-resectable pancreatic cancer. Girelli *et al*[102], based on results obtained in 50 patients, showed that RFA of

locally advanced pancreatic cancer is feasible and relatively well tolerated, with a 24% complication rate.

Postoperative observation (clinical surveillance, laboratory tests and imaging studies) is mandatory because of the potential for major or minor, early or later complications. The most frequent complications encountered in the earlier postoperative period (within 1 wk) are fluid collection, pancreatic fistula, duodenal perforation and vascular damage. At later times, digestive or abdominal bleeding, infections or abscesses are more common. Severe acute pancreatitis is a rare complication[96]: in Girelli's[102] study, there was only one case, and none were reported in Wu's study[101]. Major complications are frequently present with RFA of pancreatic head tumors, mainly owing to the closeness of the duodenum. These lesions are more difficult to treat, as reported previously[101].

While waiting for other studies to clarify its role, RFA should be performed in high-volume centers by an experienced surgical team devoted to pancreatic surgery.

CONCLUSION

Thus, as suggested by Zerbi *et al*[12], a high index of suspicion is necessary for all patients with a history of RCC, and they should be monitored lifelong to allow the early detection of recurrence.

Because of the possibility of substantial morbidity after pancreatic resection and the questionable benefit of metastasectomy in some patients, pancreatic metastasectomy should be offered only after a thoughtful and systematic selection process. Ideally, this process would involve a multidisciplinary team that includes a medical oncologist and an experienced pancreatic surgeon. Once the decision is made to proceed with resection, evidence suggests that the procedure should be performed at a high-volume center[103,104]. In agreement with Reddy *et al*[54], we suggest the following criteria for the selection of patients for pancreatic metastasectomy: primary cancer type that is associated with successful outcome, control of the primary cancer site, isolated metastases, resectability of the metastasis, and patient fitness to tolerate pancreatectomy.

Prospective studies would better address the role of surgical therapy in pancreatic metastasectomy. However, because of the rarity of these lesions, such a trial can only be performed in a multi-institutional setting and possibly only for RCC metastases because of their high relative incidence compared with other cancers.

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REVIEW

Spectrum of mucin-producing neoplastic conditions of the abdomen and pelvis: Cross-sectional imaging evaluation

Nam Kyung Lee, Suk Kim, Hyun Sung Kim, Tae Yong Jeon, Gwang Ha Kim, Dong Uk Kim, Do Youn Park, Tae Un Kim, Dae Hwan Kang

Nam Kyung Lee, Suk Kim, Department of Radiology, Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, Busan 602-739, South Korea
Hyun Sung Kim, Tae Yong Jeon, Department of Surgery, Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, Busan 602-739, South Korea
Gwang Ha Kim, Dong Uk Kim, Department of Internal Medicine, Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, Busan 602-739, South Korea
Do Youn Park, Department of Pathology, Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, Busan 602-739, South Korea
Tae Un Kim, Department of Radiology, Yangsan Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, Yangsan 626-770, South Korea
Dae Hwan Kang, Department of Internal Medicine, Yangsan Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, Yangsan 626-770, South Korea
Author contributions: Kim S contributed to study conception and design; Lee NK, Kim HS, Jeon TY, Kim GH, Kim DU, Park DY, Kim TU, and Kang DH contributed to acquisition of data, or analysis and interpretation of data; Lee NK and Kim S contributed to drafting the article or revising it critically for important intellectual content; Kim S gave final approval of the version to be published.
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Correspondence to: Suk Kim, MD, Department of Radiology, Pusan National University Hospital and Medical Research Institute, Pusan National University School of Medicine, Pusan National University, No. 1-10, Ami-Dong, Seo-Gu, Busan 602-739, South Korea. kimsuk@medimail.co.kr
Telephone: +82-51-2407354 Fax: +82-51-2447534
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Abstract

Various mucin-producing neoplasms originate in different abdominal and pelvic organs. Mucinous neoplasms differ from non-mucinous neoplasms because of the differences in clinical outcome and imaging appearance. Mucinous carcinoma, in which at least 50% of the tumor is composed of large pools of extracellular mucin and columns of malignant cells, is associated with a worse prognosis. Signet ring cell carcinoma is characterized by large intracytoplasmic mucin vacuoles that expand in the malignant cells with the nucleus displaced to the periphery. Its prognosis is also generally poor. In contrast, intraductal papillary mucinous neoplasm of the bile duct and pancreas, which is characterized by proliferation of ductal epithelium and variable mucin production, has a better prognosis than other malignancies in the pancreaticobiliary tree. Imaging modalities play a critical role in differentiating mucinous from non-mucinous neoplasms. Due to high water content, mucin has a similar appearance to water on ultrasound (US), computed tomography (CT), and magnetic resonance imaging, except when thick and proteinaceous, and then it tends to be hypoechoic with fine internal echoes or have complex echogenicity on US, hyperdense on CT, and hyperintense on T1- and hypointense on T2-weighted images, compared to water. Therefore, knowledge of characteristic mucin imaging features is helpful to diagnose various mucin-producing neoplastic conditions and to facilitate appropriate treatment.

Key words: Mucin; Neoplasm; Ultrasound; Computed tomography; Magnetic resonance; Abdomen and pelvis

Peer reviewer: Edward J Ciaccio, PhD, Research Scientist, Department of Medicine, HP 804, Columbia University, 180 Fort Washington Avenue, New York, NY 10032, United States

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INTRODUCTION

Mucinous carcinoma is a histological subtype of tumor in which at least 50% of the tumor is composed of large pools of extracellular mucin and columns of malignant cells. Although less frequent than non-mucinous carcinoma, mucinous carcinoma is associated with a worse prognosis, because it is more frequently diagnosed in the advanced stage and is associated with more frequent local recurrence, serosal invasion, lymphatic and vascular invasion, lymph node metastasis, and distant metastasis[1,2]. Signet ring cell carcinoma is composed of signet ring cells that have a vacuolated cytoplasm containing abundant intracellular mucin and a nucleus displaced to the periphery. The prognosis for signet ring cell carcinoma is generally poor, although its prognosis is still controversial[3,4]. Intraductal papillary mucinous neoplasm (IPMN) is an uncommon neoplasm arising from the bile duct and pancreas, which is characterized by a proliferation of ductal epithelium associated with ductal dilatation and variable mucin production. Distinguishing IPMN from other tumors is essential because IPMN has a better prognosis than other malignancies in the pancreaticobiliary tree[5,6].

Careful distinction of mucinous and non-mucinous tumors is important, as the clinical outcome of these entities may somewhat differ. Because abundant mucin within the tumor is the hallmark of mucinous neoplasms, mucin-producing neoplasms typically show some distinct imaging features. Therefore, familiarity with the critical imaging features of mucin-producing neoplasms in the abdomen and pelvis may facilitate an accurate diagnosis and treatment.

In this article, we classify mucin-producing neoplasms in the abdomen and pelvis into four types according to characteristic morphological features: unilocular or multilocular cystic neoplasms lining mucin-secreting epithelium that contain mucinous fluid; tumors characterized by intraluminal proliferation of mucinous neoplastic transformation of epithelium lining pancreaticobiliary ducts, which are arranged in a papillary pattern and typically produce and accumulate mucin; tumors composed of neoplastic epithelium containing intracellular mucin associated with little or no extracellular mucin; and tumors composed of abundant extracellular mucin due to mucin-secreting neoplastic epithelium. On the basis of these four types, we discuss and illustrate the clinical significance and imaging features of mucin-producing neoplasms in the abdomen and pelvis.

BASIC CONCEPTS

Mucin is a high-molecular-weight glycoprotein containing oligosaccharides attached to the mucin core protein, and is the major component of mucus lining the surface of glandular epithelium as a viscoelastic gel. Mucin is expressed by various epithelial mucosal cells, which exist in the respiratory, digestive, and urogenital tracts, and secretory epithelial surfaces of specialized organs such as the liver, pancreas, gallbladder, kidneys, salivary glands, lacrimal glands, and eyes. Mucin has a central role in maintaining homeostasis and promoting cell survival in a variety of conditions. Because the outermost area of a typical epithelial surface consists of secreted gel-forming mucin, mucin lubricates and forms a barrier that protects the mucosal epithelium from potentially noxious intraluminal substances such as air, food, enzymes, acidic pH, salt, bacteria, and viruses. Additionally, mucin gels capture and hold biologically active molecules that may incite inflammatory, repair, or healing processes following their release[7,8].

Cancer cells, especially adenocarcinomas, express aberrant forms or amounts of mucins that arise as a consequence of the deregulation of mucin core protein expression. Mucins in cancer cells contribute to carcinogenesis and tumor invasion by simultaneously disrupting existing interactions and establishing new ones. These tumors produce variable amounts of intracellular and/or extracellular mucins[9].

Mucus is composed of 95% water and 5% high-molecular-weight glycoprotein. Due to its high water content, mucin usually appears anechoic on ultrasound (US), and has computed tomography (CT) attenuation and magnetic resonance (MR) signal intensity similar to those of water. However, the imaging appearance of mucin varies depending on water and protein concentrations. Concentrated mucin increases US echogenicity, which may appear hypoechoic with fine internal echoes or in a complex echogenic pattern. Concentrated mucin also has CT attenuation values above that of water, as well as high signal intensity on T1-weighted images and low signal intensity on T2-weighted images due to shortening of both the T1 and T2 relaxation times[10]. Knowledge of these characteristic mucin imaging features is helpful to diagnose various mucin-producing neoplastic conditions and to avoid pitfalls.

MUCIN-PRODUCING NEOPLASTIC CONDITIONS

Well-circumscribed cystic neoplasms lining mucin-secreting epithelium and containing mucinous fluid

Mucinous cystic neoplasm of the pancreas: A mucinous cystic neoplasm (MCN) is lined by mucin-producing columnar epithelium. This tumor is characterized by an ovarian-like stromal component, which is essential for diagnosing MCN of the pancreas. MCN of the pancreas

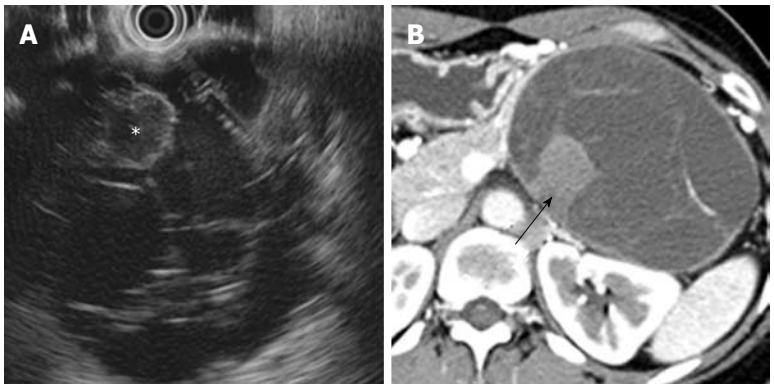

Figure 1 Mucinous cystadenoma of the pancreas in a 40-year-old woman. A: Endoscopic ultrasound image shows a complex multiloculated cystic mass in the pancreatic tail. Note a hyperechoic locule (asterisk) within the cystic tumor; B: Contrast-enhanced CT image shows a well-circumscribed multiloculated mass in the tail of the pancreas with enhancement of thin internal septa and the peripheral wall. Note a highly attenuated locule (arrow) within the cystic tumor. CT: Computed tomography.

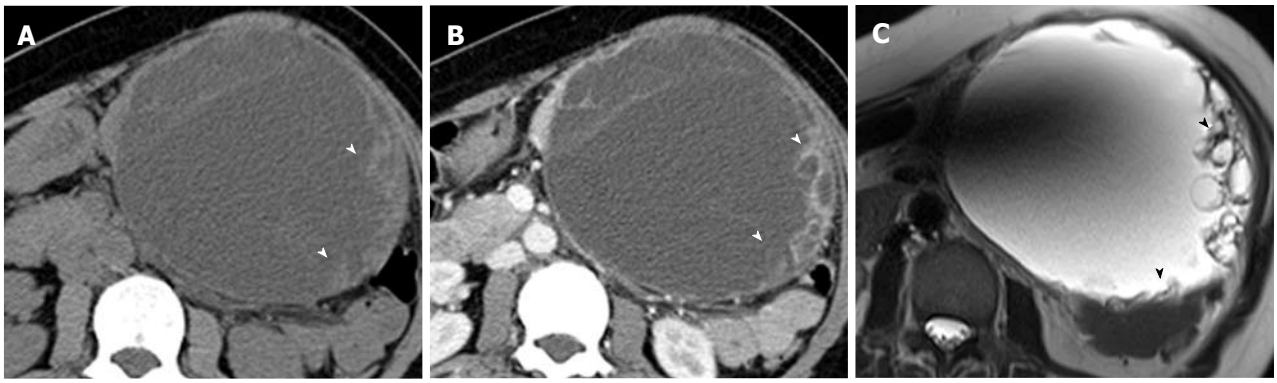

Figure 2 Mucinous cystadenocarcinoma of the pancreas in a 44-year-old woman. A, B: Non-enhanced (A) and contrast-enhanced (B) CT images show a well-circumscribed multi-septated cystic mass with enhancing soft tissue components (arrowheads) in the tail of the pancreas; C: T2-weighted MR image shows a large multilocular cystic mass with high signal intensity and peripheral soft tissue components with low signal intensity (arrowheads) in the pancreatic tail. CT: Computed tomography; MR: Magnetic resonance.

shares both clinical and pathological characteristics with biliary and ovarian mucinous tumors[11]. MCN occurs predominantly in middle-aged women, typically in the body or tail of the pancreas. Although a spectrum of MCNs from benign (mucinous cystadenoma) to malignant (mucinous cystadenocarcinoma) occurs, MCNs should always be resected because they are all potentially malignant[11].

On cross-sectional imaging, a MCN appears as a well-capsulated, unilocular, or multilocular septated cystic lesion (Figures 1 and 2). The tumor is round to oval with a smooth external margin, and the wall of the cyst is typically thick with delayed enhancement[11,12]. Peripheral calcification is seen in 10%-25% of cases and is an important characteristic of MCN that can be used to distinguish it from serous cystadenoma, which tends to have central calcification[11]. A MCN generally does not communicate with the pancreatic duct, unlike an IPMN-P. When rarely present, such a communication is due to fistula formation between the MCN and the pancreatic duct[13]. Different attenuations or signal intensities may be noted within the cystic cavity, depending on whether mucoid or hemorrhagic fluid is present. The presence of an internal enhancing soft tissue component is indicative of mucinous cystadenocarcinoma (Figure 2)[11,14].

Biliary cystadenoma and cystadenocarcinoma: Biliary cystadenomas and cystadenocarcinomas are rare tumors arising in the bile duct epithelium as multilocular cystic masses containing carcinoembryonic antigen-rich mucinous fluid. They are generally intrahepatic (85%) and occur more frequently in middle-aged women. These tumors are similar to mucinous cystic tumors that arise in the pancreas and ovary and are further subdivided into those with ovarian stroma and those without ovarian stroma. The presence of ovarian stroma may be a favorable prognostic sign. However, gross or imaging features cannot distinguish tumors with ovarian stroma from those without ovarian stroma[15,16].

The CT appearance of a biliary cystadenoma and cystadenocarcinoma includes a solitary cystic mass with a well-defined thick fibrous capsule, mural nodules, internal septa, and, rarely, capsular calcification. These appear as a multilocular cystic mass with variable signal intensities on both T1- and T2-weighted images depending on the

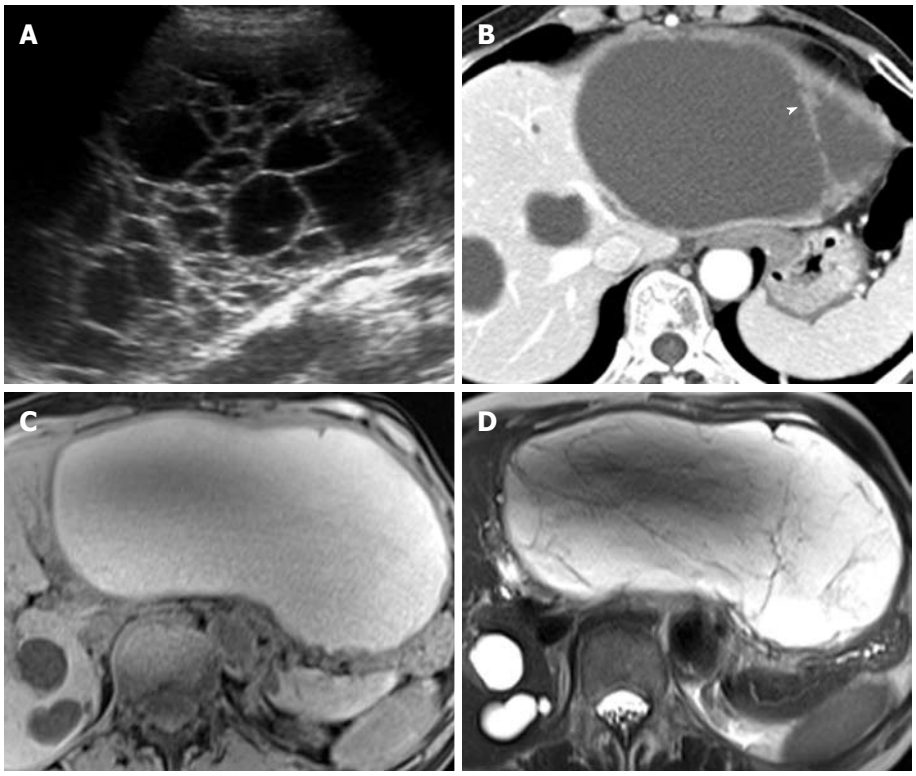

Figure 3 Biliary cystadenoma in the liver of a 55-year-old woman. A: Ultrasound image shows a multiseptated cystic mass in the liver; B: Contrast-enhanced CT image shows a well-circumscribed cystic liver mass, which has similar attenuation to that of hepatic cysts. Note the internal septa with nodular thickening (arrowhead); C, D: T1- (C) and T2-weighted (D) MR images show a multiseptated cystic mass with hyperintensity. CT: Computed tomography; MR: Magnetic resonance.

presence of mucin, hemorrhage content, or solid components (Figure 3)[17]. Internal nodularity and septation have been associated with a biliary cystadenocarcinoma, whereas septation without nodularity is associated with a biliary cystadenoma. However, these findings overlap between benign and malignant forms. Because both a biliary cystadenoma and cystadenocarcinoma are treated with total surgical excision, distinguishing these two diseases may be of little practical importance[15,16].

Biliary cystadenomas and cystadenocarcinomas morphologically resemble a cystic variant of an intrahepatic intraductal papillary neoplasm of the bile duct (IPN-B). Distinguishing these two diseases may be possible on images in which the cystic IPN-B is communicating with the intrahepatic bile ducts and the downstream bile duct is dilated due to excessive mucin, whereas biliary cystadenomas and cystadenocarcinomas do not communicate with the bile duct, and mucin is confined to the cystic mass[18].

Mucinous cystic tumors of the ovary: Mucinous cystic tumors of the ovary are the second most common type of ovarian epithelial tumor. They can be classified as adenomas, borderline malignancies, or adenocarcinomas, according to the histopathological degree of malignancy. A mucinous cystadenoma is composed of a single layer of columnar cells with abundant intracellular mucin and small basilar nuclei in the cysts. A mucinous borderline tumor is a noninvasive epithelial tumor characterized by cytologic atypia without stromal invasion. A mucinous cystadenocarcinoma has an irregular glandular structure and papillae with obvious stromal invasion[19].

On MR imaging, a mucinous cystic tumor typically appears as a large multilocular cystic mass. The loculi show variable signal intensity on both T1- and T2-weighted images (so-called "stained glass" appearance), depending on the viscosity of the materials present, such as mucin, blood products, or debris (Figures 4, 5 and 6). A unilocular cystic appearance is rare for mucinous cystic tumors. A solid component, thick septa, and a thick and irregular wall are suggestive of a malignant epithelial ovarian tumor (Figure 6)[20,21].

A primary intestinal mucinous carcinoma, most commonly from the appendix or colon, can metastasize to the ovary. Metastases to ovary are much more common than an ovarian cystadenocarcinoma. A metastatic carcinoma is often bilateral, whereas a mucinous cystadenocarcinoma is usually unilateral. However, mucinous cystadenocarcinomas mimic metastases to the ovary from a primary mucinous carcinoma on MR imaging[20,21].

The gross and radiological appearances of mucinous borderline tumors usually do not reliably distinguish them from cystadenomas or even some cystadenocarcinomas. The most frequent MR feature of mucinous borderline tumors is a predominantly cystic lesion with varying cyst and septal wall thickness. Mucinous borderline tumors and cystadenocarcinomas tend to have greater numbers of loculi on MR imaging than cystadenomas, which can be explained by more active mitosis in mucinous bor-

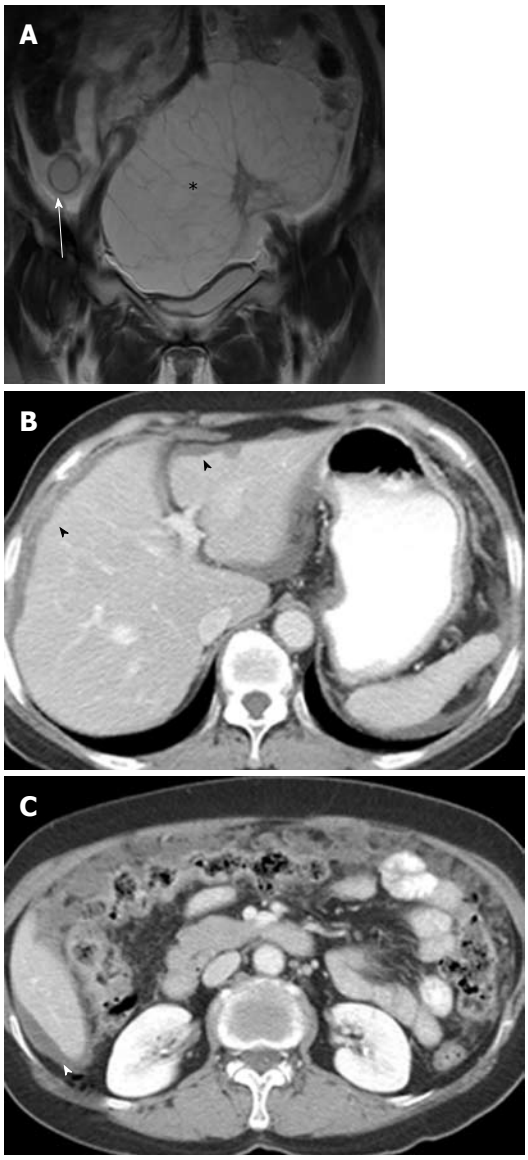

Figure 4 Coexistence of a mucinous cystadenoma of the appendix and ovary in a 63-year-old woman. A: Coronal T2-weighted MR image shows a well-defined cystic mass with hyperintensity in the appendix (arrow) and a multiloculated cystic mass in the left ovary (asterisk); B, C: Contrast-enhanced CT images show low-attenuation mucinous deposits in the peritoneal cavity and scalloping of the liver margin (arrowheads), suggestive of pseudomyxoma peritonei. CT: Computed tomography; MR: Magnetic resonance.

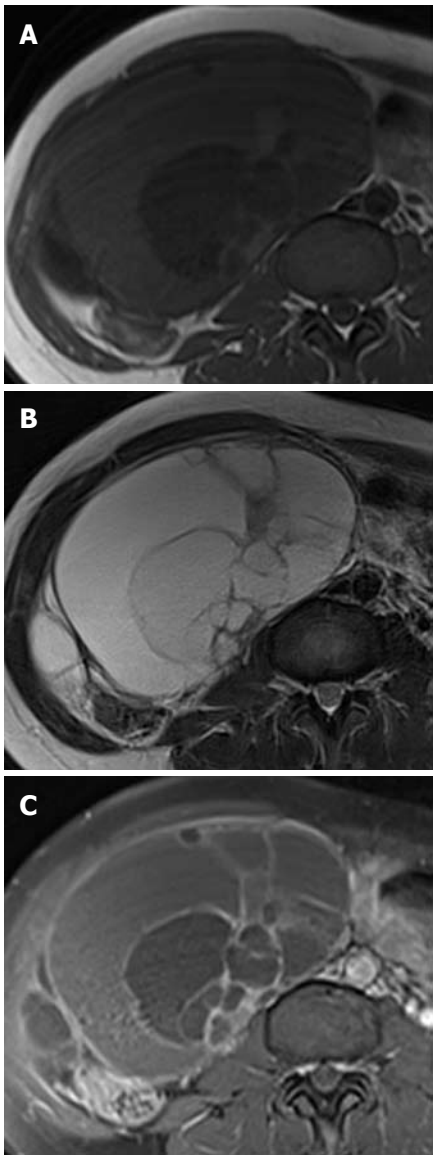

Figure 5 Mucinous borderline tumor of the right ovary in a 41-year-old woman. A, B: T1- (A) and T2-weighted (B) MR images show a cystic mass with large numbers of loculi with varying signal intensity in the right ovary; C: Contrast-enhanced fat-saturated T1-weighted MR image shows the enhancement of multiple internal septa and wall in the cystic mass. MR: Magnetic resonance.

derline tumors and cystadenocarcinomas, resulting in production of larger numbers of glands and loculi (Figures 5 and 6). Both mucinous cystadenocarcinomas and borderline tumors show a solid portion on MR imaging, which is pathologically composed of densely aggregated fine numerous loculi or diffuse tumor cell proliferation. However, the solid portion of a mucinous cystadenocarcinoma is larger and more commonly seen than in a borderline tumor. Pelvic organ invasion, implants, and lymphadenopathy may be helpful ancillary findings suggestive of malignancy[22,23].

Mucocele of the appendix: Mucocele is a descriptive term for a mucinous distension of the appendiceal lumen, regardless of the underlying pathology. A mucocele is quite rare, with a prevalence of 0.2%-0.3% among appendectomies and 8% of all appendiceal tumors. A mucocele can be caused by a variety of nonneoplastic, benign neoplastic, and malignant conditions. However, most mucoceles are associated with neoplastic epithelium. A mucinous cystadenoma is the most common type, representing 63%-84% of mucoceles. A mucinous cystadenoma is based on villous adenomatous changes in the mucin-rich epithelium, which produce marked intraluminal dilatation by mucin reaching up to 6 cm. An appendiceal perforation occurs in up to 20% of cases, with mucinous spillage into the periappendiceal area or onto the serosal surface, which leads to pseudomyxoma

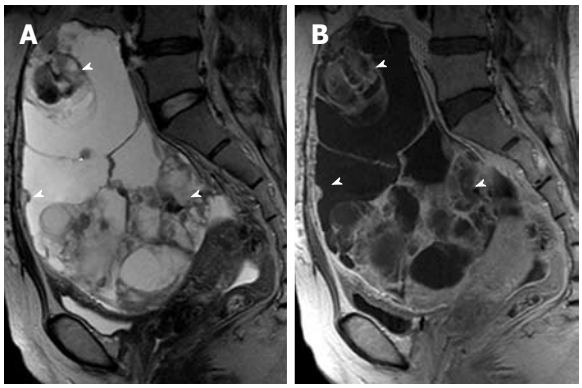

Figure 6 Mucinous cystadenocarcinoma of the left ovary in a 47-year-old woman. A: Sagittal T2-weighted MR image shows a huge multilocular ovarian cystic mass with multiple hypointense solid components (arrowheads); B: Sagittal contrast-enhanced T1-weighted MR image shows an ovarian cystic mass with enhancement of internal septa and solid components (arrowheads). MR: Magnetic resonance.

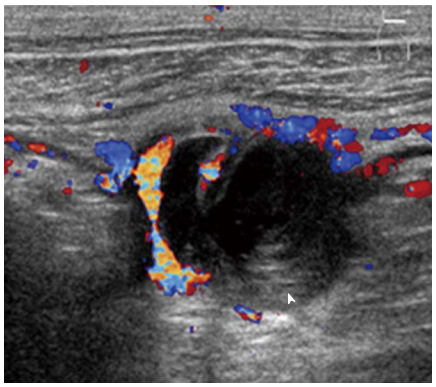

Figure 7 Mucocele of the appendix in a 54-year-old man. Ultrasound image shows an anechoic round mass in the appendix with echogenic layers (arrowhead) (the so-called "onion-skin" sign).

peritonei (PMP) and a possibly fatal outcome. A mucinous cystadenocarcinoma is less common than a cystadenoma, accounting for 11%-20% of cases. The presence of stromal invasion by neoplastic cells is indicative of an adenocarcinoma[24,25].

Imaging plays an important role when evaluating appendiceal mucocele. US usually reveals a unilocular, ovoid, anechoic mass in the region of the appendix. Internal echogenicity varies depending on the acoustic interfaces produced by the mucin, including hypoechoic masses with fine internal echoes and complex echogenic masses with acoustic enhancement. Concentric and echogenic layers within the cystic mass (the so-called "onion skin" sign) are specific for mucocele of the appendix (Figure 7). The reason for the layered appearance on US is unclear; nevertheless, it may be explained by a fluctuation in mucin secretion into the cavity along with the gradual absorption of water or by a fluctuation in the degree of excretion blockage from the cavity[24,26]. CT is the imaging modality of choice because CT depicts tissue characteristics, the anatomical relationship between the cystic mass

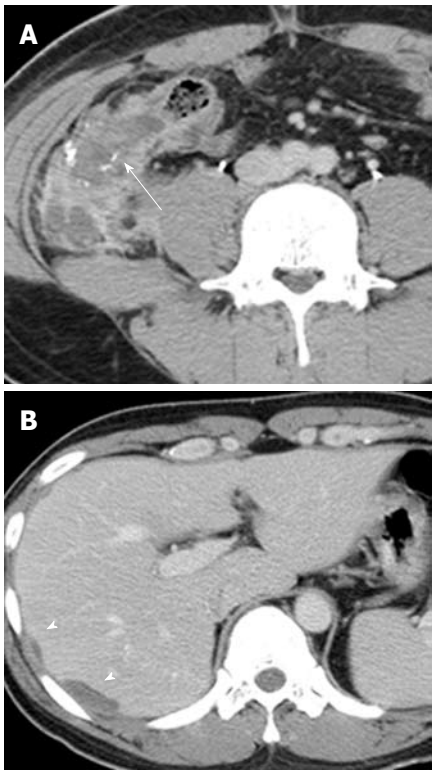

Figure 8 Mucinous cystadenocarcinoma of the appendix in a 26-year-old man. A: Contrast-enhanced CT image shows a complex hypoattenuating mass with enhancing solid portions and punctate calcifications in the appendix (arrow); B: Contrast-enhanced CT image of the upper abdomen shows loculations of fluid scalloping on the liver surface (arrowheads), providing evidence of a mass effect. CT: Computed tomography.

and cecum, and helps rule out or confirm the diagnosis. On CT, a mucocele appears as a round or tubular cystic mass with thin and enhancing walls, which is contiguous with the base of the cecum. Curvilinear mural calcification is sometimes seen in less than 50% of cases, which is suggestive of a mucocele. A mucocele is hyperintense on T2-weighted image and variably hypointense or isointense on T1-weighted image, depending on the mucin concentration (Figure 4). The presence of enhancing nodular lesions raises the possibility of a mucinous cystadenocarcinoma (Figure 8). Identifying a normal right ovary in women is also crucial to exclude a cystic ovarian neoplasm or tubo-ovarian abscess[24,25,27].

Since mucoceles usually present as a chronic noninfectious process, most are relatively asymptomatic. However, their presenting symptoms sometimes mimic acute appendicitis. The differential diagnosis between a mucocele with secondary appendicitis and appendicitis without a mucocele is important because surgical management may be altered according to the presence or absence of mucocele. CT features such as cystic dilatation of the appendix, a luminal diameter greater than 1.5 cm, and mural calcification suggest a mucocele coexisting with acute appendicitis, although there is some overlap with the diagnosis of acute appendicitis without a mucocele[28].

The treatment of choice is surgical excision, and the

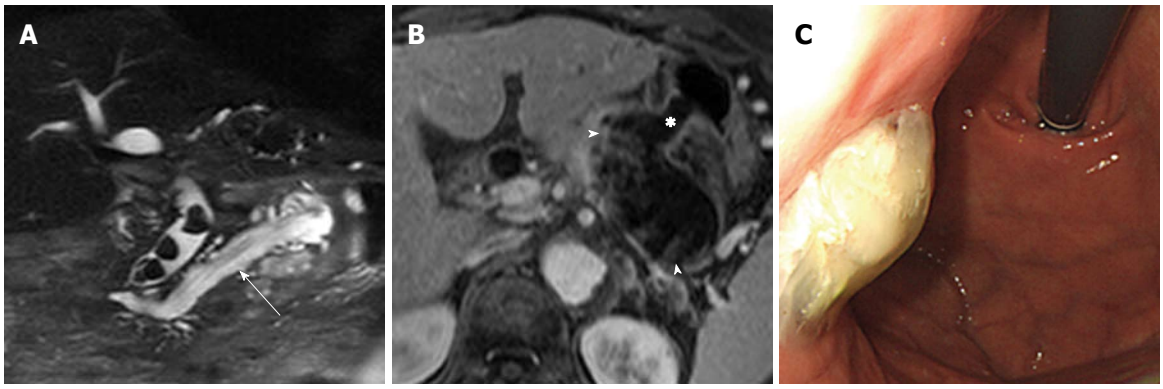

Figure 9 Intraductal papillary mucinous neoplasm of the pancreas in an 80-year-old woman. A: Coronal T2-weighted RARE MR image shows cord-like hypointense mucin in the dilated main pancreatic duct (arrow). Note multiple stones in the dilated common bile duct; B: Axial contrast-enhanced fat-saturated T1-weighted MR image demonstrates a fistula (asterisk) between the dilated main pancreatic duct and the stomach. Note mural nodules (arrowheads) within the dilated main pancreatic duct, which strongly suggest a malignant Intraductal papillary mucinous neoplasm; C: Endoscopy image reveals mucin leaking from the papilla. RARE: True rapid acquisition with relaxation enhancement; MR: Magnetic resonance.

type of surgery performed is related to the size and the histological contents of the mucocele. A full abdominal exploration is advised during surgery, because a mucocele can be associated with other tumors, particularly colonic adenocarcinoma and ovarian tumors[26].

Tumors that produce and accumulate mucin in the pancreaticobiliary tract

Intraductal papillary mucinous neoplasm of the pancreas: IPMN-P, known as one of the mucin-producing pancreatic tumors, is an uncommon pancreatic neoplasm with characteristic histology and distinctive clinicobiological behavior. It is characterized by an intraductal proliferation of mucinous cells arranged in a papillary pattern. Excessive mucin secretion in the ducts by this proliferation ultimately leads to cystic dilatation of the major duct, the second duct, or both, depending on the tumor location. Abundant mucin production is usually observed in most cases of IPMN-P. The clinical symptoms and signs of IPMN-P are due to impaired outflow of pancreatic juice, which is induced by the hypersecretion of mucin[5,29].

IPMN-P occurs most frequently in men, and the mean age at the time of diagnosis is approximately 60 years. It is most commonly located in the head or uncinate process of the pancreas. IPMN-P has a low potential for malignancy, and it has a better prognosis than other pancreatic malignancies because of slow growth rates, rare parenchymal invasion, low rates of metastatic spread, and low recurrence after resection[5,29].

Characteristic endoscopic retrograde cholangiopancreatography findings of IPMN-P include communication between a cystic lesion in the branch duct and the main pancreatic duct, intraluminal filling defects of the pancreatic duct due to the presence of mucin or a mass, and the depiction of a patulous papilla with mucin extrusion from the orifice of the papilla. CT or MR findings of IPMN-P include grapelike clustered cystic lesions reflecting focal dilatation of the branch ducts, diffuse dilatation of the main pancreatic duct, mural nodules, bulging papilla, and communication of branch duct-type IPMN with the main pancreatic duct. Although the diagnosis of malignancy in IPMN is often difficult, even with recent advanced MR techniques, several indirect findings can suggest the presence of malignancy, including the presence of mural nodules, thick septa, septal calcification, and a main pancreatic duct dilated to greater than 10 mm in diameter (Figure 9)[5].

Papillary neoplasm of the bile duct: A papillary neoplasm of the bile duct is an intraductal tumor with numerous minute frondlike papillary projections. In approximately one-third of cases, tumors produce abundant viscous mucin, resulting in intermittent and incomplete obstruction of the segmental or lobar bile ducts or the entire biliary tree. A papillary neoplasm of the bile ducts arises from the mucosa and slowly spreads along its luminal surface. Only in the late stage does it invade the bile duct, and thus prognosis is relatively favorable[6,30].

Considering the shared biliary tract and pancreatic origin, the two systems may have similar pathological features. Thus, IPN-B is the biliary counterpart of IPMN-P. In both organs, these neoplasms arise within the duct system and show a predominantly intraductal growth pattern, commonly an overproduction of mucin, and an association with invasive adenocarcinoma[30].

Based on the gross appearance of the intraductal tumors, IPN-B may be classified as an intraductal polypoid tumor, a cast-like growing tumor, a mucosal spreading growth, a cystic variant, or a floating tumor[31]. Thus, variable imaging appearances of IPN-B include an intraductal polypoid mass within a localized ductal dilatation, diffuse and marked duct ectasia with a visibly enhanced papillary mass or masses, intraductal cast-like lesions within a mildly dilated duct, diffuse and marked duct ectasia without a visible mass, or a floating tumor. The friable part of the papillary tumor may slough off and be seen in imaging as a floating tumor within the bile duct, which can be radiologically confused with a bile duct stone[32,33].

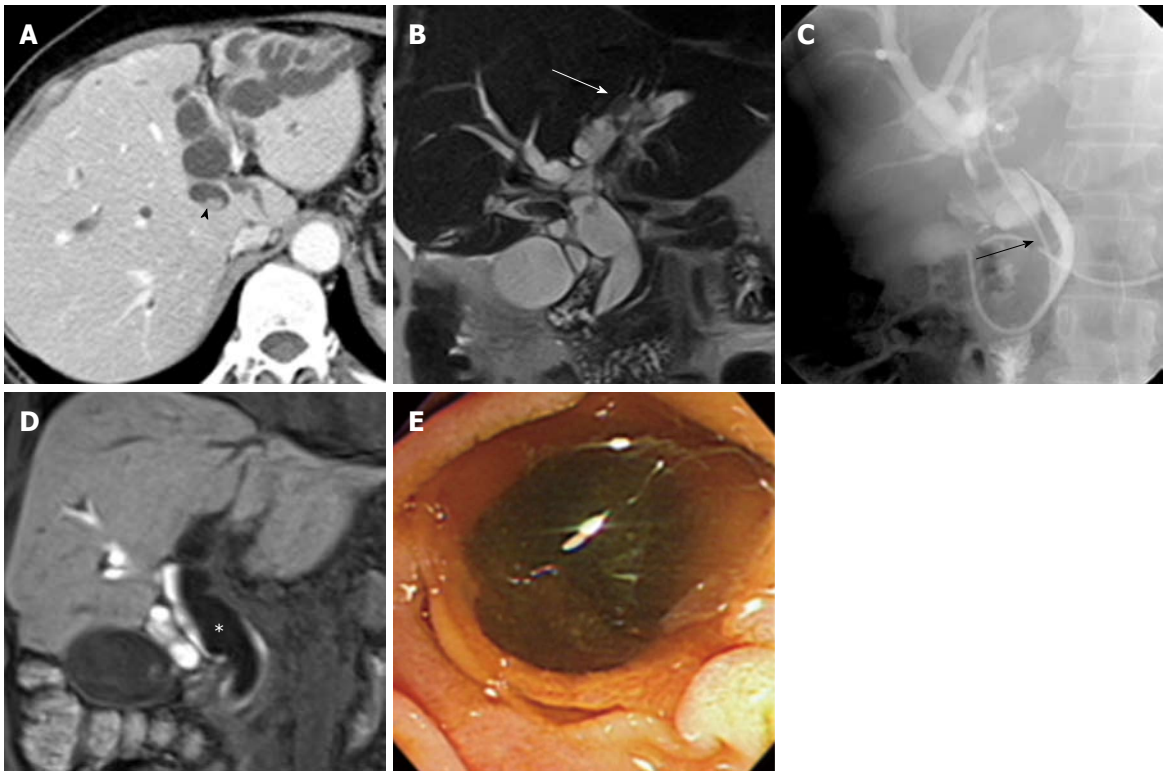

Figure 10 Intraductal papillary mucinous neoplasm of the bile duct in a 58-year-old woman. A: Contrast-enhanced CT image shows severe dilatation of the left intrahepatic ducts without visible intraductal mass. Note a small stone in the dilated intrahepatic duct (arrowhead); B: Coronal T2-weighted RARE MR image shows dilatation of the intrahepatic and extrahepatic ducts. Note the hypointense intraductal mass (arrow) in the left intrahepatic duct; C: Endoscopic retrograde cholangiogram image shows a filling defect (arrow) within the marked dilated extrahepatic bile duct, due to mucin; D: Coronal gadoxetic acid-enhanced T1-weighted MR image obtained at 60 min post-injection shows contrast-filled bile duct with an elongated, low signal intensity lesion (asterisk), which represents mucin; E: Endoscopy image reveals mucin leaking from the papilla. CT: Computed tomography; RARE: True rapid acquisition with relaxation enhancement; MR: Magnetic resonance.

CT and MR images may fail to detect mucin itself because the attenuation or signal intensity of mucin is usually similar to that of bile. A large amount of mucin can be suggested by indirect CT and MR findings, including disproportionate and severe dilatation of the bile ducts proximal or even distal to the tumor, hepatic parenchymal atrophy in the affected lobe or segments, and bulging of the papilla (Figure 10). This usually occurs in IPN-B with mucosal spreading growth without forming tangible masses, and can be explained by the production of excessive amounts of mucin and a longstanding increased ductal pressure on the adjacent hepatic parenchyma due to the partial obstruction[34].

Moreover, in our experience, mucin appears as an elongated and amorphous filling defect within an enhanced bile duct on gadobenate dimeglumine-enhanced or gadoxetic acid-enhanced MR imaging, similar to that of direct cholangiographic findings (Figure 10). The filling defect due to mucin could be confused with other intraluminal filling defects, such as a stone, blood clot, or mass. T2-weighted images can be helpful to distinguish hyperintense mucin from other intraluminal filling defects that usually appear as a signal void, or as hypointense or isointense[35].

Papillary neoplasm of the gallbladder: Mucin-producing carcinoma of the gallbladder is rare, and occurs mostly in older women. Mucin-producing carcinoma of the gallbladder histologically includes two different types: well-differentiated papillary adenocarcinoma and mucinous carcinoma. Well-differentiated adenocarcinoma (particularly papillary adenocarcinoma) usually presents as a papillary growth pattern and can produce mucin in the gallbladder lumen. A well-differentiated papillary adenocarcinoma is potentially less invasive due to its tendency toward intraluminal growth. At cross-sectional imaging, it appears as a papillary protrusion in an enlarged gallbladder[36].

Tumors composed of neoplastic epithelium containing intracellular mucin associated with little or no extracellular mucin

Signet ring cell carcinoma: Signet ring cell carcinoma is characterized by large intracytoplasmic mucin vacuoles that expand in the malignant cells and push the nucleus to the periphery, creating a "signet ring" configuration. When ⩾ 50% of the tumor is composed of cells of this type, it is classified as a signet ring cell carcinoma. More than 96% of all signet ring cell carcinomas arise in the stomach, with the rest arising in other primary organs including the rectum, colon, gallbladder, pancreas, bladder, and breast[3,4].

Signet ring cell carcinomas usually produce minimal mucosal alterations in the gastrointestinal tract, but signet

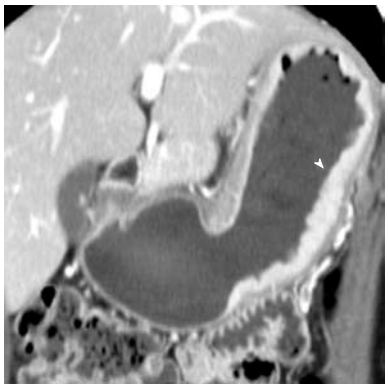

Figure 11 Signet ring cell carcinoma of the stomach in a 45-year-old woman. Coronal reformatted contrast-enhanced CT image shows diffuse gastric wall thickening with strong enhancement (arrowhead) along the lesser and greater curvature of the stomach. CT: Computed tomography.

ring cells diffusely infiltrate throughout the bowel wall and often incite a marked desmoplastic reaction in the submucosa and muscularis propria, which produces the classic pathological features of primary scirrhous carcinoma, also known as the linitis plastic type[3,4].

Gastric signet ring cell carcinoma accounts for 5%-15% of all gastric cancers, and occurs at a higher frequency in females and young patients. The prognosis of gastric signet ring cell carcinoma remains controversial. However, signet ring cell histology is generally considered a poor prognostic factor in gastric carcinoma[3]. Hyperenhancement of the involved stomach wall compared with liver attenuation on portal venous phases of contrast-enhanced CT and a predominantly thickened layer of the gastric wall are more commonly found in gastric signet ring cell carcinoma than in non-signet ring cell carcinoma (Figure 11). Hyperenhancement of the gastric lesion may correspond to intermingled loose and immature fibrosis or neovascularized signet ring cells[37].

Colorectal signet ring cell carcinoma is rare, ranging from 0.1% to 2.4% of all colorectal carcinomas. Most are localized exclusively in the rectum. This carcinoma commonly occurs in younger patients and also has a high rate of spread to the lymph nodes, ovaries, or peritoneal surface. Distant hematogenous metastasis to the liver or lung is uncommon. The most common and characteristic CT features of this tumor are a long segment of concentric wall thickening and a target appearance (Figure 12)[38]. Imaging findings of colorectal signet ring cell carcinoma are similar to those of metastatic linitis plastica originating from a primary tumor, such as breast, gastric, or bladder cancers. Metastatic linitis plastica appears as a malignant target sign consisting of thickened inner (mucosa and submucosa) and outer (serosa) layers and a relatively thin hypoattenuated middle layer (muscularis propria), or bowel wall thickening with homogeneous attenuation. In contrast, a benign target sign can be caused by submucosal edema, inflammatory infiltration, or hemorrhage and thus appears as a prominent hypoattenuating middle layer (submucosa) and thin hyperattenuating inner (mucosa) and outer layer (muscularis propria and serosa)[39].

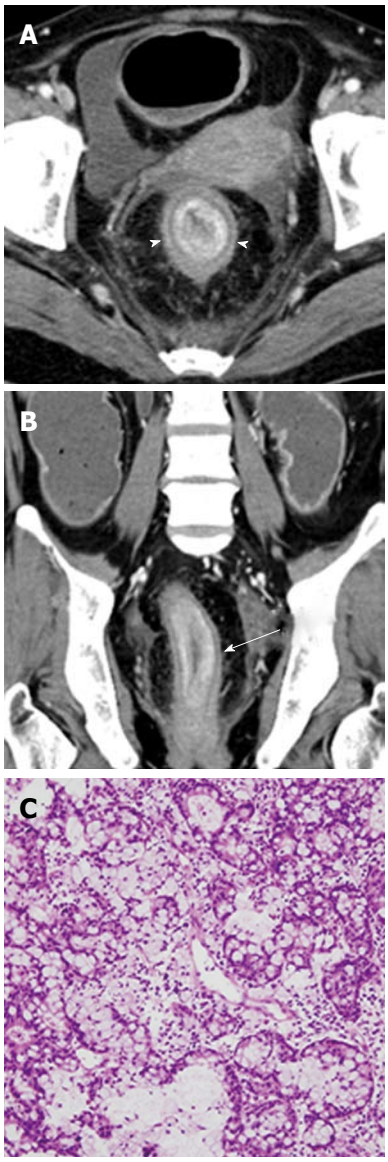

Figure 12 Signet ring cell carcinoma of the rectum in a 42-year-old man. A, B: Axial (A) and coronal reformatted (B) contrast-enhanced CT images show concentric wall thickening with malignant target sign (arrowheads in A, arrow in B) in the rectum; C: Photomicrograph image (original magnification, × 200; HE stain) shows multiple signet ring cells. CT: Computed tomography; HE: Hematoxylin and eosin.

Tumors composed of abundant extracellular mucin due to mucin-secreting neoplastic epithelium

Mucinous adenocarcinoma of the gastrointestinal tract: Adenocarcinomas in various organs can produce and secrete extracellular mucin. This extracellular mucinous material may be so copious that the malignant cells appear to float within a gelatinous pool. Mucinous carcinoma is diagnosed when extracellular mucin within the tumor is retained by more than 50% of the cells[1,2].

Mucinous carcinoma of the gastrointestinal tract is a rare subtype of adenocarcinoma that usually occurs in the stomach and colorectum. Mucinous carcinomas in

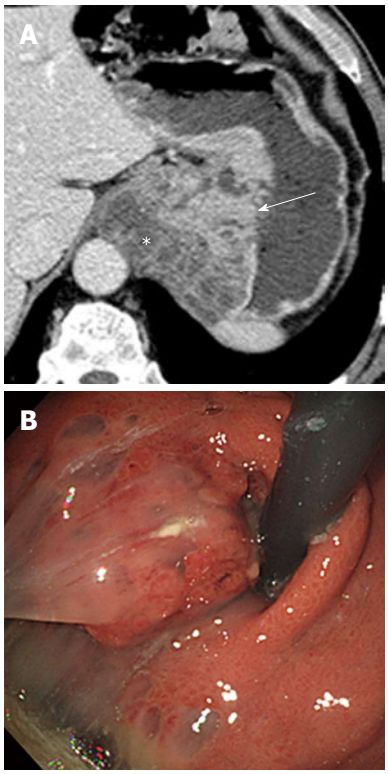

Figure 13 Mucinous carcinoma of the stomach in a 62-year-old woman. A: Contrast-enhanced CT image shows a large mass lesion in the gastric cardia containing abundant mucin pools (asterisk) and enhanced solid portions (arrow); B: Endoscopy image shows jellylike mucin leaking from the gastric mass. CT: Computed tomography.

the gastrointestinal tract have worse prognosis than non-mucinous carcinomas, because they are more frequently diagnosed in the advanced stage and are associated with deeper cancer depth, higher incidence of lymph node metastasis, lymphatic and venous permeation, and peritoneal dissemination[1,2].

Gastric mucinous carcinoma represents approximately 3% of all gastric cancers. Most gastric carcinomas are detected by endoscopy combined with biopsy. However, it is often difficult to diagnose mucinous carcinoma by biopsy because most mucinous gastric carcinomas are located predominantly in the submucosa and the frequency of mucosal involvement is somewhat low. Therefore, an accurate preoperative diagnosis of mucinous carcinomas in the gastrointestinal tract by CT or MR is important. The most common CT appearance for a gastric mucinous carcinoma is a diffuse wall thickening greater than 1 cm with preserved layering enhancement (Figure 13). A thickened hypoattenuating middle or outer layer corresponds to abundant mucin pools located in the submucosa or deeper layers, and the thin enhancing inner layer corresponds to the overlying normal mucosal layer with or without the exception of focal cancer infiltration. Miliary and punctate calcifications within the mucin pool are present and are thought to be diagnostic for mucinous adenocarcinoma[40].

Colorectal mucinous carcinoma varies from 5% to 15%

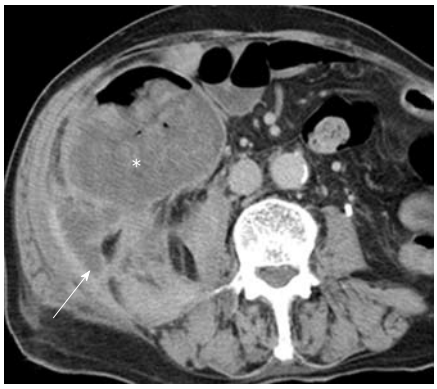

Figure 14 Mucinous adenocarcinoma of the cecum in a 69-year-old man. Contrast-enhanced CT image shows a huge eccentric hypoattenuating mass with poor enhancement of the solid portion of the cecum (asterisk). The mass has invaded the retroperitoneum (arrow). CT: Computed tomography.

of all colorectal carcinomas. Mucinous carcinoma most frequently occurs in the rectosigmoid or ascending colon. CT features indicating mucinous-type colorectal cancer include marked eccentric bowel wall thickening greater than 2 cm, heterogeneous contrast enhancement of the tumor with poor enhancement of the solid portion, a large area of hypoattenuation, and intramural calcification (Figure 14). On MR imaging, a colorectal mucinous carcinoma appears with very high signal intensity on T2-weighted images as a manifestation of the presence of extracellular mucin (Figure 15). Intratumoral congestion, abscess, necrosis and mural edema or entrapped fluids also appear with high signal intensity on T2-weighted images. A mucin tumor can be distinguished from a mimic because the tumor mucin pool may be enhanced, whereas others are not enhanced[41,42]. The enhancement pattern can be a peripheral, heterogeneous, or lacelike enhancement, corresponding to the enhancing mesh-like internal structure formed by the cells, cord, and vessels that line the pools of extracellular mucin[42].

Perianal mucinous adenocarcinoma: A perianal mucinous adenocarcinoma is a rare clinical condition that represents approximately 3% to 11% of all perianal carcinomas. Although their etiology is debatable, mucinous adenocarcinomas may originate from chronic anal fistulas, abscesses, anal glands, or intestinal duplications. This carcinoma is usually diagnosed at an advanced stage and the overall prognosis is poor[43]. CT findings suggesting a perianal mucinous adenocarcinoma include a multilocular cystic mass with peripheral calcification around the anus. It is well established that MR imaging plays an important role when evaluating a perianal mucinous carcinoma-associated fistula in ano. MR features indicating a perianal mucinous adenocarcinoma include masses filled with markedly hyperintense content on T2-weighted images, enhancing solid components, mesh-like internal enhancement, a fistula between the mass and the anus, contrast enhancement of peripheral structures or peritumoral areas, and regional areas of lymph node enlargement. A sol-

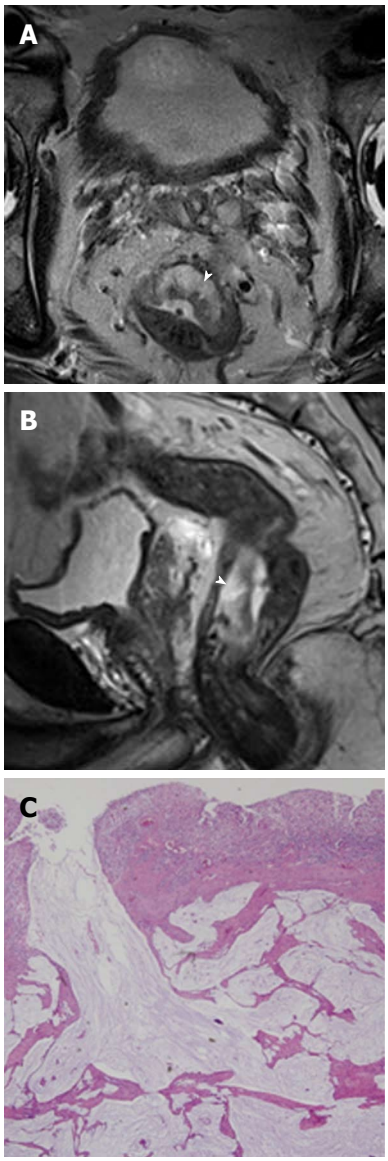

Figure 15 Mucinous adenocarcinoma of the rectum in a 63-year-old man. A, B: Axial (A) and coronal (B) T2-weighted MR images show an area of hyperintense mucin pools (arrowhead) in the rectal mass; C: Photomicrograph image (original magnification, × 40; HE stain) shows large pools of extracellular mucin and tumor cells. MR: Magnetic resonance; HE: Hematoxylin and eosin.

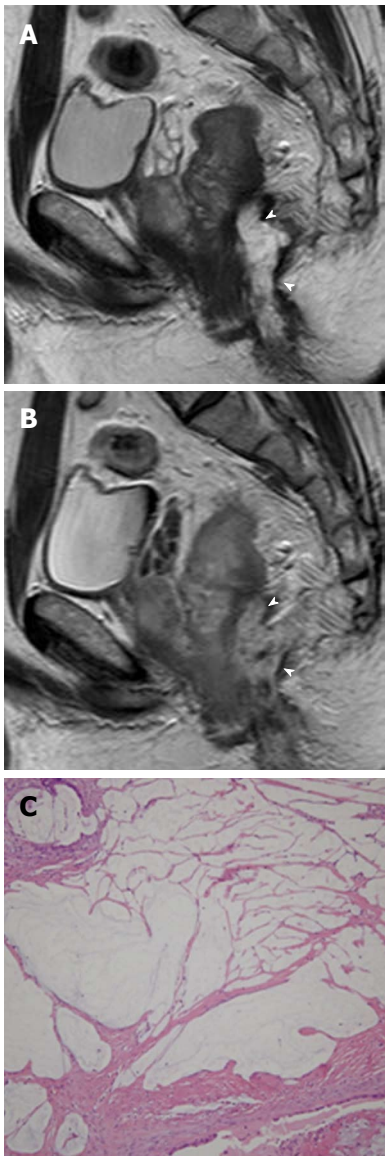

Figure 16 Perianal mucinous adenocarcinoma in a 41-year-old man. A: Sagittal T2-weighted MR image shows a hyperintense mass (arrowheads) in the perianal area; B: Sagittal contrast-enhanced T1-weighted MR image shows mesh-like internal enhancement (arrowheads) within the mass; C: Photomicrograph image (original magnification, × 100; HE stain) shows large pools of extracellular mucin and tumor cells. MR: Magnetic resonance; HE: Hematoxylin and eosin.

id enhancing component and mesh-like enhancement can be a clue to distinguish mucinous adenocarcinomas from abscesses associated with a fistula in ano (Figure 16)[43].

Urachal mucinous carcinoma: Urachal carcinoma is a rare neoplasm arising from the urachal remnant. Although a normal urachus is commonly lined by transitional epithelium, most urachal cancer is adenocarcinoma (90%), which is caused by metaplasia of the urachal mucosa into columnar epithelium followed by a malignant transformation. Approximately 70% of urachal carcinomas are mucinous adenocarcinomas which contain variable amounts of extracellular mucin. Although the prognosis for urachal carcinoma is slightly better than that of nonurachal adenocarcinoma, its prognosis is generally poor because this tumor arises in a clinically silent anatomical location and is generally discovered only after local invasion or metastatic disease. A characteristic CT feature of urachal carcinoma is a midline supravesical solid and partly cystic mass due to mucin produced by the tumors (Figure 17). Psammomatous calcification may occur in 50%-70% of cases. Extension of a urachal carcinoma along the Retzius space helps distinguish it from other vesical carcinomas. T2-weighted images are helpful for detecting the area of the mucin pool within the tumor[44].

Adenoma malignum: Adenoma malignum, also known as a minimal deviation adenocarcinoma, is a rare subtype of

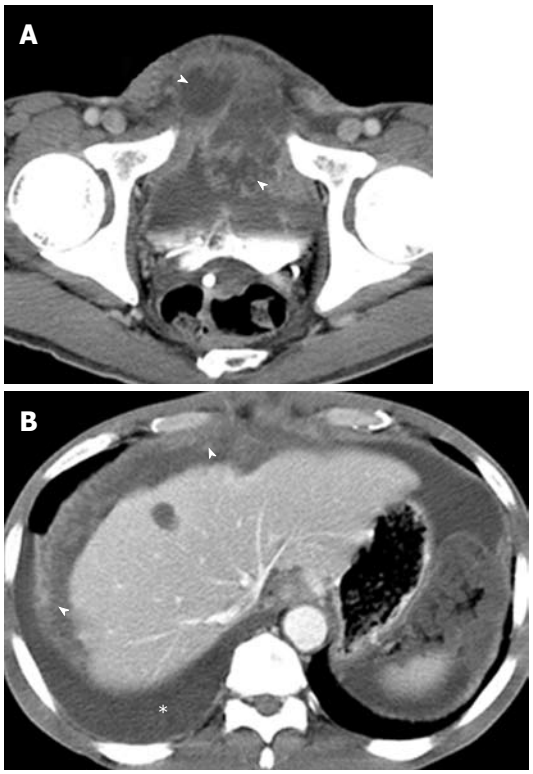

Figure 17 Intraperitoneal spread of mucinous adenocarcinoma of the urachus (also called peritoneal mucinous carcinomatosis) in a 57-year-old man. A: Contrast-enhanced CT image shows a midline supravesical mass with heterogeneous attenuation. Within the mass are scattered low-attenuation areas (arrowheads), which represent mucin; B: Contrast-enhanced CT image of the upper abdomen shows low-attenuation mucinous ascites scalloping the liver margin. Note the right pleural effusion (asterisk) and diffuse nodular thickening of the peritoneum (arrowheads). CT: Computed tomography; MR: Magnetic resonance.

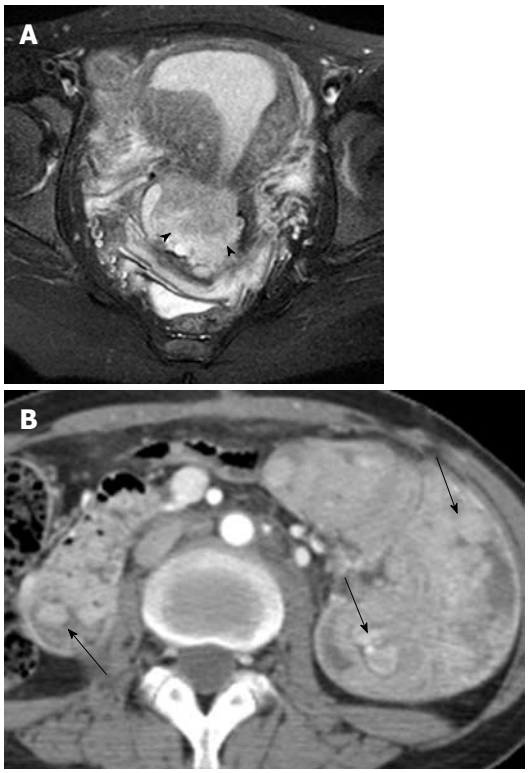

Figure 18 Adenoma malignum of the cervix in a 30-year-old woman with Peutz-Jeghers syndrome. A: Axial T2-weighted MR image shows a multicystic lesion with a solid component (arrowheads) in the uterine cervix; B: Contrast-enhanced CT image shows multiple polyps (arrows) in the duodenum and small bowel causing intussusceptions. CT: Computed tomography; MR: Magnetic resonance.

mucinous adenocarcinoma, representing about 3% of all cervical adenocarcinomas. Adenoma malignum is often associated with Peutz-Jeghers syndrome and mucinous tumors of the ovary. Despite the presence of well-differentiated histopathological features, the prognosis is unfavorable because of early dissemination into the peritoneal cavity and early distant metastasis[45].

On MR imaging, adenoma malignum is characterized by a multicystic lesion, demonstrating very high signal intensity on T2-weighted images, with some solid enhancing components in the deep cervical stroma (Figure 18). However, these MR findings are often confused with those of some other pseudoneoplastic lesions such as deep nabothian cysts, florid endocervical hyperplasia, or papillary endocervicitis, because some portions of these pseudoneoplastic cervical lesions are thickened or accompanied by solid components with enhancement, reflecting the inflammatory process in the cervix stroma or congestion of the small vessels[45].

Mucinous carcinoma of the gallbladder: Mucinous carcinoma of the gallbladder has a massive mucus pool within the carcinoma tissue, tends to growth invasively, and is associated with a poor prognosis. A mucinous carcinoma appears as a focal multilocular hypodense lesion with rim-like enhancement on contrast-enhanced CT. A hyperechoic mass on US and a near-water density on non-enhanced CT pathologically reflect a tumor containing a massive mucin pool with fibrous septa (Figure 19). Calcification within the tumor is often seen. Movable spotty and hyperechoic debris on US or movable filling defects on cholangiography inside the gallbladder lumen or biliary tree, which reflect hypersecretion of mucin, are helpful for diagnosing a mucin-producing carcinoma of the gallbladder. Dilatation of the cystic duct, intrahepatic ducts, or common bile duct is sometimes seen[36].

Mucinous type of cholangiocarcinoma: Mucinous carcinoma is the rarest histological type of cholangiocarcinoma. Mucinous carcinoma appears as an extremely hypodense mass with marginal or septal enhancement on contrast-enhanced CT, and as an extremely hypointense and hyperintense mass on T1- and T2-weighted images, respectively. Its radiological characteristics reflect large mucinous lakes throughout the tumor without mucin excretion into the bile duct[46].

PSEUDOMYXOMA PERITONEI

PMP is an uncommon clinical condition characterized by

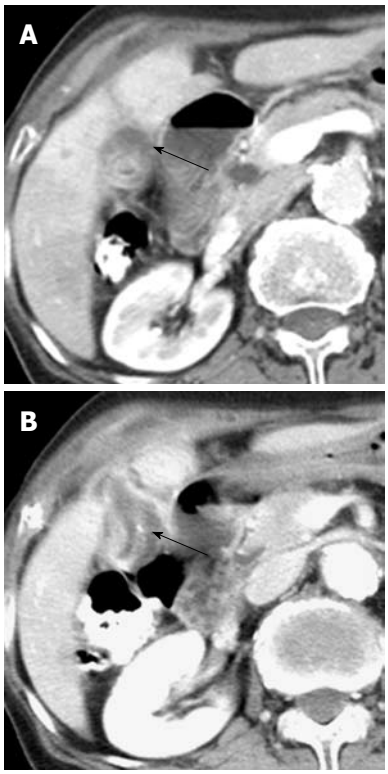

Figure 19 Mucinous carcinoma of the gallbladder in a 77-year-old woman. A: Contrast-enhanced CT image shows localized wall thickening (arrow) in the body of the gallbladder containing a suspicious mucin pool; B: Contrast-enhanced CT image inferior to (A) shows a punctate calcification and localized wall thickening (arrow) in a mildly distended gallbladder. CT: Computed tomography.

accumulation of copious gelatinous materials throughout the peritoneal cavity. It is found in one of 5000 laparotomies, and occurs more commonly in women than men. PMP occurs when mucin-producing lesions rupture into the peritoneal cavity. PMP due to rupture of an appendiceal mucocele is the most common. Mucinous tumors arising from the ovary, gastrointestinal tract, pancreas, and urachus may also cause PMP. However, its origin may not be clear due to extensive organ involvement. Coexistence of mucinous tumors of the appendix and ovary are frequently observed in most women with PMP (Figure 4). Although relationship of PMP to other tumors continues to be controversial, ovarian tumors may represent a secondary deposit from appendiceal tumors[47]. Although most pseudomyxomas are in the peritoneal cavity, they may occur in the retroperitoneum. Pseudomyxoma retroperitonei is caused by rupture of a retrocecal appendiceal mucocele into the retroperitoneal space and fixation of the lesion to the posterior abdominal wall[48].

PMP may be classified into three clinicopathological categories: disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis (PMCA), and PMCA with features intermediate between DPAM and PMCA or with discordant features. DPAM is characterized by peritoneal lesions composed of abundant extracellular mucin containing scant mucinous epithelium with little cytologic atypia or mitotic activity, whereas PMCA is characterized by peritoneal lesions composed of more abundant mucinous epithelium with the architectural and cytologic features of carcinoma. Because DPAM is histologically benign, its prognosis is better than that of PMCA[49].

On CT, PMP appears as ascites with attenuation slightly higher than water. It initially accumulates at sites of relative stasis such as the pouch of Douglas/rectovesical pouch, the right and left subphrenic space, or the surface of the liver and spleen. Septa, curvilinear or amorphous calcification, areas of soft tissue attenuation due to solid elements within mucinous material, or compressed mesentery are seen more commonly within PMP as the volume of disease increases. Scalloping of the visceral surface, particularly the liver, is the diagnostic feature that distinguishes mucinous from simple ascites (Figures 4, 8 and 17). Although CT findings of DPAM and PMCA overlap considerably, PMCA tends to be more frequently accompanied by coexistent pleural masses or effusion, lymphadenopathy, and diffuse peritoneal infiltration such as omental cakes (Figure 17)[49].

CONCLUSION

Various mucin-producing neoplasms originate in different abdominal and pelvic organs. Distinguishing mucinous from non-mucinous tumors is important because of the differences in clinical outcome. Imaging modalities play a critical role in differentiating these two entities. Due to high water content, mucin has a similar appearance to water on both CT and MR imaging, except when thick and proteinaceous, and then it tends to be hyperdense compared to water and hyperintense on T1- and hypointense on T2-weighted images. A correct diagnosis of mucin-producing neoplasms is possible when these imaging features are identified on cross-sectional imaging. Because imaging features of mucin can have a similar appearance to water in both CT and MR imaging, the differentiation of mucinous from non-mucinous neoplastic conditions *via* only imaging features of mucin is sometimes problematic. In addition, further studies with appropriate quantitative and statistical analyses are required to confirm whether these imaging findings can be helpful in the correct diagnosis of mucin-producing neoplasms. However, the imaging appearance of mucin-producing neoplasms differs somewhat depending on the organ of origin; additional information about the distinctive imaging features of mucin-producing neoplasms according to tumor location may also facilitate accurate diagnosis and treatment.

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ORIGINAL ARTICLE

Lipopolysaccharide induces and activates the Nalp3 inflammasome in the liver

Michal Ganz, Timea Csak, Bharath Nath, Gyongyi Szabo

Michal Ganz, Timea Csak, Bharath Nath, Gyongyi Szabo, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, United States
Author contributions: Ganz M, Szabo G designed the research; Ganz M, Csak T and Nath B performed the research; Ganz M analyzed the data; and Ganz M and Szabo G wrote the paper.
Supported by NIH grant RO1 DK075635
Correspondence to: Gyongyi Szabo, MD, PhD, Department of Medicine, University of Massachusetts Medical School, LRB215, 364 Plantation Street, Worcester, MA 01605, United States. gyongyi.szabo@umassmed.edu
Telephone: +1-508-8565275 Fax: +1-508-8564770
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Abstract

AIM: To examine the activation of the Nalp3 inflammasome and its downstream targets following lipopolysaccharide (LPS)-induced stimulation in the liver.

METHODS: Six-to-eight-week-old C57BL/6 chow fed mice were injected intraperitoneally with 0.5 μg/g bodyweight LPS and sacrificed 2, 4, 6, 18 or 24 h later. LPS-induced liver damage was confirmed by a biochemical assay to detect alanine aminotransferase (ALT) levels. To determine if LPS stimulation in the liver led to activation of the inflammasome, real-time quantitative polymerase chain reaction was used to evaluate the mRNA expression of components of the Nalp3 inflammasome. Enzyme-linked immunosorbent assays were used to determine the protein expression levels of several downstream targets of the Nalp3 inflammasome, including caspase-1 and two cytokine targets of caspase-1, interleukin (IL)-1β and IL-18.

RESULTS: We found that LPS injection resulted in liver damage as indicated by elevated ALT levels. This was associated with a significant increase in both mRNA and protein levels of the proinflammatory cytokine tumor necrosis factor (TNF)-α in the liver, as well as increased levels of TNFs in serum. We showed that LPS stimulation led to upregulation of mRNA levels in the liver for all the receptor components of the inflammasome, including Nalp3, Nalp1, pannexin-1 and the adaptor molecule apoptosis-associated speck-like, caspase recruitment domain-domain containing protein. We also found increased levels of mRNA and protein for caspase-1, a downstream target of the inflammasome. In addition, LPS challenge led to increased levels of both mRNA and protein in the liver for two cytokine targets of caspase-1, IL-1β and IL-18. Interestingly, substantial baseline expression of pre-IL-1β and pre-IL-18 was found in the liver. Inflammasome and caspase-1 activation was indicated by the significant increase in the active forms of IL-1β and IL-18 after LPS stimulation.

CONCLUSION: Our results show that the Nalp3 inflammasome is upregulated and activated in the liver in response to LPS stimulation.

Key words: Endotoxin; Nod-like receptor; Interleukin-1β; Interleukin-18; Caspase-1

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INTRODUCTION

The endotoxin lipopolysaccharide (LPS), a component of Gram-negative bacteria, plays an important role in

acute liver injury as well as chronic liver diseases including fatty liver associated with either alcohol consumption or metabolic syndrome and obesity[1,2]. LPS has also been implicated in insulin resistance as well as in steatohepatitis in non-alcoholic fatty liver disease[3-5]. Increasing evidence suggests that gut-derived LPS through the gut-liver axis affects the extent of liver damage in many different types of inflammatory liver diseases[6]. LPS is a prototypical ligand for the pattern recognition receptor (PRR), Toll-like receptor 4 (TLR4). TLR4 induces downstream signaling *via* the MyD88 adapter molecule and induces production of proinflammatory cytokines through activation of the regulatory factor nuclear factor (NF)-κB[6]. In the liver, TLR4 is expressed in both parenchymal and immune cells, thereby providing potential for LPS-induced activation[7].

Studies have shown that the Nalp3 inflammasome is activated by both pattern associated molecular patterns (PAMPs), including LPS and bacterial RNA, and danger-associated molecular patterns (DAMPs)[8,9]. The Nalp3 inflammasome is a caspase-1 activating multiprotein complex, which has been implicated in numerous inflammatory processes and human diseases including gout, pseudogout, contact hypersensitivity, and most recently Alzheimer's disease[10,11]. Furthermore, mutations in the *Nalp3* gene that lead to gain-of-function mutations, result in several hereditary syndromes including: Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and chronic infantile neurological cutaneous and articular syndrome[12].

In response to stimulation by either DAMPs or PAMPs, Nalp3 interacts with pro-caspase-1 through the adaptor molecule [apoptosis-associated speck-like, caspase recruitment domain (CARD)-domain containing protein (ASC)] to form the inflammasome, which leads to activation of caspase-1. Active caspase-1, previously known as interleukin (IL)-1β converting enzyme, a heterodimer of p20 and p10 subunits, is the central effector protein of the inflammasome complex and promotes the cleavage of pro-IL-1β, pro-IL-18 and pro-IL-33 to their biologically active, mature forms[13]. In addition, active caspase-1 has been shown to cleave other substances, such as caspase-7 and sterol regulatory element-binding proteins, therefore playing a pivotal role in cell death and survival[14].

The mRNA levels of the components the Nalp3 inflammasome are expressed in the eye, heart and lung in response to LPS stimulation[15]. Although mRNA levels of IL-1β have also been shown to increase in the liver in response to LPS stimulation, the role of the Nalp3 inflammasome in the liver in response to LPS stimulation has not been determined[16]. In the liver, the Nalp3 inflammasome is upregulated upon acetaminophen-induced toxicity[17] and the contribution of Nalp3 has been described in *Propionibacterium acnes* and LPS-induced acute liver injury[18]; however, a role for Nalp3 in other liver conditions has not been identified.

Although the role of the proinflammatory cytokine, tumor necrosis factor (TNF)α, has been extensively studied in both alcoholic and non-alcoholic fatty liver disease, the role of IL-1β and the inflammasome has yet to be explored. LPS plays an important role in liver disease; elevated LPS levels are detected in the portal and systemic blood of patients with alcoholic and non-alcoholic fatty liver disease[19,20]. Furthermore, there is increased sensitivity to LPS in both alcoholic liver disease and fatty liver disease[21]. Here, we examined if LPS induce the Nalp3 inflammasome in the liver. In this study, we showed that LPS stimulation led to induction of the components of the Nalp3 inflammasome at both the mRNA and protein level in the liver and this is associated with increased IL-1β production.

MATERIALS AND METHODS

Mice

Six-to-eight-week-old wild-type C57BL/6 chow fed mice received intraperitoneal injections of 0.5 μg/g LPS in PBS (Sigma, St Louis, MO, United States) (three mice per group) for 2, 4, 6, 18 and 24 h. Serum was separated from whole blood and stored at -80 ℃. Liver samples were snap frozen in liquid nitrogen or stored in RNAlater (Qiagen Sciences, Germantown, MD, United States) for RNA extraction. All animals received proper care in agreement with animal protocols at the University of Massachusetts Medical School Institutional Animal Use and Care Committee.

Biochemical alanine aminotransferase assay

Serum alanine aminotransferase (ALT) was determined using a kinetic method (DTEK LLC, Bensalem, PA, United States).

RNA analysis

RNA was purified from livers using the RNeasy kit (Qiagen Sciences) and on-column DNA digestion. cDNA was transcribed with the Reverse Transcription System (Promega, Madison, WI, United States). Real-time quantitative polymerase chain reaction (qPCR) was performed using iCycler (Bio-Rad Laboratories, Hercules, CA, United States) as described previously[22]. Primer sequences are shown in Table 1.

Cytokine enzyme-linked immunosorbent assay measurements

Whole cell lysates were extracted from liver tissue as previously described[22]. These lysates were assayed for mature IL-1β (R and D Systems, Minneapolis, MN, United States), TNF-α, total IL-18, and total IL-1β (BD Biosciences, United States). Serum was also assayed for mature IL-1β (R and D Systems).

Statistical analysis

Statistical significance was determined using Student's *t*

Table 1 Real-time polymerase chain reaction primers

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
TNF-a	GAA GTT CCC AAA TGG CCT CC	GTG AGG GTC TGG GCC ATA GA
IL-1β	TCT TTG AAG TTG ACG GAC CC	TGA GTG ATA CTG CCT GCC TG
IL-1RA	TCA GAT CTG CAC TCA ATG CC	CTG GTG TTT GAC CTG GGA GT
Caspase-1	AGA TGG CAC ATT TCC AGG AC	GAT CCT CCA GCA ACT TC
ASC	GA GCT GCT GAC AGT GCA AC	GCC ACA GCT CCA GAC TCT TC
Nalp3	AGC CTT CCA GGA TCC TCT TC	CTT GGG CAG TTT CTT TC
Nlrc4	TGG TGA CAA TAG GGC TCC TC	CTG TTC CCT TTG CTC ACC TC
Nalp1	TGG CAC ATC CTA GGG AAA TC	TCC TCA CGT GAC AGC AGA AC
Pannexin-1	TGT GGC TGC ACA AGT TCT TC	ACA GAC TCT GCC CCA CAT TC

TNF: Tumor necrosis factor; IL: Interleukin; ASC: Apoptosis-associated speck-like, caspase recruitment domain-domain containing protein.

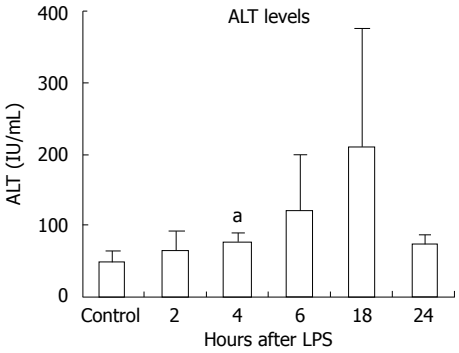

Figure 1 Lipopolysaccharide stimulation results in liver damage. C57BL/6 wild-type chow-fed mice (three per group) were injected intraperitoneally with lipopolysaccharide (LPS) for 2, 4, 6, 18 or 24 h. Serum was separated from whole blood and analyzed for alanine aminotransferase (ALT), which was significantly increased after 4 h of LPS stimulation. Mean ± SD are shown. $^{a}P < 0.01$.

test (two-tailed distribution). Data are presented as mean ± SE and were considered significant at $P < 0.05$. ALT statistical significance was determined using the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test.

RESULTS

LPS induces liver injury and inflammatory cytokines

Gut-derived LPS plays a role in several inflammatory liver diseases[6]. To evaluate the effects of LPS on the liver, we injected wild-type mice with LPS for 2, 4, 6, 18 or 24 h and monitored ALT levels to evaluate liver injury. We found increased ALT levels in sera from mice injected with LPS at the 4-18-h time points, reaching statistically significant levels at 4 h, indicating LPS-induced liver damage (Figure 1).

It has been shown that LPS-induced signaling through TLR4 results in production of proinflammatory cytokines, one of which, TNF-α, has been linked to liver damage[23,24]. We identified significant elevation in liver TNF-α mRNA expression at all time points after LPS stimulation compared to the unstimulated controls (Figure 2A). TNF-α protein levels were undetectable in liver tissue from unstimulated controls but were significantly increased at all time points after LPS stimulation with a maximal increase at 2 h (Figure 2B).

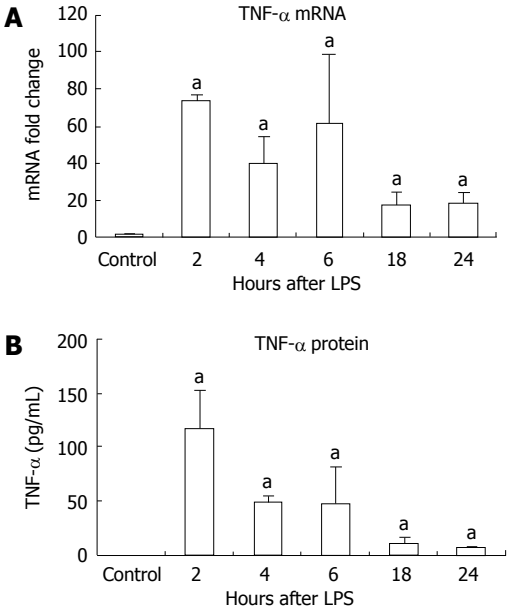

Figure 2 Inflammation in the liver is seen after lipopolysaccharide stimulation. A: Tumor necrosis factor (TNF)-α mRNA in liver tissue was significantly increased at all time points after lipopolysaccharide (LPS) stimulation. TNF-α mRNA was analyzed by real-time quantitative polymerase chain reaction and normalized to 18S. The values are shown as a fold change to the non-stimulated LPS control; B: Similarly, TNF-α protein was significantly elevated in liver tissue following LPS stimulation as detected by enzyme-linked immunosorbent assay. Protein levels were normalized to total protein concentration in each tissue sample. Mean ± SD are shown. $n = 3$ for each group (except at 4 h LPS stimulation, where $n = 2$ due to an outlier), $^{a}P < 0.01$.

LPS upregulates mRNA expression of Nalp3 inflammasome components

For complete function, the inflammasome requires expression of its various components including Nalp3, ASC (the adapter molecule), and caspase-1[8,10]. We investigated the mRNA levels of the different types of inflammasomes, including the Nalp1 and Nlrc4 inflammasomes. Our examination revealed a significant increase in liver mRNA of *Nalp3* after ≥ 4 h LPS stimulation, with a peak at 6 h (Figure 3A). Upregulation of the inflammasome was not limited to *Nalp3*, and there was a significant increase in *Nalp1* (sevenfold) and *Nlrc4* (3.5-fold) mRNA at 24 h compared to the unstimulated controls ($P < 0.01$, $P = 0.04$, respectively, data not shown). We also analyzed the mRNA of other components of the inflammasome including the pyrin and CARD-domain-containing adaptor ASC; caspase-1, an inflammatory caspase; and pannexin-1, a hemi-channel that is recruited upon activation of the P2X7 receptor and is a necessary component for inflammasome activation[25]. The mRNA levels of *ASC* gradually increased at each time point after LPS stimulation (Figure 3B), whereas caspase-1 levels peaked at 6 h and pannexin-1 mRNA levels peaked at 6 h (Figure 3C and D).

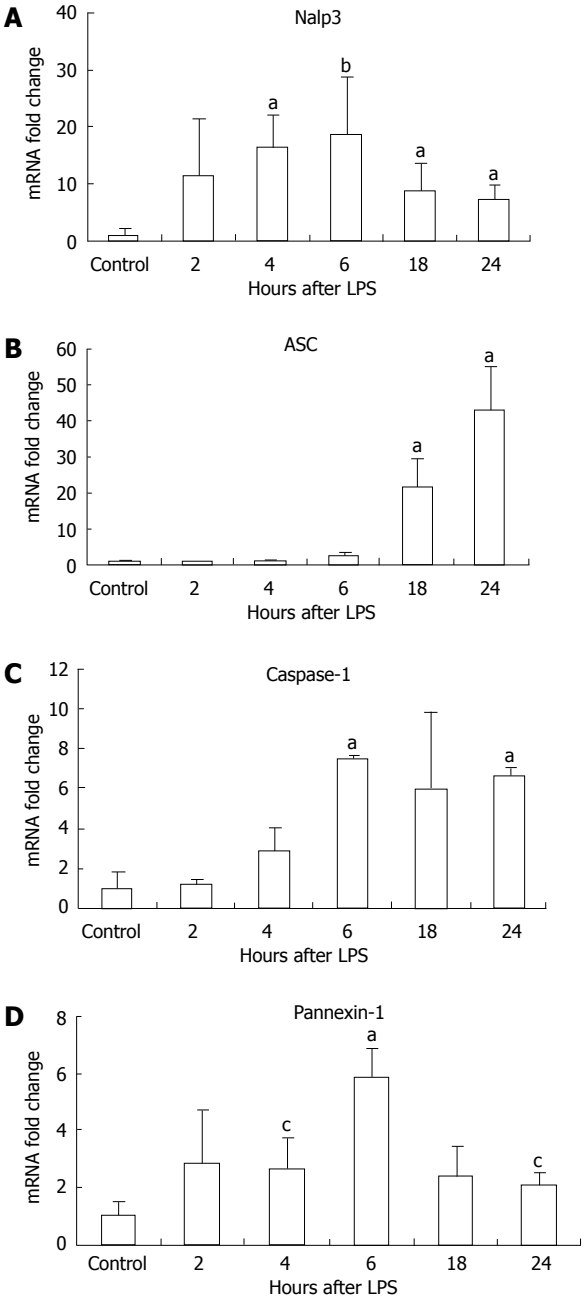

Figure 3 Lipopolysaccharide stimulation increased Nalp3 inflammasome mRNA expression in liver tissue. Liver RNA analysis of Nalp3 (A), apoptosis-associated speck-like, caspase recruitment domain-domain containing protein (ASC) (B), caspase-1 (C) and pannexin-1 (D) were analyzed by real-time quantitative polymerase chain reaction. The values were normalized to 18S and are shown as a fold increase to the non-lipopolysaccharide (LPS) stimulated control. Mean ± SD are shown. $n = 3$ per group, $^{a}P < 0.01$, $^{b}P < 0.03$, $^{c}P < 0.05$.

Activation of the inflammasome results in increased levels of total IL-18, total IL-1β, and mature IL-1β

LPS stimulation has been shown to increase levels of pro-IL-1β transcripts[26]. However, production of the biologically active *IL-1β* and IL-18 requires the mature caspase-1 to cleave the precursor forms into their mature forms[27]. First, we evaluated mRNA levels of *IL-1β* and *IL-18* and found that *IL-1β* mRNA levels were significantly increased at all time points post-LPS stimulation

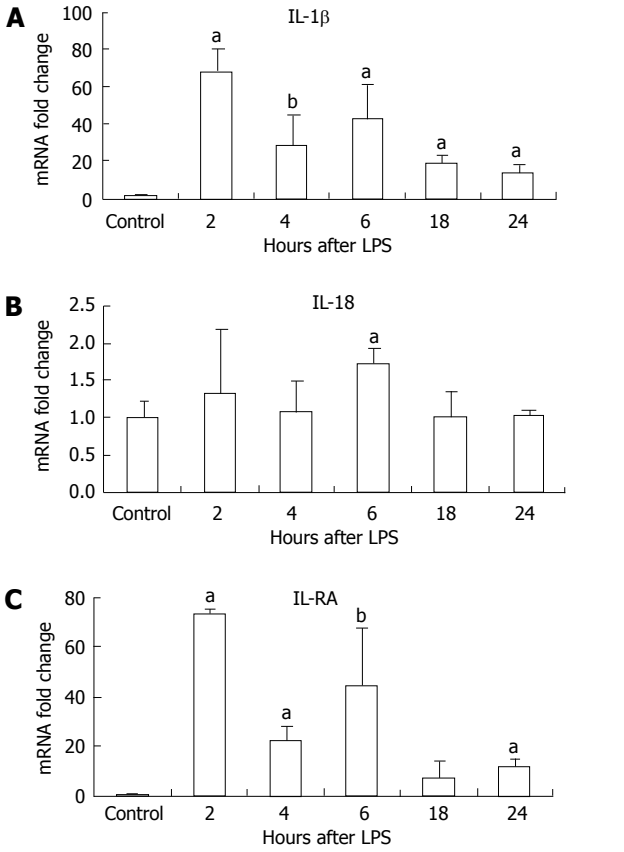

Figure 4 Lipopolysaccharide stimulation increased interleukin (IL)-1β and IL-18 mRNA, and IL-1RA transcription in the liver. Liver mRNA levels of IL-1β (A), IL-18 (B) and IL-1RA (C) were analyzed by real-time quantitative polymerase chain reaction and normalized to 18S. Mean ± SD are shown. $n = 3$ per group [except at 2 h lipopolysaccharide (LPS) stimulation, where $n = 2$ due to outlier], $^{a}P < 0.01$, $^{b}P < 0.03$.

peaking at 2 h (Figure 4A). Induction of *IL-18* mRNA by LPS was modest but reached statistical significance at the 6-h time point (Figure 4B). There was also induction of the mRNA for the IL-1 receptor antagonist after LPS stimulation in the liver (Figure 4C).

We sought to determine whether the increase in mRNA correlated with increased protein production for IL-1β and IL-18. We found basal expression of pro-IL-1β protein in the livers of unstimulated control mice and this was significantly upregulated at 2 h after LPS stimulation (Figure 5A). More importantly, we observed a significant increase in mature IL-1β protein in liver tissue at all time points post-LPS stimulation compared to the controls, in which no mature IL-1β was detected (Figure 5B). Consistent with inflammasome activation, we found increased serum IL-1β levels in mice after stimulation with LPS for 2 and 6 h (Figure 5C). Investigation of IL-18, which is also cleaved by caspase-1, revealed a significant increase in total IL-18 in the liver at all time points following LPS injection (Figure 5D).

DISCUSSION

Although many molecules and compounds activate the

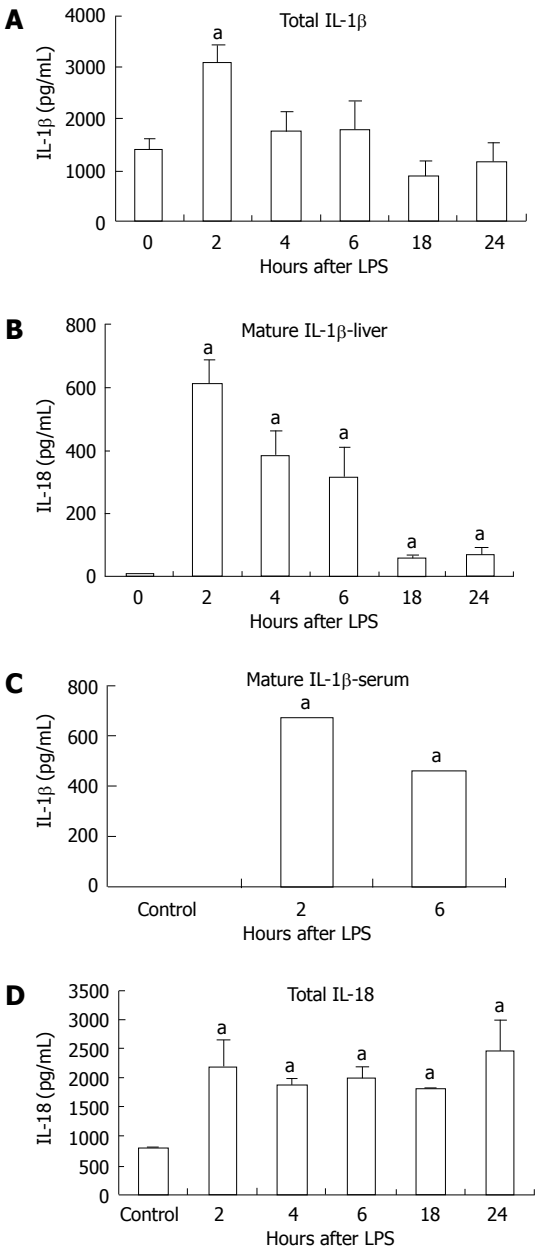

Figure 5 Lipopolysaccharide increased interleukin-1β and interleukin-18 protein. Protein level was detected in liver tissue by enzyme-linked immunosorbent assay for total-interleukin (IL)-1β (pro-IL-1β and cleaved IL-1β) (A), cleaved IL-1β in the liver tissue (B),cleaved IL-1β in the serum (C) and total IL-18 (pro-IL-18 and cleaved IL-18) (D) in liver tissue. The values shown are the fold change compared to non-Lipopolysaccharide (LPS) stimulated control. Protein levels were normalized to total protein concentrations in each tissue sample. Mean ± SD are shown. $n = 3$ per group (except at 2 h LPS stimulation, where $n = 2$ due to an outlier), $^{a}P < 0.01$.

Nalp3 inflammasome both *in vitro* and *in vivo*[8], the role of Nalp3 inflammasome in a healthy liver following LPS stimulation has not been determined. Our novel findings demonstrate upregulation of the Nalp3 inflammasome in the liver in response to LPS stimulation, both at the mRNA and protein levels. We also show for the first time that the components of the inflammasome pathway, including ASC and caspase-1, are upregulated in the liver after LPS challenge and this results in functional activation of the inflammasome and caspase-1, indicated by IL-1β and IL-18 secretion.

LPS is recognized by TLR4, which induces an intracellular signaling cascade leading to activation of NF-κB, as well as the production of pro-IL-1β and pro-IL-18[28]. Previous studies have suggested that signaling through the TLRs alone is insufficient to generate the production of mature IL-1β and IL-18, and that a second signal is needed that leads to ionic perturbation[29]. These perturbations may include ATP signaling through the P2X7 channel, leading to changes in intracellular potassium concentrations and membrane perturbations. The physiological importance of ATP in IL-1β secretion is unclear and the second signal that leads to cleavage of pro-IL-1β in the liver is still under investigation[30]. *In vitro* studies have shown that a second signal is needed in addition to LPS to obtain cleavage of pro-IL-1β[31]. It is possible that a DAMP released by injured cells could act as the second signal. *In vivo* studies, however, have shown that LPS alone is sufficient to increase cleavage of IL-1β and IL-18[31]. Our novel data demonstrate that, after *in vivo* LPS challenge, the inflammasome is activated in the liver and this results in increased inflammasome function and IL-1β secretion. We found that LPS stimulation not only increased *IL-1β* mRNA levels and the expression of pro-IL-1β protein, but it significantly increased the levels of the 18-kDa form of mature IL-1β in the liver. Secretion of mature IL-1β also resulted in elevated IL-1β in the serum. Interestingly, we found significant levels of pro-IL-1β protein expressed in the liver without exogenous stimulation. This suggests that the exogenous LPS probably provided the second signal for IL-1β production. The mechanisms for the presence of pro-IL-1β protein in the liver remain to be determined, however, one possibility is stimulation *via* PAMPs, such as LPS, which are being constantly supplied by the gut through the portal vein to the liver[6].

Cleavage of pro-IL-1β is mediated by activated caspase-1; consistent with this, we found increased levels of IL-1β protein in the serum after LPS challenge. The other target of the caspase-1 complex and inflammasome activation is IL-18. Consistent with LPS-induced inflammasome and caspase-1 activation, we found an increase in IL-18 mRNA and total IL-18 levels in the liver. Due to limitations of the detection assay, we could not distinguish between the mature form of IL-18 and pro-IL-18, but the increase in total IL-18 after LPS stimulation was most likely due to an increase in both.

We found elevated basal levels of pro-IL-1β in the liver, and pro-IL-1β was further increased by LPS with the highest levels at 2 h following stimulation. It is likely that LPS-induced inflammasome activation can cleave the pre-existing pro-IL-1β to the mature form by caspase-1. In addition to activation of the Nalp3 inflammasome, our data suggests that other types of caspase-1-activating inflammasomes, Nalp1 and Nlrc4, may also play a role in caspase-1 activation and subsequent IL-1β cleavage in the liver. Our data showed that both *Nalp1* and *Nlrc4* mRNA

levels were upregulated in the liver following LPS stimulation. This suggests that other inflammasome-activating signals lead to full activation in the liver.

The contribution of the Nalp3 inflammasome to inflammation in the liver in response to LPS stimulation may have implications for several different liver diseases. Increased gut-derived LPS has been shown to contribute to liver inflammation in alcoholic and non-alcoholic fatty liver diseases, as well as other forms of liver damage such as hepatitis C virus infection[32]. In healthy subjects, the liver plays a central role in elimination of gut-derived endotoxins and other pathogens to maintain immune homeostasis[33]. Thus, it is tempting to speculate that gut-derived ligands of TLRs constantly entering the liver could contribute to the high basal expression of pro-IL-1β found in the liver in our experiments. The high basal expression of pro-IL-1β represents the first step of inflammasome activation, and this appears to provide a pre-activated state where even single LPS stimulation can result in inflammasome activation, as seen in the liver after *in vivo* LPS challenge in our experiments. The specific role of Nalp3 and the inflammasome remains to be evaluated in different types of liver diseases where inflammation is related to LPS and other pathogen-derived or endogenous danger signals.

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COMMENTS

Background

The endotoxin lipopolysaccharide (LPS) is a component of Gram-negative bacteria, and plays an important role in both acute liver injury as well as chronic liver diseases, including fatty liver associated with either alcohol consumption or metabolic syndrome and obesity. Several pathogen-associated molecular patterns, including LPS, induce inflammasome activation, an intracellular multiprotein complex leading to release of inflammatory cytokines and cell death.

Research frontiers

Inflammasomes are required for effective clearance of several viral and bacterial pathogens. In addition, inflammasomes have been reported to play a major role in several diseases with sterile inflammation such as gout, Alzheimer's disease, atherosclerosis and type 2 diabetes. Here, the authors show the contribution of the Nalp3 inflammasome to LPS-induced inflammation in the liver.

Innovations and breakthroughs

Recently, the contribution of the Nalp3 inflammasome to *Propionibacterium acnes* plus LPS-induced acute liver injury has been described. Here, authors show the increased expression and role of the inflammasome in healthy liver following LPS stimulation.

Applications

The contribution of the Nalp3 inflammasome to inflammation in the liver in response to LPS stimulation may have implications for several liver diseases, including alcoholic and non-alcoholic fatty liver disease, as well as hepatitis C virus infection where the role of gut-derived endotoxin has been shown previously. A better understanding of the pathological mechanism may help future attempts to develop effective therapy.

Terminology

Nalp3, also called cryporin is a nucleotide oligomerization domain-like receptor that forms one of the most characterized inflammasome complexes.

Peer review

This is a well done study that examines the role of the inflammasome in the liver following LPS exposure.

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ORIGINAL ARTICLE

Autophagy-related proteins Beclin-1 and LC3 predict cetuximab efficacy in advanced colorectal cancer

Gui-Fang Guo, Wen-Qi Jiang, Bei Zhang, Yu-Chen Cai, Rui-Hua Xu, Xu-Xian Chen, Fang Wang, Liang-Ping Xia

Gui-Fang Guo, Wen-Qi Jiang, Bei Zhang, Yu-Chen Cai, Rui-Hua Xu, Xu-Xian Chen, Fang Wang, Liang-Ping Xia, State Key Laboratory of Oncology, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China
Gui-Fang Guo, Bei Zhang, Xu-Xian Chen, Fang Wang, Liang-Ping Xia, VIP Region, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China
Wen-Qi Jiang, Rui-Hua Xu, Medical Oncology Department, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China
Author contributions: Guo GF, Jiang WQ, and Xia LP designed the research; Guo GF, Cai YC, Chen XX and Wang F performed the research; Zhang B and Xu RH analyzed the data; Guo GF wrote the paper and Xia LP revised the paper.
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Correspondence to: Liang-Ping Xia, Associated Professor of Medicine, State Key Laboratory of Oncology, Cancer Center, Sun Yat-Sen University, 651 East Dongfeng Road, Guangzhou 510060, Guangdong Province, China. xialiangping@163.com
Telephone: +86-20-87343107 Fax: +86-20-87343392
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Abstract

AIM: To investigate the utility of Beclin-1 and LC3, two autophagy-related proteins, in predicting the cetuximab efficacy in advanced colorectal cancer (ACRC).

METHODS: The data of 85 patients with ACRC treated at the Sun Yat-sen University Cancer Center from March 1, 2005 to December 31, 2008 were studied, including 45 cases treated with cetuximab-containing chemotherapy and 40 cases treated with non-cetuximab-containing chemotherapy. Beclin-1 and LC3 expression was evaluated by immunohistochemistry, and KRAS status was evaluated by polymerase chain reaction.

RESULTS: Beclin-1 and LC3 expression in ACRC was significantly correlated (r = 0.44, P < 0.01); however, LC3 was more highly expressed in cancerous tissues than in normal tissues (Z = -2.63, P < 0.01). In the cetuximab-containing chemotherapy group, patients with low LC3 expression had higher objective response rates (ORRs) than those with high LC3 expression (52.9% *vs* 17.9%, P = 0.01), and patients with low Beclin-1 expression had a longer median progression-free survival (PFS) than their counterparts with higher Beclin-1 expression (9.0 mo *vs* 3.0 mo, P = 0.01). However, neither of these predictive relationships was detected in the group treated with non-cetuximab-containing chemotherapy. Patients with wild-type KRAS had higher ORRs (42.3% *vs* 9.1%, P = 0.049) and disease control rates (DCRs) (73.1% *vs* 36.4%, P = 0.035), and longer median PFS (5.5 mo *vs* 2.5 mo, P = 0.02) than those with mutant KRAS in the cetuximab-containing chemotherapy group. Neither Beclin-1 (P = 0.52) nor LC3 (P = 0.32) expression was significantly correlated with KRAS status.

CONCLUSION: Patients with low Beclin-1 expression had a longer PFS than those with high Beclin-1 expression, and patients with low LC3 expression had a higher ORR in ACRC patients treated with cetuximab-containing chemotherapy.

Key words: Beclin-1; Cetuximab; Colorectal neoplasms; Drug therapy; LC3

Peer reviewer: Shivananda Nayak, PhD, Department of Preclinical Sciences, Biochemistry Unit, Faculty of Medical Sciences, The University of The West Indies, Building 36, EWMSC, Mount Hope, Trinidad and Tobago

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide. Although therapeutic strategies have improved in recent years, the fact that 25% of patients present with advanced colorectal cancer (ACRC) at diagnosis and an additional 25% eventually progress to this stage makes it especially challenging to treat. Both the Irinotecan-based[1] and Oxaliplatin-based regimens[2] have achieved a median overall survival of 20 mo in these patients; however, further improvement has been difficult due to their serious adverse cytotoxic events. The introduction of cetuximab, a monoclonal antibody targeting epidermal growth factor receptor (EGFR), to standard chemotherapy has increased the median overall survival to 24 mo in the patients with chemotherapy-naïve or refractory ACRC[3,4]. Beneficial effects were also observed in Chinese patients with ACRC[5,6]. Although wild-type KRAS is the precondition recommended by national comprehensive cancer network (NCCN) guidelines for the administration of cetuximab to patients with ACRC, at least half of the patients[7,8] with wild-type KRAS do not benefit from this drug. Thus, it is vital to identify new predictive markers for cetuximab efficacy.

Autophagy is a catabolic process involving the degradation of a cell's own unnecessary, injured, or aged proteins and organelles and the subsequent recycling of degraded products to maintain survival. However, excessive autophagy leads to cell death, characterized by the presence of autophagic vacuoles[9]. In addition to the physiological role of autophagy, this process is also involved in many pathological conditions, including myopathy, neuronal degeneration, infectious disease, and cancer[10]. Since autophagy was first observed in yeast, more than 20 autophagy-related genes (*Atg*) have been identified[11], many of which are conserved in mammals. As an autophagosomal orthologue of yeast Atg8, microtubule-associated protein 1 light chain 3 (LC3), including LC3-Ⅰ and LC3-Ⅱ, plays a crucial role in autophagosome formation. In particular, LC3-Ⅱ is a specific marker of the autophagic process since it directly correlates with the number of autophagosomes[12]. Additionally, Beclin-1 is an essential modifier of the autophagic process and has been implicated in tumor development, including breast, ovarian, and prostate tumors in humans, which have allelic loss of Beclin-1[13]. Combined with other biochemical factors, Beclin-1 can be used to monitor autophagy[13].

It was reported in the journal cancer cell that autophagic death appears in colon cancer cells when the protein level of EGFR is decreased as a result of transient transfection with EGFR siRNA. Moreover, it was found that autophagic death was independent of EGFR tyrosine kinase activity, but depended on SGLT-1, a new pathway downstream of EGFR that controls the glucose metabolism essential for cell life[14]. In a previous study, our group also found that the SGLT-1 expression level was related to the clinical stage of CRC[15]. Cetuximab, a chimeric monoclonal antibody targeted against the extracellular domain of EGFR, may have a similar effect as EGFR siRNA, such that it also simultaneously induces autophagic death by the SGLT-1 pathway and apoptosis by the inhibition of a tyrosine kinase pathway in colon cancer cells. Until now, the relationship between the efficacy of cetuximab and autophagy in colorectal cancerous tissues was uncertain. To clarify this issue, we investigated autophagy activity by assaying the expression of Beclin-1 and LC3 in colorectal cancerous tissues, determined the correlation between Beclin-1 and LC3 expression and the efficacy of cetuximab in patients with ACRC, and evaluated the association between KRAS status and the expression of Beclin-1 and LC3.

MATERIALS AND METHODS

Patients

Eighty-five ACRC patients with definitive pathological diagnoses, paraffin-embedded pathology specimens and complete clinicopathologic information who received papillary chemotherapy in the Sun Yat-Sen University Cancer Center from March 1, 2005, to December 31, 2008, were enrolled in this study. Two study arms were used. The first arm included 45 patients who received cetuximab-containing chemotherapy. The other arm of 45 patients was selected randomly from the ACRC patients who had received papillary chemotherapy without cetuximab. Five patients in the second arm were excluded because their paraffin-embedded specimen blocks were not available. Thus, only 40 cases were ultimately entered. These patients had local relapse or distant metastasis when they began papillary chemotherapy with or without cetuximab. Some had stage Ⅱ or Ⅲ cancer at the initial visit. Cancer cell-free surgical margins obtained from 28 CRC patients were used as controls.

Immunohistochemical staining for Beclin-1 and LC3

The formalin-fixed, paraffin-embedded pathology specimens of 85 CRC tissue samples and 28 normal colorectal tissue samples were all successfully examined. The diagnosis was microscopically confirmed by a pathologist. All hematoxylin-eosin stained specimens from the 85 CRC cases contained cancerous tissues, whereas all 28 control samples were cancer free. Five-micrometer sections were cut from the paraffin blocks and placed on glass slides. The slides were dried in an incubator at 60 ℃ for 60 min, deparaffinized in xylene, and then rehydrated in an ethanol series. After washing in water, antigen retrieval with citrate buffer was performed at high temperature and pressure. The sections were cooled for 20 min, washed twice with Phosphate buffered solution (PBS) for 5 min each, and then incubated in serum for 10 min. The primary antibody, either Beclin-1 (1:100) or LC3 (1:400) (Beclin-1 from Cell Signal, United States; LC3 from Novus Biologicals, United States), was diluted in 1% PBS and incubated for 45 min after the serum was tipped. The slides were then washed twice with PBS and incubated with the anti-rabbit secondary antibody (Invitrogen, United States) for 30 min. After an additional two washes in PBS, the slides were incubated with di-

Table 1 Comparison of baseline clinicopathological characteristics between cetuximab-containing chemotherapy group and non-cetuximab-containing group *n* (%)

Clinical factors	Total	Cetuximab chemotherapy	Non-cetuximab chemotherapy	*P* value
Gender				0.85
Male	54 (63.5)	29 (64.4)	25 (62.5)	
Female	31 (36.5)	16 (35.6)	15 (37.5)	
Age (yr), median (range)	50 (12-79)			0.56
Risk group (40-60)	46 (54.1)	23 (51.1)	23 (57.5)	
Non-riskgroup (< 40)	39 (45.9)	22 (48.9)	17 (42.5)	
Family history of tumor				0.63
Yes	12 (14.1)	9 (20.0)	3 (15.0)	
No	73 (85.9)	36 (80.0)	17 (85.0)	
Tumor site				0.89
Rectum	24 (28.2)	13 (28.9)	11 (27.5)	
Colon	61 (71.8)	32 (71.1)	29 (72.5)	
Primary clinical stage				0.86
Ⅱ	12 (14.1)	5 (11.1)	7 (17.5)	
Ⅲ	25 (29.4)	15 (33.3)	10 (25.0)	
Ⅳ	48 (56.5)	25 (55.6)	23 (57.5)	
Histological grade				0.93
Well differentiation	6 (7.1)	3 (6.7)	3 (7.5)	
Moderate differentiation	57 (67.1)	30 (66.6)	26 (65.0)	
Poor differentiation	22 (25.9)	12 (26.7)	11 (27.5)	

aminobenzidine (DAB, Invitrogen, United States) for 10 min to visualize immunolabeling. After washing, the sections were counterstained with hematoxylin (Invitrogen, United States). Squamous cell cancer of the cervix and neurons from the cerebral cortex were used as positive controls for Beclin-1 and LC3, respectively, according to the manufacturer's instructions for each antibody. PBS was used as the negative control instead of the primary antibody on each slide for both Beclin-1 and LC3.

Hscore assessment

This method of assigning an histological score (Hscore) has been previously described[16,17]. Two independent pathologists with no knowledge of the clinical data scored all immunohistochemical staining of Beclin-1 and LC3, according to the staining intensity and the percentage of positively stained tumor cells. Staining intensities were classified into 4 grades: 0 (pale yellow or no staining), 1 (yellow), 2 (deep yellow) and 3 (brown). The percentage of positively stained tumor cells was scored in 4 grades: 0 (0%-10%), 1 (10%-25%), 2 (25%-50%) and 3 (50%-100%). The intensity and percentage of positively stained tumor cells were scored after counting at least 10 high-power fields at 400 ×. Mean Hscores were calculated as follows: [(Intensity reader 1 × Percentage reader 1) + (Intensity reader 2 × Percentage reader 2)]/2.

Polymerase chain reaction analysis of KRAS mutation

Six sections (5-μm thick) from the formalin-fixed, paraffin-embedded blocks were used for genomic DNA extraction using the QIAamp DNA Paraffin-Embedded Tissue Kit (QIAGEN) according to the manufacturer's instructions. The quality and concentration of the extracted DNA were determined by ultraviolet spectrophotometry. DNA quality was analyzed using a polymerase chain reaction (PCR) reaction (20 μL) that contained 10 μL master mix, 0.15 μL glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F, 0.15 μL GAPDH-R, 7.7 μL ddH_2O and 2 μL DNA. Products were visualized by electrophoresis on a 2% agarose gel. KRAS mutations were detected by a PCR (25 μL) composed of 12.5 μL PCR mix, 0.5 μL Primer A, 0.5 μL Primer B, 0.3 μL Probe 1 [FAM (carboxyfluorescein, blue) maker], 0.6 μL Probe 2 [VIC (Aequoria Victoria, green) maker], 8.1 μL ddH_2O and 2.5 μL DNA. The KRAS primers are as follows[18]: forward, 5'-AAGGCCTGCTGAAAATGAC-3'; and reverse, 5'-TGGTCCTGCACCAGTAATATG-3'. Samples for the control assay with a cycle threshold (Ct) below 35 were considered positive, whereas samples with Ct ⩾ 38 were scored as negative (wild-type).

Statistical analysis

All statistics were calculated using SPSS for Windows, version 17.0. Nonparametric tests were used to compare the expression of Beclin-1 and LC3 between colorectal cancerous tissues and normal tissues. Correlations of Beclin-1 and LC3 Hscore with KRAS status were assessed by Spearman's correlation analysis. The Chi-square test was used to compare the baseline characteristics of the cetuximab-containing chemotherapy and the non-cetuximab-containing chemotherapy, and analyze the influence of Beclin-1 and LC3 expression on the objective response rates (ORR) and disease control rates (DCR) of the two groups. Kaplan-Meier curves and Cox regression models were used as univariate and multivariate analysis tools, respectively, to evaluate progression-free survival (PFS) and overall survival (OS). Significance was defined as $P \leqslant 0.05$. All *P* values were two-sided.

PFS was calculated as the time lapsed between the date of treatment and the date of relapse or progressive disease. Patients with no signs of relapse were censored at the time of last follow-up or death. OS was calculated from the day of diagnosis until death or the last follow-up.

RESULTS

Baseline characteristics of the 85 patients with ACRC, including gender, age, primary clinical stage, tumor site, family history of tumor and histological grade, are listed in Table 1. All clinicopathologic characteristics of the patients receiving cetuximab-containing chemotherapy or non-cetuximab-containing chemotherapy were equivalent. By the time of the final follow-up (December 1, 2010), 57 patients had died, 27 were alive [performance status (PS) ⩽ 1 in 20 patients, PS = 2 in five patients, and PS = 3 in two patients], and one patient was lost to follow-up during a median follow-up time of 34.0 mo (2.0-137.0 mo).

Expression of Beclin-1 and LC3 in CRC tissues

The expression of Beclin-1 and LC3 was successfully evaluated in all 85 CRC tissues and 28 normal colorectal

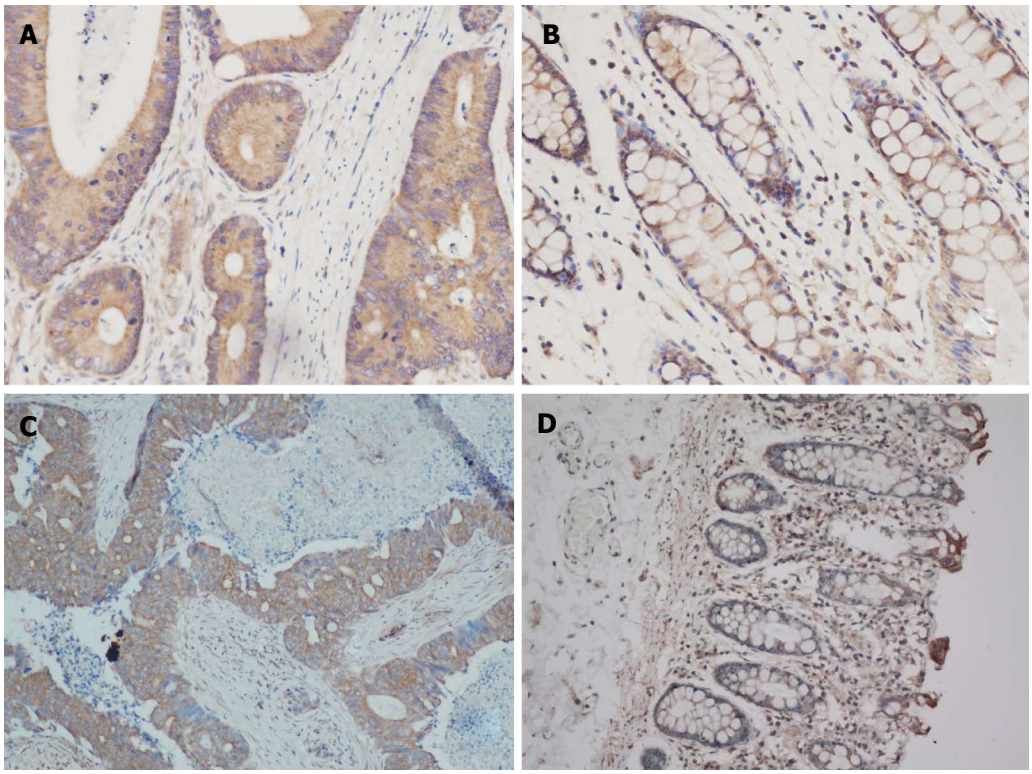

Figure 1 Immunohistochemical photomicrographs of Beclin-1 and LC-3 in colorectal cancer tissues and colorectal normal tissues (200 ×). A, B: Beclin-1 displayed strongest cytoplasm staining in colorectal cancer (CRC) tissues and weak positive staining in normal tissues: C, D: LC3 displayed intense granular staining of the cytoplasm in CRC tissues but absent in normal tissues.

Table 2 Expression of Beclin-1 and LC3 in colorectal cancer and colorectal normal tissues *n* (%)

Hscores	Beclin-1 expression		LC3 expression	
	CRC tissues	Colorectal normal tissues	CRC tissues	Colorectal normal tissues
0	5 (5.9)	2 (7.1)	4 (4.7)	2 (7.1)
1	2 (2.4)	0 (0.0)	4 (4.7)	1 (3.6)
2	8 (9.4)	3 (10.7)	9 (10.6)	8 (28.6)
3	18 (21.2)	7 (25.0)	14 (16.5)	5 (17.9)
4	4 (4.7)	1 (3.6)	5 (5.9)	3 (10.7)
6	28 (32.9)	13 (46.4)	28 (32.9)	8 (28.6)
9	20 (23.5)	2 (7.1)	21 (24.7)	1 (3.6)
Z /P (K-M test)	-0.94/0.35		-2.63/0.00	

K-M test was used to compare the expression of Beclin-1 or LC3 between CRC tissues and colorectal normal tissues. *P* value ≤ 0.05 was considered statistically significant. CRC: Colorectal cancer.

tissues. The Hscore consisting of seven grades was used to assess the tumor tissue. The expression of Beclin-1 and LC3 in cancerous and normal tissues is shown in Figure 1. The Hscore of LC3 expression in cancerous tissues was higher than that in normal tissues ($P < 0.01$), while Beclin-1 expression was not significantly different ($P = 0.35$) (Table 2).

Association of Beclin-1 and LC3 expression and KRAS status with efficacy of cetuximab-containing chemotherapy

Expression of Beclin-1 and LC3 correlated with short-term efficacy: For the 45 patients who had ever received cetuximab-containing chemotherapy, the Chi-square test was used to analyze the different ORRs and DCRs of the regimen, in patients with tumors exhibiting low or high Beclin-1 and LC3 expression (Table 3). The patients with low LC3 expression had a higher ORR than those with high LC3 expression (52.9% *vs* 17.9%, $P = 0.01$). Beclin-1 expression had no influence on ORR (26.7% *vs* 33.3%, $P = 0.65$). Neither Beclin-1 (80.0% *vs* 56.7%, $P = 0.12$) nor LC3 (76.5% *vs* 57.2%, $P = 0.19$) expression affected the DCR. In this whole group, the median PFS was 3.0 mo. The median PFS of the patients with low and high Beclin-1 expression was 9.0 mo and 3.0 mo, respectively ($P = 0.01$) (Figure 2). The median PFS of the patients with low and high LC3 expression was 3.0 mo and 4.0 mo, respectively ($P = 0.62$).

Association between KRAS status and short-term efficacy: Among the 45 patients treated with cetuximab-containing chemotherapy, 37 patients were successfully tested for *KRAS* gene mutations. The *KRAS* mutation rate in CRC patients was 29.7% (11/37). The *KRAS* mutations were all in codon 12. Nine cases had the G12D mutation (12 GGT→GAT, Gly→Asp), one case had the G12C mutation (12 GGT→TGT, Gly→Cys), and one case had the G12S mutation (12 GGT→AGT, Gly→Ser). The patients with wild-type KRAS had higher ORR (42.3% *vs* 9.1%, $P = 0.049$), DCR (73.1% *vs* 36.4%, $P = 0.035$) and PFS (5.5 mo *vs* 2.5 mo, $P = 0.02$) than those with *KRAS* mutations (Table 4 and Figure 3).

Table 3 Correlation between Beclin-1 and LC3 expression with objective response rate and disease control rate in cetuximab-containing chemotherapy group and non-cetuximab-containing group in patients with advanced colorectal cancer *n* (%)

	Beclin-1 expression		LC3 expression	
	Low	High	Low	High
Cetuximab-containing chemotherapy				
CR	0 (0)	0 (0)	0	0
PR	4 (26.7)	10 (33.3)	9 (52.9)	5 (17.9)
SD	8 (53.3)	7 (23.3)	4 (23.5)	11 (39.3)
PD	3 (20.0)	13 (43.3)	4 (23.5)	12 (42.8)
Non-cetuximab-containing chemotherapy				
CR	0 (0)	0 (0)	0 (0)	0 (0)
PR	9 (42.9)	7 (36.8)	7 (43.7)	9 (37.5)
SD	9 (42.9)	9 (47.4)	6 (37.5)	12 (50.0)
PD	3 (14.2)	3 (15.8)	3 (18.8)	3 (12.5)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progression disease.

Table 4 Objective response rate and disease control rate of patients with wild type KRAS and KRAS mutation in cetuximab-containing chemotherapy group *n* (%)

Effect	Wild type KRAS	Mutant type KRAS	Total
CR	0 (0)	0 (0)	0
PR	11 (42.3)	1 (9.1)	12 (32.4)
SD	8 (30.8)	3 (27.3)	11 (29.7)
PD	7 (26.9)	7 (63.6)	14 (37.8)
Total	26 (70.3)	11 (29.7)	37 (100)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progression disease.

Effects of KRAS, Beclin-1 and LC3 on OS: The median OS in this study cohort was 40.0 mo. The most common potential factors affecting OS, including gender, age, primary clinical stage, tumor site, family history of tumor, histological grade, the expression of Beclin-1, LC3 and KRAS status, were analyzed as shown in Table 5. Only clinical stage ($P < 0.01$) and histological differentiation ($P = 0.049$) were found to be predictive factors of OS by univariate analysis. When the clinical stage, histological differentiation, Beclin-1 expression, LC3 expression, Hscore and KRAS status were subjected to multivariate analysis, only TNM stage retained its prognostic value ($P < 0.01$) (Table 6). Only 37 cases were included in the COX regression model because the KRAS status of eight cases was not tested successfully.

Correlation of Beclin-1 and LC3 expression and KRAS status: Spearman's correlation analysis revealed that Beclin-1 and LC3 expression levels in CRC exhibited a correlation coefficient of 0.44 ($P < 0.01$). However, neither Beclin-1 nor LC3 expression was related to KRAS status ($P = 0.52$ and $P = 0.32$, respectively).

Association of Beclin-1 and LC3 expression with efficacy of non-cetuximab-containing chemotherapy

For patients treated with non-cetuximab-containing chemotherapy, the ORRs and DCRs of the first-line chemotherapy were compared among patients exhibiting low and high expression of Beclin-1 and LC3. Neither Beclin-1 expression (ORR = 42.9% and 36.8%, $P = 0.70$; DCR = 85.7% and 84.2%, $P = 0.89$ in low and high Beclin-1 expression groups, respectively) nor LC3 expression (ORR = 43.7% and 37.5%, $P = 0.69$; DCR = 81.2% and 87.5%, $P = 0.59$ in the low and high LC3 expression groups, respectively) affected the ORR or DCR (Table 3). In addition, neither low nor high Beclin-1 or LC3 expression influenced PFS (7.0 mo *vs* 7.0 mo, $P = 0.29$; 7.0 mo *vs* 7.0 mo, $P = 0.60$) in this group, which had a median PFS of 7.0 mo. In addition, no significant associations were found between OS and Beclin-1 (33.0 mo *vs* 24.5 mo, $P = 0.67$) or LC3 expression (33.0 mo *vs* 29.0 mo, $P = 0.84$).

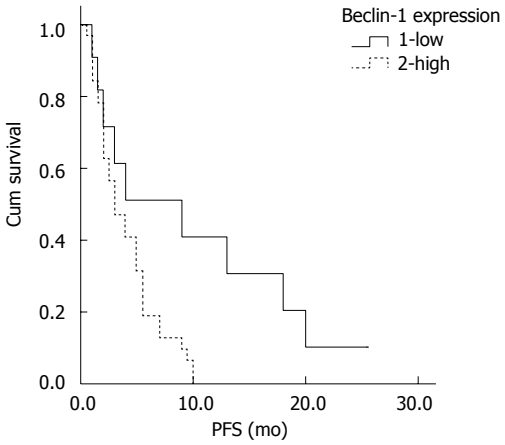

Figure 2 Progression-free survival of advanced colorectal cancer patients with low and high Beclin-1 expression in cetuximab-containing chemotherapy group. The median PFS of the patients with low and high Beclin-1 expression (a low expression defined as immuhistochemistry score < 6.0 and a high expression defined as immuhistochemistry score ⩾ 6.0.) was 9.0 mo and 3.0 mo respectively ($P = 0.01$). PFS: Progression-free survival.

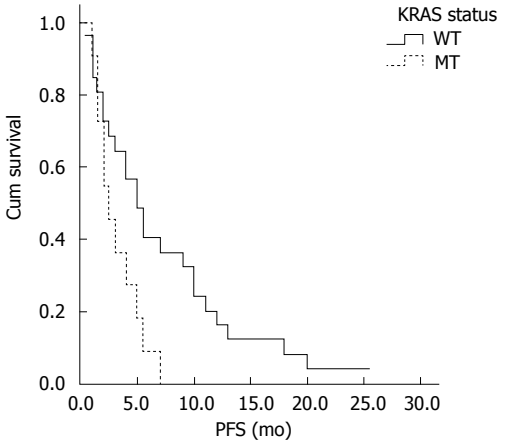

Figure 3 Progression-free survival of advanced colorectal cancer patients with KRAS wild type and patients with KRAS mutation treated by cetuximab-containing chemotherapy. Thirty-seven of 45 patients were successfully tested *KRAS* gene in cetuximab-containing chemotherapy group, the median PFS of patients with KRAS wild type was significantly higher than that of patients with KRAS mutation (5.0 mo and 2.5 mo, respectively, $P = 0.02$). PFS: Progression-free survival.

Table 5 Survival analysis of 45 patients with advanced colorectal cancer treated by cetuximab-containing chemotherapy

Clinical factors	*n* (%)	MOS (mo)	*P* value
Gender			0.93
Male	29 (64.4)	52.5	
Female	16 (35.6)	33	
Age (yr), median (range)			
Risk group (40-60)	23 (51.1)	40	0.38
Non-riskgroup (≤ 40 or > 60)	22 (48.9)	35	
Family history of tumor			0.23
No	36 (80.0)	40	
Yes	9 (20.0)	42	
Tumor site			0.88
Colon	32 (71.1)	42	
Rectum	13 (28.9)	42	
Primary clinical stage			< 0.01
Ⅱ	5 (11.1)	137	
Ⅲ	15 (33.3)	43	
Ⅳ	25 (55.6)	22	
Histological grade			0.049
Well and moderate diff	34 (75.6)	43	
Poor diff	11 (24.4)	23	
Beclin-1 expression			0.75
Low	15 (33.3)	42.5	
High	30 (66.7)	35	
LC3 expression			0.27
Low	17 (37.8)	42.5	
High	28 (62.2)	33	
KRAS status			0.73
Wild type	28 (71.8)	43	
Mutation	11 (28.2)	22	

MOS: Median overall survival; Diff: Differentiation.

Table 6 Multivariate analysis of 37 patients with advanced colorectal cancer treated by cetuximab-containing chemotherapy

	B	SE	Wald	*P* value	OR	95.0% CI for OR	
						Lower	Upper
Primary clinical stage	1.52	0.43	12.63	0.00	4.55	1.97	10.51
Histological grade	-0.06	0.54	0.01	0.92	0.95	0.33	2.72
Beclin-1 expression	-0.09	0.08	1.14	0.29	0.92	0.78	1.08
LC3 expression	0.01	0.10	0.01	0.92	1.01	0.83	1.22
KRAS status	0.52	0.46	1.27	0.26	1.68	0.68	4.15

Thirty-seven of 45 cases were included in COX regression model because the KRAS status of 8 patients was not available. B: Regression coefficient; SE: Standard Error; OR: Odds ratio; CI: Confidence Interval.

DISCUSSION

The expression of Beclin-1 and LC3 was evaluated in 85 patients with ACRC. Forty-five of those patients had been treated with cetuximab-containing chemotherapy, and 40 patients received non-cetuximab-containing chemotherapy at the Sun Yat-Sen University Cancer Center. The two treatment groups were merged because the baseline characteristics with potential prognostic influence, including gender, age, primary clinical stage, tumor site, family history and histological grade, were equivalent. Levels of Beclin-1 and LC3 in ACRC tissues were significantly correlated ($P < 0.01$); however, only LC3 was more highly expressed in cancerous tissues than in normal tissues ($P < 0.01$). The reason for the expression heterogeneity of these two autophagy-related proteins could be the followings: (1) mutations in Belcin-1 are rarely present in gastrointestinal cancers[19,20], such that Beclin-1 expression is predicted to be almost identical in colorectal cancerous tissues and normal tissues; or (2) autophagy might be induced by factors other than Beclin-1, e.g., SGLT1[14], such that LC3 overexpression, usually considered a hallmark of autophagy, might not always occur in conjunction with Belcin-1 overexpression[21]. Indeed, the status of Beclin-1 expression in colorectal cancer had not been satisfactorily evaluated before the present work. One study investigating 103 colorectal and 60 gastric carcinoma tissues by immunohistochemistry indicated increased expression of Beclin-1 in malignant gastrointestinal epithelial tissues compared with normal mucosal epithelial tissues[22]. However, decreased mRNA and protein levels of Beclin-1 in colorectal cancerous tissues were also reported in China[20]. Although immunohistochemical detection of LC3 cannot distinguish LC3-Ⅰ and LC3-Ⅱ, it can still serve as an indicator of autophagic activity since LC3-Ⅱ is a dominant form of LC3[23]. The present study demonstrated that most colorectal cancerous tissues had higher levels of LC3 expression than most normal colorectal tissues, which generally exhibited low levels of LC3 (Table 2). This finding was supported by the results of a study involving 163 gastrointestinal cancer patients in whom LC3 was differentially expressed in the cytoplasm of cancer cells, but not in noncancerous epithelial cells[24]. Based on these findings, we speculate that normal cells exhibit a basal level of autophagy in order to maintain cellular homeostasis. Furthermore, increased autophagy in colorectal cancer cells may also play a crucial role in tumor survival; this finding was consistent with previous studies[25,26].

We also found that the patients with low LC3 expression had higher ORRs than those with high LC3 expression ($P = 0.01$) in the cetuximab-containing chemotherapy group; however, LC3 did not correlate with ORR in the non-cetuximab-containing chemotherapy group. Furthermore, the median PFS of patients with low and high Beclin-1 expressions was significantly different ($P = 0.01$) in the cetuximab-containing chemotherapy group; however, Beclin-1 expression could not predict PFS in the non-cetuximab-containing chemotherapy group. In summary, neither LC3 nor Beclin-1 correlated with treatment outcome in the non-cetuximab-containing chemotherapy group, but low expression of each protein correlated with good outcomes, based on ORR or median PFS, in the cetuximab-containing chemotherapy group. These data indicates that low autophagy levels are strongly correlated with good efficacy of cetuximab. To our knowledge, this is the first report that is based on clinical data. Our findings are in accordance with the idea that autophagy protects colon cancer cells from the apoptotic effects of cetuximab[27,28].

We also determined whether Beclin-1 and/or LC3,

two autophagy-related proteins, were as useful as *KRAS* gene mutations[3] in predicting the efficacy of cetuximab-containing regimens in ACRC. *KRAS* gene status is considered the gold standard to predict cetuximab efficacy in patients with ACRC. Cetuximab was specially designed to block the tyrosine kinase pathway in which KRAS is involved, and induce apoptosis; however, autophagy, another pathway downstream of EGFR, was also recently found to be influenced by cetuximab[27,28]. Consistent with other studies[7,29,30], our results showed that patients with wild-type KRAS had higher ORRs and DCRs and longer median PFS. We then attempted to predict the cetuximab treatment outcome by combining KRAS status with Beclin-1 and LC3 expression levels; however, this was not possible because neither of the autophagy-related proteins was correlated with KRAS status. Additionally, we found that KRAS status was a more powerful biomarker than either Beclin-1 or LC3 for predicting the efficacy of cetuximab-containing chemotherapy in ACRC. Although the OS of subgroups with low LC3 expression, low Beclin-1 expression and wild-type KRAS was longer than that of their counterparts with high LC3 expression, high Beclin-1 expression and mutant KRAS (Table 5), none of the three biomarkers was powerful enough to predict the OS of the ACRC patients treated with cetuximab by univariate or multivariate analysis. More importantly, the autophagy-related proteins, Beclin-1 and LC3, predicted drug efficacy independent of KRAS status, possibly because the autophagic pathway is distinct from the tyrosine kinase pathway that regulates KRAS. Panitumumab, an entirely human monoclonal antibody specific to EGFR, was also reported to affect colon cancer cell proliferation independent of KRAS mutation status, possibly through the induction of autophagy[31]. Though the nature of autophagy's involvement in a cell's response to cytotoxic chemotherapy or cetuximab treatment has been controversial, our findings imply that autophagy makes a mild to moderate contribution to the anti-tumor effects of cetuximab, but it is not as critical as KRAS. We believe that the autophagy could be used to enhance and/or predict cetuximab efficacy.

Obviously, the heterogeneity of Beclin-1 and LC3 expression and their limited ability to predict the efficacy of cetuximab may restrict their utility. The findings discussed herein may be associated with our small patient sample. Beclin-1 is only an autophagy modifier, and varying levels of this protein do not always indicate the presence or absence of autophagy; however, LC3 expression is a hallmark of autophagy initiation.

Based on our data, we conclude that low levels of autophagy were associated with high anti-tumor activity of cetuximab-containing chemotherapy because patients with low LC3 expression exhibited higher ORRs. Similarly, patients with tumors expressing low levels of Beclin-1 had longer median PFS. Wild-type KRAS was strongly correlated with good outcomes in terms of ORR, DCR and PFS. Importantly, Beclin-1 and LC3 expression predicted cetuximab treatment outcomes while they were not related to the KRAS status, indicating that autophagy might offer another potential avenue to enhance and/or predict cetuximab efficacy in patients with ACRC.

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COMMENTS

Background

At least half of the advanced colorectal cancer (ACRC) patients with wild-type KRAS do not benefit from cetuximab. Thus, it is vital to identify new predictive markers for cetuximab efficacy. Autophagic death occurred in colon cancer cells when epidermal growth factor receptor (EGFR) was blocked with EGFR siRNA. Both of cetuximab and EGFR siRNA targeted the extracellular domain of EGFR, which indicates that autophagy may be involved in cetuximab antitumor activity and predict cetuximab efficacy in patients with ACRC. LC3 plays a crucial role in autophagosome formation and Beclin-1 is an essential modifier of the autophagic process, so LC3 and Beclin-1 can be used to monitor autophagy.

Research frontiers

Autophagy protects colon cancer cells from the apoptotic effects of cetuximab, however, Panitumumab, another antibody specific to EGFR, was also reported to affect colon cancer cell proliferation independent of KRAS mutation status, possibly through the induction of autophagy. What the exact role autophagy plays in ACRC and whether autophagic markers, such as Beclin-1 and LC3, can predict efficacy of cetuximab, are still unknown.

Innovations and breakthroughs

Based on clinical data, this study found for the first time that low levels of autophagy were associated with high anti-tumor activity of cetuximab-containing chemotherapy in ACRC patients.

Applications

The study results suggest that autophagy might offer another potential avenue to enhance and/or predict cetuximab efficacy in patients with ACRC.

Terminology

Autophagy: A catabolic process involving the degradation of the cells to maintain survival, but excessive autophagy leads to cell death. In recent years, it has been found that autophagy is involved in cancer process and treatment; Microtubule-associated protein 1 light chain 3 (LC3): It includes LC3-Ⅰ and LC3-Ⅱ, and plays a crucial role in autophagosome formation; Beclin-1: An essential modifier of the autophagic process and being implicated in tumor development.

Peer review

It is an interesting article and authors have done extensive work and written well with important figures and tables.

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BRIEF ARTICLE

Early experience of the compression anastomosis ring (CAR™ 27) in left-sided colon resection

Jung-Yeon Lee, Jin-Hee Woo, Hong-Jo Choi, Ki-Jae Park, Young-Hoon Roh, Ki-Han Kim, Hak-Yoon Lee

Jung-Yeon Lee, Jin-Hee Woo, Hong-Jo Choi, Ki-Jae Park, Young-Hoon Roh, Ki-Han Kim, Hak-Yoon Lee, Department of Surgery, Dong-A University Medical Center, Busan 602-715, South Korea

Author contributions: Choi HJ, Lee JY and Park KJ designed the research; Choi HJ performed surgery on the study subjects; Lee JY, Roh YH and Kim KH collected and analyzed the data; Lee JY and Woo JH wrote the paper; Park KJ and Lee HY reviewed and revised the paper; All authors read and approved the final manuscript.

Correspondence to: Hong-Jo Choi, MD, FACS, Department of Surgery, Dong-A University Medical Center, 3-1 Dongdaeshin-Dong, Seo-Gu, Busan 602-715, South Korea. hjchoi1@dau.ac.kr
Telephone: +82-51-2405146 Fax: +82-51-2479316

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Abstract

AIM: To evaluate clinical validity of the compression anastomosis ring (CAR™ 27) anastomosis in left-sided colonic resection.

METHODS: A non-randomized prospective data collection was performed for patients undergoing an elective left-sided colon resection, followed by an anastomosis using the CAR™ 27 between November 2009 and January 2011. Eligibility criteria of the use of the CAR™ 27 were anastomoses between the colon and at or above the intraperitoneal rectum. The primary short-term clinical endpoint, rate of anastomotic leakage, and other clinical outcomes, including intra- and postoperative complications, length of operation time and hospital stay, and the ring elimination time were evaluated.

RESULTS: A total of 79 patients (male, 43; median age, 64 years) underwent an elective left-sided colon resection, followed by an anastomosis using the CAR™ 27. Colectomy was performed laparoscopically in 70 patients, in whom two patients converted to open procedure (2.9%). There was no surgical mortality. As an intraoperative complication, total disruption of the anastomosis occurred by premature enforced tension on the proximal segment of the anastomosis in one patient. The ring was removed and another new CAR™ 27 anastomosis was constructed. One patient with sigmoid colon cancer showed postoperative anastomotic leakage after 6 d postoperatively and temporary diverting ileostomy was performed. Exact date of expulsion of the ring could not be recorded because most patients were not aware that the ring had been expelled. No patients manifested clinical symptoms of anastomotic stricture.

CONCLUSION: Short-term evaluation of the CAR™ 27 anastomosis in elective left colectomy suggested it to be a safe and efficacious alternative to the standard hand-sewn or stapling technique.

Key words: Compression anastomosis; Colon; Anastomotic leakage; CAR™ 27

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INTRODUCTION

A well-vascularized tension-free anastomosis is of paramount importance for successful colorectal surgery and the hand-sewn or stapling technique is currently the most common surgical standard for restoration of bowel continuity. However, both techniques inevitably leave foreign materials within tissue, evoking an inflammatory reaction, thus potentially resulting in a number of anastomosis-related adverse effects. In particular, permanent presence of metallic foreign material after stapled anastomosis often contributes to the reduced size of the anastomotic lumen, and may be responsible for frequent postoperative stricture[1].

Anastomotic leakage still remains a serious problem associated with substantial morbidity and mortality. Although considerable variations are seen among surgeons, an overall clinically apparent anastomotic leakage proximal to the peritoneal reflection of the rectum ranges in frequency from 3.4% to 6%[2,3]. For anterior resection of the rectum, clinical leakage rate is known to be higher-between 2.9% and 15.3%[4-6]. In particular, not only increasing surgical morbidity and mortality, this complication may be associated with worse survival and higher recurrence after curative resection of colorectal cancer[7,8]. These drawbacks associated with conventional anastomotic techniques naturally lead surgeons to investigate a more ideal concept of the anastomosis. As an alternative to the end-to-end circular stapling device, a novel compression anastomosis ring (CAR™ 27; NiTi Surgical Solutions, Netanya, Israel) was introduced recently. It is made of shape-memory alloy of nickel-titanium (Nitinol), which is temperature dependent. Because this device is staple-free, there is no puncturing injury of the bowel wall, no risk of anastomotic bleeding, and it does not leave any permanent foreign material within the body. Within 6 to 11 d, the entire device together with the necrotized tissue detaches and is naturally eliminated from the body during bowel movements, leaving a wide and patent sutureless end-to-end anstomosis[9].

The aims of the current study were to present early clinical experience of the CAR™ 27 device in colorectal or colocolic end-to-end anastomosis after left-sided colon resection, and to evaluate the safety, efficacy, and technical feasibility of the device.

MATERIALS AND METHODS

Patients

A non-randomized prospective data collection was performed for patients undergoing an elective left-sided colon resection, followed by an anastomosis using the CAR™ 27 for various left-colonic etiologies. Eligibility criteria of the use of the CAR™ 27 were anastomoses between the colon and at or above the intraperitoneal rectum. The surgical procedures were performed by a single surgeon (HJ Choi). Between November 2009 and January 2011, a total of 79 anastomoses were constructed by use of the CAR™ 27. Preoperatively, patients were informed of the method of bowel anastomosis. Data on patient demographics, surgical indications, operative procedure, perioperative course, and outcome were recorded prospectively on data sheets. The primary short-term clinical endpoint was the rate of anastomotic leakage, and other clinical outcomes, including intra- and postoperative complications, length of operation time and hospital stay, and the ring elimination time were recorded. No postoperative radiologic contrast study was performed routinely, and anastomotic leakage was diagnosed clinically.

Figure 1 NiTi compression anastomosis ring (CAR™ 27). It consists of firing device, ring loader, polyethylene anvil, and nitinol ring (Courtesy of NiTi™ Surgical Solutions, Netanya, Israel).

Description and use of the CAR™ 27

Nitinol is a temperature-dependent shape memory alloy of nickel-titanium because it expands and flexes when cooled, but resumes its shape and size when it returns to its normal temperature[10]. Nitinol leaf springs are used in the CAR™ 27 to maintain a continuous pressure at the anastomosis independent of the thickness of tissue within the anastomosis. By cooling the ring in cold saline for about 30 s, the nitinol leaf springs flatten within the ring. When an anastomosis is created, the ring can accommodate to the different thicknesses of tissue within the anastomosis[11]. At body temperature, the nitinol leaf springs begin to return to their original shape, which closes the gap gradually until the trapped tissue becomes necrotic. As a result, healthy tissue connects the bowel ends at the ring's outer perimeter to restore the bowel continuity.

The CAR™ 27 instrument consists of two main components, a main firing device and detachable compression elements (Figure 1). Compression elements are composed of a polyethylene anvil ring and a nitinol leaf spring-containing metal ring. Its use is similar to that of current curved circular staplers and it was manipulated according to the manufacturer's instructions. In brief, the detachable anvil ring was inserted into the proximal bowel lumen and secured by a purse-string suture, when an anastomosis was ready after colon resection. After immersing the mounted ring with its loader in cold saline for several minutes, the ring was loaded to the distal end of the firing device. The firing device was then inserted transanally up to the stapled end of the colon or upper rectum. A thin trocar shaft emerged from the instrument to pierce the distal stapled end and was assembled to the shaft of the detachable anvil head by sliding one into the other. To deploy the CAR™ 27 from the housing onto

Table 1 Patient demographic and diagnosis

	Compression anastomosis ring (*n* = 79)
Gender	
Male	43
Female	36
Median age (yr, range)	64 (30-82)
Primary diagnosis	
Neoplasm	
Sigmoid colon	54
Rectosigmoid junction	17
Descending colon	4
Carcinomatosis (cervical cancer)	1
Rectal prolapse	1
Sigmoid colonic ulcer	1
Sigmoid volvulus	1

Table 2 Surgical and pathologic data

	CAR (*n* = 79)
Median operation time (min, range)	172.5 (110-430)
Median postoperative hospital stay (d, range)	7 (4-29)
Type of surgery (laparoscopic/open)	
Anterior resection	65(2)[1]/8
Left hemicolectomy	4/1
Resection rectopexy	1/0
Combined procedure	
TAH + BSO	2
VATS pneumonectomy	1
Cholecystectomy	1
Total hysterectomy	1
Postoperative pathology for neoplasm	
Adenoma	2
Stage 0	2
Stage Ⅰ	21
Stage Ⅱ A	30
Stage Ⅲ A	2
Stage Ⅲ B	15
Stage Ⅲ C	1
Stage Ⅳ A	2
Metastatic adenosquamous carcinoma	1

CAR: Compression anastomosis ring; TAH: Total abdominal hysterectomy; BSO: Bilateral salpingo-oophorectomy; VATS: Video-assisted thoracic surgery. [1]Number in parenthesis is cases of conversion to open procedure.

the tissue, the operating knob was rotated until it could not be turned any further and the indicator line was visible, and the cutting trigger and the cutting handle were squeezed simultaneously. When fired, the device holds the two ends of tissue together with circumferentially placed barbed points, which penetrate through the tissue, holding it to the polyethylene anvil ring. After firing, the cutting trigger and the cutting handle were released and the instrument was withdrawn by pulling gently out of the rectum and anus. Because this device is temperature-dependent, warm saline was instilled around the anastomosis immediately after firing for quicker and securer return to its pre-deformed shape. Completeness of the anastomosis was confirmed by an air-leak test.

RESULTS

A total of 79 patients (male, 43) underwent an elective left-sided colon resection, followed by an anastomosis using the CAR™ 27. Patient demographics and primary diagnoses for a colectomy are shown in Table 1. Patients ranged in age from 30 to 82 (median, 64). The majority of patients (95%) received left-sided colon surgery for neoplastic etiologies. Surgical and pathologic data are shown in Table 2. Laparoscopic colectomy was performed in 70 patients, in whom two patients who underwent a laparoscopic anterior resection for sigmoid colon cancer were converted to open procedure (2.9%); one for bulky tumor with uterine adhesions and the other for physiologic adhesions with marginal artery injury. Combined procedures were performed in 5 patients [2, total abdominal hysterectomy (TAH) and bilateral salpingo-oophorectomy; 1, video-assisted thoracic surgery (VATS) wedge pneumonectomy; 1, cholecystectomy; 1, TAH]. Table 3 shows intra- and postoperative complications. There was no surgical mortality. There was an intraoperative complication associated with immature manipulation of the CAR™ 27 anastomosis. This 67-year old male received a laparoscopic anterior resection for sigmoid colon cancer. Immediately after deployment of the CAR™ 27 by firing, the proximal segment of the anastomosis was pulled upward to check perfectness of the anastomosis and the proximal side of the anastomosis was stripped off circumferentially. To solve this intraoperative problem, the device was dismantled and another new CAR™ 27 anastomosis was constructed. Premature enforced tension over the anastomosis with the CAR™ 27 still in the cold temperature might be a factor. One patient with sigmoid colon cancer showed anastomotic leakage after 6 d postoperatively. This 33-year-old female received a second laparoscopic exploration, and primary suture closure of the anastomotic defect (with the CAR™ 27 left) and temporary diverting ileostomy were performed. Postoperatively, all patients were informed that the ring would be expelled with bowel movement within 14 d after surgery. Practically, the exact date of expulsion of the ring could not be recorded because most patients were not aware that the ring had been expelled with stool. In the patient who received a diverting ileostomy for anastomotic leakage, the ring was not expelled and retained within the colon until 9 d after an ileostomy closure (Figure 2). Postoperatively, no patient manifested clinical symptoms suggestive of anastomotic stricture. Colonoscopy at 6-month follow-up showed a wide and patent anastomosis constructed by the CAR™ 27 (Figure 3).

Table 3 Intra- and postoperative complications

	CAR (*n* = 79)
Premature enforced anastomotic disruption	1
Anastomotic leak	1

CAR: Compression anastomosis ring.

DISCUSSION

As a secure anastomosis is the crucial step for success-

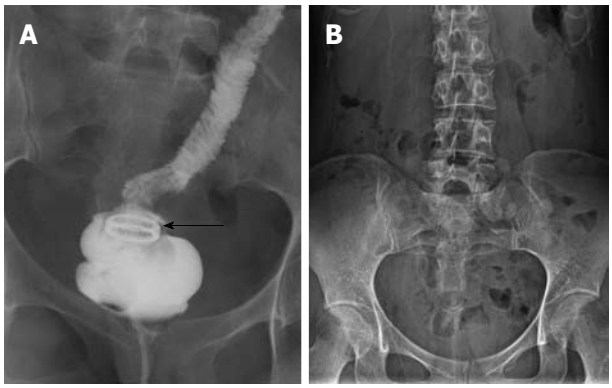

Figure 2 Abdominal radiographs of the patient complicated by anastomotic leakage. A: Gastrografin study showed complete healing of the anastomotic leakage with the ring (arrow) retained at the anastomosis a week before ileostomy takedown; B: The ring was eliminated from the body spontaneously and was no longer seen on the radiograph nine days after the ileostomy closure.

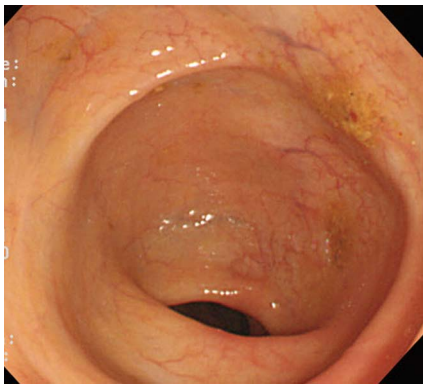

Figure 3 Colonoscopic view of the CAR™ 27 anastomosis at 6 mo. It showed complete healing with a wide and patent anastomosis. CAR™ 27: Compression anastomosis ring.

ful colorectal surgery, search for the ideal concept of the anastomosis has been the subject of surgical interest since the 19th century. The goal has been to devise an innovative method to eliminate the risk of anastomosis-associated leakage. The concept of compression anastomosis is that two bowel ends are compressed together by a sutureless device, preventing leakage and facilitating the natural healing process in the compressed region. The idea of compression anastomosis was first devised by Denan who developed two metal rings kept in position by a spring in a canine model in 1826. In 1892, Murphy[12] introduced "Murphy's button," which consisted of a pair of metal rings that compressed circular segments of intestine, leading to tissue necrosis. But it was not popular in clinical use because of too tight compression that led to peripheral ischemia and premature necrosis. It took another 100 years for a new compression anastomosis device to be put into wide use. In 1985, Hardy *et al*[13] developed a biofragmentable anastomosis ring (Valtrac™ BAR; Covidien, Mansfield, MA, United States) which is made of absorbable polyglycolic acid and radiopaque barium sulfate. To prevent tissue ischemia and to accommodate tissue thickness, margins of two identical rings are scalloped in shape and three gap-widths in the closed position (1.5, 2.0 and 2.5 mm) are available. A number of studies, including prospective randomized trials confirmed that the Valtrac™ BAR was a safe and reliable compression anastomotic device in both elective and emergency surgery[14-17]; however, intraoperative problems have been reported, including failure of purse-string sutures, inappropriate selection of the size of the Valtrac™ BAR with partial or full-thickness tear of the bowel wall, and failure of snap or shattering of the device[16,17].

Stepping forward, a novel compression anastomotic device made of nitinol (Nickel Titanium Naval Ordinance Laboratory), a shape memory alloy of nickel-titanium was introduced recently. Nitinol has two physical properties: temperature-dependent shape memory and super-elasticity. When cooled down, it has lower mechanical properties and becomes pliable, and then it becomes stable and returns to its pre-deformed shape at room temperature[10]. Super-elastic leaf springs made of nickel-titanium alloy are used in the NiTi CAR™ 27 in order to maintain a continuous pressure at the anastomosis independent of the thickness of tissue within the anastomosis[11]. The reparative healing process outside the ring produces an intact anastomosis before detachment and expulsion of the ring, leaving no foreign material within the body. The safety of this device has been demonstrated in animal studies[9,18]. Compared to other compression devices clinically, distinctive theoretical merits of the Nitinol are that it can accommodate different thickness around the circumference of the anastomosis and that it provides a constant, continuous, and uniform pressure over the pressed tissue along its compressing perimeter[19]. The CAR™ 27 was approved by the US Food and Drug Administration (FDA) for the use in intestinal anastomoses in August, 2006 and by the Korea FDA in May, 2009.

Clinical experiences with use of the CAR™ 27 in colonic anastomoses are still very scarce. Preliminary results of a phase Ⅱ, prospective, clinical trial by D'Hoore *et al*[20] were promising. In 10 patients who underwent high anterior resection or left colectomy, no anastomotic leakage occurred, and only three patients noticed passage of the ring through the anal canal. A recent prospective multicenter study comparing CAR™ 27 anastomosis (10 patients) with stapled anastomosis (13 patients) also demonstrated that the safety and efficacy of colorectal anastomosis using the CAR™ 27 in human was comparable to standard stapled anastomosis[19]. This study was first human multicenter trial but the sample size was too small to draw definite conclusions. The most recent non-randomized, prospective pilot study of the CAR™ 27 device in 23 patients undergoing a left-sided colectomy experienced an anastomotic leakage in one patient (4.3%) and stricture in two (8.6%)[21]. The current study has the largest sample size ever presented, demonstrating clinical safety relevant to CAR™ 27 anastomosis in both laparo-

scopic and open left-sided colectomies. A systemic review (Cochrane Database) of nine studies involving 1233 patients (622 stapled and 611 hand-sewn) found that overall rate of clinical leaks was 6.3% and 7.1%, respectively[22]. Valtrac™ BAR instead of staples is a standard method of intraperitoneal colonic anastomosis in open surgery in our practice to minimize anastomotic stricture. The overall rate of anastomotic leak after the Valtrac™ BAR colonic anastomosis in our series (632 patients) was 0.6%[23]. Compared to these results, leakage in one patient (1.3%) in this study implies that CAR™ 27 anastomosis is as safe and efficacious as conventional hand-sewn or stapling anastomosis. In addition, it is financially superior to end-to-end (EEA) stapling device (around $324 *vs* $360).

Issues associated with the CAR™ 27 anastomosis may deserve mentioning. Our experience of an intraoperative total anastomotic disruption associated with premature enforced tension over the anastomosis might reflect learning-curve error. Since this experience, it is our technical policy to leave the CAR™ 27 anastomosis soaked in warm saline for a few minutes, allowing the ring to recover to body temperature. Tulchinsky *et al*[19] recommended digital removal of the ring when it is not expelled spontaneously in a diverted patient. This seems to be an unnecessary maneuver. Moreover, in higher anastomosis from the anus, manual removal is not possible and forced instrumental removal of the retained ring may be rather harmful. Although the ring was not expelled and retained during the diversion in the leaked patient in this study, there were no problems associated with the retained ring during the diversion (134 d), and it expelled spontaneously after the ileostomy closure. In this sense, no specific maneuver is necessary and restoration of fecal stream after takedown of the diversion would be enough to eliminate the ring exteriorly.

To date, the clinical indication for the CAR™ 27 anastomosis is restricted to high level anastomoses at or above the anterior resection and it is not indicated in low rectal or anal anastomoses. Considering its superior technologic properties, anastomosis using the CAR™ 27 appears to be able to be performed safely in low anterior resection of the rectum. In addition, further prospective large-scale studies might be imperative to determine if the CAR™ 27 anasomosis can be constructed safely in diseased bowel such as Crohn's colitis, and if it can be performed liberally in emergency surgery.

In conclusion, short-term evaluation of the CAR™ 27 anastomosis in patients undergoing laparoscopic or open elective left colectomy proved to be a safe and efficacious alternative to the standard hand-sewn or stapling technique. Along with its technical superiorities, a prospective large-scale clinical trial would confirm the validity of the CAR™ 27 in diverse clinical settings.

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COMMENTS

Background

A secure anastomosis is a crucial step for successful colorectal surgery and the hand-sewn or stapling technique is currently the most common surgical standard method of anastomosis. However, both techniques inevitably leave foreign materials within tissue, evoking an inflammatory reaction, thus potentially resulting in a number of anastomosis-related adverse effects.

Research frontiers

To overcome safety limits in hand-sewn and stapled techniques, the concept of compression anastomosis is introduced. A novel compression anastomotic device (CAR™ 27), made of a shape memory alloy of nickel-titanium, has two peculiar physical properties: temperature-dependent shape memory and superelasticity.

Innovations and breakthroughs

This is the largest study to evaluate the safety, efficacy, and technical feasibility of the CAR™ 27 anastomosis in left-sided colon resection. Short-term evaluation of the CAR™ 27 anastomosis in elective left colectomy is a safe and efficacious alternative to the standard hand-sewn or stapling technique. Among 79 patients, only one (1.3%) was complicated by anastomotic leakage in one patient, and it is financially superior to stapling device.

Applications

The study results suggest that the CAR™ 27 anastomosis can be applied both in laparoscopic and open left colectomy with safety. Despite encouraging results, clinical indication of the CAR™ 27 is, to date, limited to intraperitoneal anastomosis, and it is not indicated in low rectal or anal anastomoses. Considering its superior technologic properties, one of promising indications would be its use after a low anterior resection of the rectum. In addition, the potential expansion of its indications to patients with inflammatory bowel disease or with impaired healing for reasons such as radiation awaits further prospective clinical evaluations.

Terminology

Temperature-dependent shape memory of the device is the property that it has lower mechanical properties and becomes supple when cooled down, and then it becomes stable and returns to its pre-deformed shape at room temperature.

Peer review

This is a well written paper on the use of the compression anastomosis ring in left-sided colonic anastomosis and the results are comparable to stapled anastomosis.

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BRIEF ARTICLE

Analysis of ABC (D) stratification for screening patients with gastric cancer

Tomohiro Kudo, Satoru Kakizaki, Naondo Sohara, Yasuhiro Onozato, Shinichi Okamura, Yoshikatsu Inui, Masatomo Mori

Tomohiro Kudo, Satoru Kakizaki, Shinichi Okamura, Masatomo Mori, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan
Naondo Sohara, Yasuhiro Onozato, Department of Endoscopy and Endoscopic Surgery, Shirakawa Clinic, Maebashi, Gunma 371-8511, Japan
Yoshikatsu Inui, Inui Clinic of Internal Medicine, Takasaki, Gunma 371-8511, Japan
Author contributions: Kudo T, Sohara N and Onozato Y performed the majority of experiments and collected data; Kakizaki S designed the study and wrote the manuscript; Okamura S, Inui Y and Mori M gave vital suggestions.
Correspondence to: Satoru Kakizaki, MD, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan. kakizaki@showa.gunma-u.ac.jp
Telephone: +81-27-2208127 Fax: +81-27-2208136
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Abstract

AIM: To evaluate the value of ABC (D) stratification [combination of serum pepsinogen and *Helicobacter pylori* (*H. pylori*) antibody] of patients with gastric cancer.

METHODS: Ninety-five consecutive patients with gastric cancer were enrolled into the study. The serum pepsinogen Ⅰ (PG Ⅰ)/pepsinogen Ⅱ (PG Ⅱ) and *H. pylori* antibody levels were measured. Patients were classified into five groups of ABC (D) stratification according to their serological status. Endoscopic findings of atrophic gastritis and histological differentiation were also analyzed in relation to the ABC (D) stratification.

RESULTS: The mean patient age was (67.9 ± 8.9) years. Three patients (3.2%) were classified into group A, 7 patients (7.4%) into group A′, 27 patients (28.4%) into group B, 54 patients (56.8%) into group C, and 4 patients (4.2%) into group D, respectively. There were only three cases in group A when the patients taking acid proton pump inhibitors and those who had undergone eradication therapy for *H. pylori* (group A′) were excluded. These three cases had mucosal atrophy in the grey zone according to the diagnostic manual of ABC (D) stratification. Histologically, the mean age of the patients with well differentiated adenocarcinoma was significantly higher than that of the patients with poorly differentiated adenocarcinoma ($P < 0.05$). There were no differences in the pattern of atrophy in the endoscopies between the well differentiated and poorly differentiated groups.

CONCLUSION: ABC (D) stratification is a good method for screening patients with gastric cancers. Endoscopy is needed for grey zone cases to check the extent of mucosal atrophy.

Key words: Gastric cancer; *Helicobacter pylori*; Pepsinogen; ABC (D) stratification; Cancer screening

Peer reviewer: Hai-Yong Han, PhD, MS, BS, Division of Clinical Translational Research, The Translational Genomics Research Institute, 445 N Fifth Street, Phoenix, AZ 85004, United States

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INTRODUCTION

Gastric cancer remains the second leading cause of can-

cer death in Japan, although its mortality has continued to decrease for decades[1]. Screening systems or methods to detect early gastric cancers have contributed to the decrease in gastric cancer deaths[2,3]. Mass screening for gastric cancer by X-ray examination was introduced in the 1960s and has been epidemiologically confirmed to be effective for reducing gastric cancer mortality[2,3]. However, the decreases in efficiency have been thought to result from the decreased coverage rates and a tendency for the same participants to undergo gastric cancer screening repeatedly. Therefore, a more effective screening system, focusing on high-risk patients, is needed.

Helicobacter pylori (*H. pylori*) infection and subsequent atrophic gastritis have been regarded as risk factors for gastric cancer[4-6]. The combination of serum pepsinogen (PG) and *H. pylori* antibody [ABC (D) stratification] has been suggested to serve as a useful predictive marker for patients with gastric cancers[4-6]. This combination of serum markers would represent a much simpler and less invasive method than endoscopy, and therefore, would be suitable for a large general population. A large scale study by Ohata *et al*[4] reported that the combination of serum PG and anti-*H. pylori* antibody provide a good method for predicting the development of gastric cancer. Mizuno *et al*[6] also reported the usefulness of this screening method in a population-based cohort study.

However, the studies concerning the use of ABC (D) stratification only focused on patients with no disease or an unknown disease status. Analyses of the ABC (D) stratification of patients with confirmed gastric cancer are lacking. The aim of this study was to evaluate the ABC (D) stratification so as to provide information that might be useful for screening patients with gastric cancers.

MATERIALS AND METHODS

Patients and study design

Ninety-five consecutive patients with gastric cancer were enrolled in the study. All patients were diagnosed as having gastric cancer at Shirakawa Clinic (Maebashi, Japan) between November 2007 and October 2009. IgG antibodies to *H. pylori* and the levels of pepsinogen I (PG I) and II (PG II) were measured and classified by ABC (D) stratification. Group A consisted of patients with normal PG and *H. pylori* antibody (-); group B had normal PG and *H. pylori* (+); group C had atrophic PG and *H. pylori* (+); and group D had atrophic PG and *H. pylori* (-). Group A' included patients with normal PG who were *H. pylori* (-) after *H. pylori* eradication therapy or who were being treated with proton pump inhibitors.

A data collection sheet was designed to obtain the relevant clinical information about the patients for review. All of the patients provided written informed consent before receiving the examination. Olympus XQ260 or N260 (Olympus Optical Co, Tokyo, Japan) instrument was used for endoscopic examination. When gastric cancer was suspected by routine endoscopy, chromoendoscopy with indigo carmine and a biopsy were performed. All cases were histologically confirmed to have gastric cancer. According to the endoscopic gastric mucosal findings, the patients were classified into three categories: those without atrophy, those with close-type atrophy, and those with open-type atrophy[7].

Serological tests and definition of ABC (D) stratification

H. pylori infection was determined by enzyme-linked immunosorbent assay (ELISA) kits (E-plate EIKEN *H. pylori*, Eiken Chemical Co., Ltd., Tokyo, Japan). Subjects with $\geqslant$ 10 U/mL were classified as having *H. pylori* infection. Those with < 10 U/mL were regarded as being *H. pylori* negative. The sensitivity and specificity of this assay for *H. pylori* infection were 100% and 93.8%, respectively. The levels of PG I and PG II were measured using the E-plate EIKEN Pepsinogen I and the E-plate EIKEN Pepsinogen II (Eiken Chemical Co., Ltd., Tokyo, Japan), respectively. Atrophic gastritis was diagnosed according to the serum PG I and PG II criteria proposed by Miki and others[8-10]. Briefly, the serum PG status was defined as atrophic when the criteria of both serum PG I level $\leqslant$ 70 ng/mL and a PG I /PG II ratio $\leqslant$ 3.0 were simultaneously fulfilled. All other cases were classified as normal. These criteria have a sensitivity of 70.5% and a specificity of 97%.

Statistical analysis

Differences between the groups were analyzed by Fischer's exact probability test and Mann-Whitney's *U* test when a significant difference was obtained by the Kruskal-Wallis test. A *P* value less than 0.05 was considered to be significant.

RESULTS

Patient characteristics

Ninety-five patients were diagnosed as having gastric cancer at our institution between November 2007 and October 2009. The mean age of the patients was (67.9 ± 8.9) years (range, 38-83, median 69). There were 72 male and 23 females. According to ABC (D) stratification, there were 3 (3.2%) patients in group A, 7 patients (7.4%) in group A', 27 (28.4%) in group B, 54 (56.8%) in group C, and 4 (4.2%) in group D, respectively (Table 1). There were no significant differences in the mean age, sex ratio, location of the tumor, macroscopic findings or histological type among the five groups. According to endoscopic findings, two cases (2.1%) had no atrophy, 21 (22.1%) had closed-type atrophy, and 72 (75.8%) had open-type atrophy. The relationship between ABC (D) stratification and the endoscopic atrophic border are shown in Table 2. There were no significant differences in the endoscopic atrophic border pattern among the five groups. There were 35 patients with closed-type or open-type atrophy in the PG negative group.

Representative cases in each group

Figure 1 shows the representative cases of gastric cancer

Table 1 Characteristics of the patients with gastric cancer in this study

Characteristics		All patients	A	B	C	D
No. of patients		95	10	27	54	4
Age (yr)	Mean (range)	67.9 ± 8.9 (38-83)	69.2 ± 10.0 (48-79)	65.5 ± 9.0 (43-77)	68.4 ± 7.6 (38-83)	70.3 ± 6.9 (63-78)
	Median	69	70.5	67	69.5	70
Sex	Male/female	72/23	5/5	19/8	45/9	3/1
Location	U/M/L	19/42/34	2/6/2	2/14/11	15/22/17	0/0/4
Macroscopic type	Elevated/flat/depressed	51/4/40	5/0/5	12/0/15	31/3/20	3/1/0
Differentiation	Well diff/poorly diff	76/19	8/2	18/9	47/7	3/1

U: Upper thirds of the stomach; M: Middle; L: Lower; Well diff: Well or moderately differentiated adenocarcinoma; Poorly diff: Poorly differentiated adenocarcinoma or signet ring cell carcinoma.

Table 2 Relationship between ABC (D) stratification and endoscopic atrophic border in patients with gastric cancers

	Endoscopic atrophic border			
ABC (D) stratification	Non	Closed type	Open type	Total
A: *H. pylori* (-) PG (-)	1	3	6	10 (10.6%)
B: *H. pylori* (+) PG (-)	1	7	19	27 (28.4%)
C: *H. pylori* (+) PG (+)	0	10	44	54 (56.8%)
D: *H. pylori* (-) PG (+)	0	1	3	4 (4.2%)
Total	2 (2.1%)	21 (22.1%)	72 (75.8%)	95 (100%)

H. pylori: *Helicobacter pylori*; PG: Pepsinogen; Non: No atrophic change.

in groups A-D. Figure 1A shows a 70-year-old male with *H. pylori* (-) and PG (-). However, open- type atrophy was found in endoscopy. He should have been classified into group D because of the extent of mucosal atrophy. The PG Ⅰ level was low (28.5 ng/mL), although PG was negative based on the PG Ⅰ/PG Ⅱ ratio (3.9). Figure 1A' shows a 48-year-old female with *H. pylori* (-) and PG (-). She had no atrophy in the endoscopic findings. This case received post-eradiation therapy for *H. pylori*. The patient was *H. pylori* negative with the titer of the *H. pylori* antibody under 10 U/mL. However, the antibody titer was 7.6 U/mL, so she was not completely *H. pylori* negative. Figure 1B shows a 72-year-old male with *H. pylori* (+) and PG (-). He had closed-type atrophy in the endoscopic findings. Figure 1C shows a 71-year-old male with *H. pylori* (+) and PG (+). This case was positive for both *H. pylori* and PG. He had open-type atrophy in the endoscopic examinations. Figure 1D presents a 69-year-old male with *H. pylori* (-) and PG (+). He had open-type atrophy in endoscopy. Because of the progression of mucosal atrophy, we concluded that this patient was likely negative for *H. pylori* because the organism cannot live in the atrophic mucosa.

Gastric cancers arising from group A and group A'

A summary of the patients with gastric cancer arising from group A and group A' is shown in Table 3. Among 10 patients, there was one case without atrophy, 3 cases of closed-type atrophy, and 6 cases of open-type atrophy. There were only 3 (3.2%) cases in group A when the patients in group A' (who was taking acid proton pump inhibitors and/or had received eradication therapy for *H. pylori*) were excluded. These 3 cases had mucosal atrophy and were classified into the grey zone pattern based on the diagnostic manual of ABC (D) stratification. Eight of the 10 patients were classified into group A or A' because their serum PG Ⅰ/PG Ⅱ ratio was greater than 3. The serum PG Ⅰ levels of the 8 patients were ≤ 70 ng/mL.

Histological analysis

Histologically, there were 76 (80.0%) cases of well or moderately differentiated adenocarcinomas (well differentiated group) (Table 4). There were 19 (20.0%) cases of poorly differentiated adenocarcinomas or signet ring cell carcinomas (poorly differentiated group). The mean age of the patients in the well differentiated group [(70.2 ± 5.8) years] was significantly higher than that of the poorly differentiated group [(61.4 ± 12.9) years, $P < 0.05$]. There were no differences in the sex ratio or the pattern of atrophy in endoscopy between the well differentiated group and the poorly differentiated group. The proportion of group C patients tended to be higher in the well differentiated group, although it did not reach statistical significance. It was supposed that the patients with well differentiated adenocarcinomas would shift from group B to C as they aged.

The relationship between ABC (D) stratification and the endoscopic atrophic border according to the histological differentiation is shown in Tables 5 and 6. There were no differences in the distribution of the endoscopic atrophic border between the well differentiated and poorly differentiated groups.

DISCUSSION

A screening program with an upper gastrointestinal series has been confirmed to be effective for reducing mortality from gastric cancer in Japan[2,3]. Since the X-ray with photofluorography was first introduced in the 1960s, it has played a key role in gastric cancer screening[11,12]. However, the existing program by the X-ray was introduced prior to the discovery of *H. pylori* and documentation of its carcinogenicity. Only approximately 13% of the target population participated in the program[13]. Given these drawbacks, it is necessary to establish an effective screening system, focusing on high-risk status such as *H. pylori* infection and atrophic gastritis. The combined use of

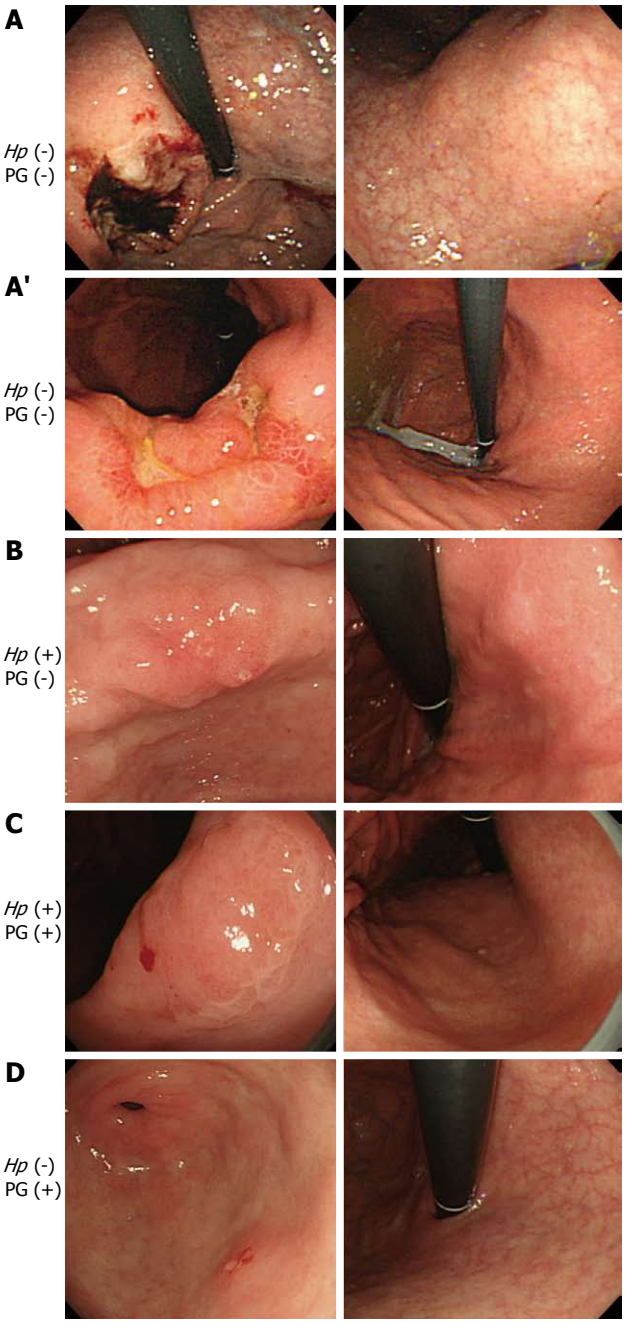

Figure 1 Representative cases of gastric cancer classified into groups A-D. A: A representative case of gastric cancer stratified into group A. This case should have been classified into group D because of the mucosal atrophy; A': A representative case with group A' gastric cancer; B: A representative case of gastric cancer from group B; C: A representative case of gastric cancer in group C; D: A representative case of gastric cancer from group D. PG: Pepsinogen; *Hp*: *Helicobacter pylori*.

serum anti-*H. pylori* antibodies and PG measurement has been reported to be a useful screening method for gastric cancers[4], and several recent reports have confirmed the usefulness of this screening method[5,6]. However, most of these reports targeted the general population during routine health check-ups. The ABC (D) stratification of patients who have already been diagnosed with gastric cancer has not been sufficiently investigated. In this study, we evaluated the ABC (D) stratification of patients with confirmed gastric cancers.

Rapid progress has been made in the studies on stomach carcinogenesis since the discovery of *H. pylori*. The relationship between atrophic gastritis and gastric cancer has been confirmed epidemiologically. Uemura *et al*[14] reported that only *H. pylori*-infected subjects developed gastric cancer among a group of patients with organic or functional gastroduodenal disorders. Fukase *et al*[15] reported a randomized controlled trial in which *H. pylori* eradication contributed to the reduction of metachronous gastric cancer after endoscopic resection of early gastric cancer. Not surprisingly, the combination of *H. pylori* infection determined by serum anti-*H. pylori* antibodies and atrophic gastritis determined by serum PG levels is promising for diagnosing gastric cancer[4-6].

Mizuno *et al*[6] reported that the atrophy-positive *H. pylori*-positive group (group C in ABC (D) stratification system) had a moderately high hazard ratio of 11.23, while the atrophy-positive *H. pylori*-negative group (group D) had a markedly higher hazard ratio of 14.81. These two groups are therefore considered the most appropriate candidates for gastric cancer screening. It is well known that anti-*H. pylori* antibody production may be reduced when atrophy progresses because *H. pylori* does not survive very well in the intestinal metaplasia mucosa[6]. As group D represents the status of severe atrophic gastritis with marked intestinal metaplasia, it is the highest-risk group for developing gastric cancer. In addition, the atrophy-negative *H. pylori*-positive group (group B) had a relatively high hazard ratio of 4.20[6]. This group represents the status of *H. pylori*-induced active gastritis without extensive atrophy, which is thought to be one of the factors that contribute to the diffuse-type gastric cancer. As diffuse-type gastric cancer grows and invades faster than the intestinal type, this group is considered to be a candidate for a gastric cancer screening program. In this study, there were only 3 cases in group A when group A' patients were excluded. Seven patients in group A' were receiving proton pump inhibitor therapy or had previously been treated for eradicating *H. pylori* infection. As a result, precise medical interviews, such as prescription of proton pump inhibitors and a past history of *H. pylori* eradication are needed. Three group A cases had mucosal atrophy upon endoscopic examination. Based on this mucosal atrophy, these cases should be classified into group D. The pepsinogen levels of these patients are shown in Table 3. In this study, the serum PG status was defined as atrophic when the criteria of both serum PG I level $\leqslant$ 70 ng/mL and a PG I /PG II ratio $\leqslant$ 3.0 were simultaneously fulfilled. These criteria have a sensitivity of 70.5% and a specificity of 97%[16]. The serum PG I levels of the 3 group A cases were all $\leqslant$ 70 ng/mL. They were classified into normal PG because they had a PG I /PG II ratio > 3.0. This indicates that special attention should be paid to avoiding false negative cases of atrophic gastritis. None of the gastric cancer patients in our study were both *H. pylori* (-) and without atrophic gastritis. Ohata *et al*[4] reported a study with a cohort of 4655 healthy asymptomatic subjects (average age,

Table 3 Summary of 10 patients with gastric cancer in group A and group A'

Case	Stratification	Age (yr)	Sex	Location	Macroscopic type	Differentiation	PG Ⅰ/PG Ⅱ levels (ng/mL)	PG Ⅰ/PG Ⅱ ratio	Endoscopic findings	PPI prescription	*H. pylori* eradication therapy
1	A	70	M	U	Borrmann Ⅱ	Poor	28.5/7.3	3.9	Open type	No	No
2	A'	48	F	M	Ⅱ c + Ⅲ	Signet ring cell carcinoma	55.9/8.2	6.8	Closed type	No	Yes
3	A	68	M	M	Ⅱ a + Ⅱ c	Well	64.1/11.8	5.4	Open type	No	No
4	A	78	F	L	Ⅱ c	Well	13.6/4.2	3.2	Open type	No	No
5	A'	67	F	L	Ⅱ c	Well	46.4/10.8	4.3	Closed type	Yes	No
6	A'	79	F	M	Ⅱ a + Ⅱ c	Well	86.2/10.9	7.9	Closed type	Yes	No
7	A'	77	M	M	LST-G	Well	384.9/54.3	7.1	Non	Yes	No
8	A'	77	F	U	Ⅱ c	Well	17.3/5.2	3.3	Open type	No	Yes
9	A'	57	M	M	Ⅱ c	Well	36.9/6.8	5.4	Open type	No	Yes
10	A'	71	M	M	Ⅱ a + Ⅱ c	Well	38.6/7.9	4.9	Open type	No	Yes

H. pylori: *Helicobacter pylori*; PG: Pepsinogen; U: Upper thirds of the stomach; M: Middle; L: Lower; PPI: Proton pump inhibitor; Non: No atrophic change.

Table 4 Histological analysis and ABC (D) stratification based on the status of endoscopic atrophic border

	All patients	Well diff	Poorly diff	*P* value
No. of patients	95	76	19	
Male:female	72:23	58:18	14:5	NS
Age (yr, mean ± SD)	67.9 ± 8.9	70.2 ± 5.8	61.4 ± 12.9	< 0.05
ABC (D) stratification				
A: *H. pylori* (-) PG (-)	10 (10.6%)	8 (10.5%)	2 (10.6%)	NS
B: *H. pylori* (+) PG (-)	27 (28.4%)	18 (23.7%)	9 (47.3%)	
C: *H. pylori* (+) PG (+)	54 (56.8%)	47 (61.8%)	7 (36.8%)	
D: *H. pylori* (-) PG (+)	4 (4.2%)	3 (3.9%)	1 (5.3%)	
Endoscopic atrophic border				
Non	2 (2.1%)	2 (2.6%)	0 (0%)	NS
Closed type	21 (22.1%)	15 (19.7%)	6 (31.6%)	
Open type	72 (75.8%)	59 (77.6%)	13 (68.4%)	

H. pylori: *Helicobacter pylori*; PG: Pepsinogen; Non: No atrophic change; Well diff: Well or moderately differentiated adenocarcinoma; Poorly diff: Poorly differentiated adenocarcinoma or signet ring cell carcinoma; NS: Not significant.

Table 5 Relationship between ABC (D) stratification and endoscopic atrophic border in histological differentiation, well differentiated adenocarcinoma

	Endoscopic atrophic border			
ABC (D) stratification	Non	Closed type	Open type	Total
A: *H. pylori* (-) PG (-)	1	2	5	8 (10.5%)
B: *H. pylori* (+) PG (-)	1	5	12	18 (23.7%)
C: *H. pylori* (+) PG (+)	0	7	40	47 (61.8%)
D: *H. pylori* (-) PG (+)	0	1	2	3 (3.9%)
Total	2 (2.6%)	15 (19.7%)	59 (77.6%)	76 (100%)

H. pylori: *Helicobacter pylori*; PG: Pepsinogen; Non: No atrophic change.

49 years) who were followed up for a mean period of 7.7 years. No cancer developed in the *H. pylori* (-)/normal PG group during their study period[4].

Graham and Asaka[17] proposed an eradication program for gastric cancer. Under their proposal, all adults would receive non-invasive testing for *H. pylori* infection and atrophic gastritis. All *H. pylori* infected patients would have confirmed *H. pylori* eradication[17]. Those with atrophic gastritis would be considered for further evaluation and possible surveillance[17]. The cases that were *H. pylori* (-) and had no atrophy would be excluded from the follow-up program. However, additional attention should be paid to mucosal atrophy, because the sensitivity of PG only 70.5%. Since the ultrathin transnasal endoscopy can be used for health check-ups, gastric cancer screening with ABC (D) stratification in combination with endoscopy may represent a useful screening system. In conclusion, our findings suggest that a combination screening for the *H. pylori* antibody titer and serum PG status may therefore be useful for predicting the development of gastric cancer. However, additional attention should be paid to avoiding false negatives for the patients who are taking acid proton pump inhibitors and those who have received prior eradication therapy for *H. pylori*. Endoscopy is needed for grey zone cases to accurately determine the mucosal atrophy status.

Table 6 Relationship between ABC (D) stratification and endoscopic atrophic border in histological differentiation, poorly differentiated adenocarcinoma

	Endoscopic atrophic border			
ABC (D) stratification	Non	Closed type	Open type	Total
A: *H. pylori* (-) PG (-)	0	1	1	2 (10.6%)
B: *H. pylori* (+) PG (-)	0	2	7	9 (47.4%)
C: *H. pylori* (+) PG (+)	0	3	4	7 (36.8%)
D: *H. pylori* (-) PG (+)	0	0	1	1 (5.3%)
Total	0 (0%)	6 (31.6%)	13 (68.4%)	19 (100%)

H. pylori: *Helicobacter pylori*; PG: Pepsinogen; Non: No atrophic change.

COMMENTS

Background

Gastric cancer remains the second leading cause of cancer death in Japan, although its mortality has continued to decrease for decades. Screening systems or methods to detect early gastric cancers have contributed to the decrease in gastric cancer deaths. The combination of serum pepsinogen (PG) and *Helicobacter pylori* (*H. pylori*) antibody [ABC (D) stratification] can serve as a useful predictive marker for diagnosing patients with gastric cancers.

Research frontiers

Recent studies concerning the use of ABC (D) stratification have focused on pa-

tients with either no disease or an unknown disease. There have so far been few analyses of ABC (D) stratification of patients confirmed to have gastric cancer.

Innovations and breakthroughs

ABC (D) stratification may be useful for predicting the development of gastric cancer. However, additional attention should be paid to avoiding false negatives for patients who are taking acid proton pump inhibitors and those who have received prior eradication therapy for *H. pylori*. Endoscopy is needed to evaluate grey zone cases to accurately determine the mucosal atrophy status.

Applications

Combination screening for the *H. pylori* antibody titer and serum PG status [ABC (D) stratification] is a good method for screening patients with gastric cancers.

Terminology

ABC (D) stratification is a screening method for patients with gastric cancer using a combination of the *H. pylori* antibody titer and serum PG status.

Peer review

This manuscript describes the evaluation of ABC (D) stratification in a group of patients confirmed to have gastric cancer. The authors found that ABC (D) stratification correlated closely with the disease status, but noted possible false negatives due to the fact that some patients have previously received treatments aimed at eradicating *H. pylori*. Overall, the study is well designed and the analysis approach is sound. Although the findings were somewhat expected based on previous studies, the study does provide further support for the use of the ABC (D) stratification system. The findings of this study would therefore be of interest to other researchers in this field if published.

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BRIEF ARTICLE

Morphological effects of autoclaved diet on the myenteric neurons of rats

Patrícia O Gonçalez, Naianne K Clebis, Renata B Mari, Karina M Gagliardo, Sandra R Stabille, Haroldo G Faria, Edson A Liberti, José Roberto Kfoury Jr

Patrícia O Gonçalez, Karina M Gagliardo, José Roberto Kfoury Jr, Department of Surgery, School of Veterinary Medicine and Animal Sciences, University of São Paulo, 05508-270 São Paulo, Brazil
Naianne K Clebis, Department of Morphology of the Centre of Biomedical Sciences, Federal University of Rio Grande do Norte, 59078-400 Natal, Brazil
Renata B Mari, Department of Biology, Paranaense University, Paranavaí, 87502-210 Paraná, Brazil
Sandra R Stabille, Department of Morphology, Maringá State University, 87020-900 Paraná, Brazil
Haroldo G Faria, Central Vivarium, Maringá State University, 87020-900 Paraná, Brazil
Edson A Liberti, Department of Anatomy, Biomedical Sciences Institute, University of São Paulo, 05508-270 São Paulo, Brazil
Author contributions: Gonçalez PO, Clebis NK, Mari RB contributed equally to this work; Gonçalez PO, Clebis NK, Mari RB, Stabille SR, Faria HG designed research; Gonçalez PO, Clebis NK, Mari RB, Gagliardo KM acquired, analyzed and interpreted the data; Stabille SR, Faria HG lent technical support; Liberti EA critically revised the manuscript; Kfoury Jr JR supervised the study.
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Correspondence to: José Roberto Kfoury Jr, Assistant Professor, Department of Surgery, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Av Prof Dr. Orlando Marques de Paiva 87, 05508-270 São Paulo, Brazil. jrobertok@usp.br
Telephone: +55-11-30911308 Fax: +55-11-30911308
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Abstract

AIM: To evaluate the effect of autoclaved diet on the jejunum neurons of the myenteric plexus of rats during their growth.

METHODS: The experimental groups were made up of rats going through weaning whose mothers received either an autoclaved or a non-autoclaved diet during gestation and lactation, and rats that were fed the same diet as their mothers during the post-weaning period. In order to measure the neurons' body profile and to quantify the number of neurons per area, preparations were stained by the nicotinamide adenine dinucleotide-diaphorase method.

RESULTS: No significant changes were observed in rats' body weight or in the number of neurons regardless of the diet used ($P > 0.05$). There was a decrease in the jejunum-ileum length in rats treated with an autoclaved diet ($P < 0.05$). An increase in the neuronal cross-sectional area was seen in rats that had received the autoclaved diet, an effect that was significant for animals undergoing weaning. In addition, all observed factors showed significant differences when related to the age of the animals.

CONCLUSION: The autoclaved diet did not alter the quantity of neurons, but increased their cell body area, suggesting changes similar to those observed in protein deficiency.

Key words: Myenteric neurons; Jejunum; Morphometry; Diet; Autoclaving

Peer reviewer: Seng-Kee Chuah, MD, Division of Hepatogastroenterology, Chang Kaohsiung Gang Memorial Hospital, 123, Ta-Pei Road, Niaosung Hsiang, Kaohsiung 833, Taiwan, China

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INTRODUCTION

The nutritional makeup of the diet provided to laboratory animals and the procedures used for its preparation, storage, and sterilization must be evaluated not only to ensure the proper development of the animal, but also to avoid contamination with pathogenic microorganisms[1-3].

Animal facilities have traditionally used the recommended autoclaving conditions of 120 ℃ for 15 min to sterilize commercial diet, as this is an easy, safe and low cost process[4]. However, this exposure to high temperature may compromise the components of the ration, destroying vitamins and proteins and affecting the nutritional value of the diet[2,4,5].

Proteins may become more chemically reactive after autoclaving, leading to their degeneration or even to reaction with other substances, and compromising their digestibility, functionality and nutritional value[4,6].

These characteristics have been evaluated through the KOH protein solubility test, which has been shown to be a good indicator of the reduction of the amount of protein in feed[6-9].

It is known that animal tissues are not homogeneously affected by protein deficiency[10]. Thus, tissues that show low rates of metabolism or cell renewal are later compromised[11]. Different conditions of protein malnutrition have been shown to cause changes in the amount and size of neurons in the myenteric plexus of the segment of the gastrointestinal tract of rats of different ages[12-18]. The results indicated that factors such as nutritional quality of the diet and animal age might interfere with functional morphology of the myenteric plexus, resulting in impairment of the function of the digestive system and, consequently, the performance of the animal.

For these reasons, in addition to the concern of maintaining the composition of the diet provided to laboratory animals, the integrity of nervous system elements that control the activities of the digestive system, such as the myenteric plexus, is important for animal nutrition and production, since a structural impairment of this plexus in animals with some type of protein deficiency should not be ignored.

Thus, considering the key role of the jejunum (and consequently the myenteric plexus) in the process of nutrient absorption[19], and the practice of autoclaving for diet sterilization in the care of laboratory animals, the myenteric neurons of the jejunum of rats fed autoclaved rations during pre- and post-weaning periods were qualitatively and quantitatively evaluated.

MATERIALS AND METHODS

Animals and diet

Four female Wistar rats from the central animal facility of the Maringa State University, were housed separately in polypropylene boxes equipped with an automatic feeder and drinker, kept in a temperature-controlled (22 ℃) and photoperiod regulated room (12 h of light and 12 h of darkness). They received commercial water and food for rats (Nuvilab® CR1-Nuvital) ad libitum. After an adaptation period of one week, the animals were impregnated and divided into two groups, where two females made part of the control group (CG) and were maintained with a non-autoclaved diet (ND) with a soluble protein level of approximately 80.85% in KOH[20] (Figure 1). The remaining two females integrated into the experimental group (EG) and were fed with the same diet, except that the diet was autoclaved (120 ℃ for 15 min), leading to protein levels around 47.36% [autoclaved diet (AD)]. The composition of the diet is shown in Table 1.

At birth, the offspring of each female were equalized to five young males, which were divided into the following groups according to diet and periods of life: (1) CG21, 21-day-old animals whose mothers were maintained with ND during pregnancy and nursing; (2) EG21, 21-day-old animals whose mothers were maintained with AD during pregnancy and nursing; (3) CG70, animals whose mothers were maintained with AD during pregnancy and nursing and were then maintained with the same diet until 70 d of age; and (4) EG70, animals whose mothers received AD during pregnancy and nursing and were then maintained with the same diet until 70 d of age.

After weighing, the animals were euthanized by cervical dislocation and laparotomized in order to remove the jejunum.

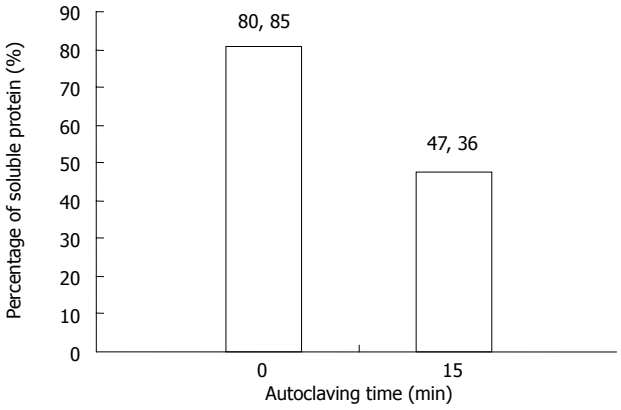

Figure 1 Percentage levels of soluble protein in KOH found in commercial diet according to the autoclaving time.

Table 1 Chemical composition of the Nuvilab CR1-Nuvital® commercial diet used as a benchmark in rat feeding-São Paulo -2004

Nutrients	%
Dry matter	89.88
Crude protein	22.23
Crude Fiber	5.73
Calcium	0.92
Phosphorous	0.87
Crude energy (kcal/kg)	3976

Source: Laboratory of animal Nutrition from the Universidade Estadual de Maringá, Maringá, PR, Brazil.

Table 2 Animals fed with an autoclaved diet (EG21 and EG7) compared to animals in the CG21 and CG70

Group	Body weight (g)	No. of neurons	Cell body area (μm^2)
CG21	46.8 ± 1.6[1]	1061.0 ± 50.72[1]	230.0 ± 10.9[1]
EG21	47.2 ± 1.7[1]	1168.0 ± 71.42[1]	282.1 ± 7.0[2]
CG70	237.4 ± 10.3[2]	881.4 ± 38.96[2]	347.4 ± 13.4[3]
EG70	252.4 ± 11.1[2]	969.4 ± 82.03[2]	377.4 ± 22.0[3]

Average and standard deviation of body weight (g), number of neurons and cell body area of reactive NADH-diaphorase myenteric neurons present in 8.96 mm2 of prepared membrane of the jejunum of rats during weaning periods (21 d), whose mothers received non-autoclaved rations (CG21) and autoclaved rations (EG21) during periods of pregnancy and nursing and of rats during post-weaning periods (70 d) fed with autoclaved feed (CG70) and non-autoclaved feed (EG70). Average followed by different numerals 1, 2, 3 in the same column differ ($P < 0.05$) by the Kruskal-Wallis test.

Nicotinamide adenine dinucleotide-diaphorase histochemical technique

The jejunum was initially washed in Krebs solution, ligated with cotton threads at its extremities and its lumen was filled with a syringe needle until slightly distended. After incubation in Krebs solution at room temperature for 15-30 min, the specimens were transferred to a permeabilizing agent (0.3% Triton-X in Krebs solution) for 60 s and then submitted to three 10 min washes in Krebs solution.

The specimens were then incubated for 60 min at 20 min in 20 mL of a medium containing 0.5 mg/mL nitro blue tetrazolium (Sigma-Aldrich) in distilled water (5 mL), 0.1 mol/L sodium phosphate buffer (5 mL, pH 7.3), distilled water (10 mL) and 0.5 mg/mL β-nicotinamide adenine dinucleotide (reduced form)[21].

The reaction was stopped by immersion in 10% buffered formalin solution in which the viscera were fixed (24 h minimum). Fragments of each jejunum about 1 cm in length were opened longitudinally. The mucosal and submucosal layers of these fragments were removed and the specimens were thoroughly washed in distilled water. Finally, whole-mount preparations were laid in glycerol on a microscope slide and sealed with Entellan (Merck KGaA, Darmstadt, Germany).

Morphoquantitative analysis

The neuronal density and the profile areas of the nerve cell bodies were measured by examining the whole-mount preparations under a binocular microscope at 400x magnification. For each specimen, all neurons present in 40 microscopic fields (0.224 mm^2 each) were counted (total area of 8.96 mm^2). The profiles of 80 random nerve cell perikarya from each specimen were obtained on a semiautomatic device for morphometry analysis (Image pro Plus, 3.01). The data were expressed as means ± SD and compared by Kruskal-Wallis test. The level of significance was set at $P < 0.05$.

Bioethics

All experimental procedures were reviewed and approved by the Bioethics Committee of the School of Medicine and Veterinary of the University of São Paulo.

RESULTS

The animals fed with an autoclaved diet (EG21 and EG70) showed an increase in body weight of 0.83% and 6.3%, respectively, compared to animals in the CG21 and CG70 (Table 2). However, there was no statistically significant difference ($P > 0.05$) when comparing groups of same age (21 d and 70 d).

Through the nicotinamide adenine dinucleotide (NADH)-diaphorase reaction, it was verified that the myenteric plexus was organized in elongated ganglia containing neurons of different sizes in all studied groups. These ganglia were scattered and arranged in parallel in the same direction as the muscle bundles of the circular layer of the muscular coat of jejunum.

The number of myenteric neurons present in 8.96 mm^2 of jejunum differed between the 21- and 70-day-old animals, with lower amounts present in the 70-day-old animals ($P < 0.05$) (Table 2). However, when comparing the same age groups, (CG21 and EG21; CG70 and EG70), the number of neurons was shown to not change after use of autoclaved diet. Animals from the EG21 and EG70 groups showed an increase of 9.2% and 9% in the number of neurons when compared with the CG21 and CG70 groups, respectively, but this increase did not reach statistical significance ($P > 0.05$).

The area of the neuron cell bodies ranged from 105.1 μm^2 to 553.9 μm^2 in the CG21 group and from 101.1 μm^2 to 640.7 μm^2 in the EG21 group. In the CG70 group, the dimensions ranged from 95.2 μm^2 to 713.2 μm^2 and from 97.3 μm^2 to 843 μm^2 in the EG70 group.

The average size of myenteric neurons was smaller ($P < 0.05$) for younger animals (CG21 and EG21) compared to the 70-day-old animals (CG70 and EG70). The neurons from the CG70 and EG70 groups showed an increase in their average area of around 51% and 33.8%, respectively, when compared to their control groups (CG21 and EG21) (Table 2).

Statistically, it was found that the average area of the neuronal cell body differed between animals from the CG21 and EG21 groups ($P < 0.05$), with higher values for animals from EG21, whose mothers received an autoclaved diet during pregnancy and nursing (Table 2). Although it was verified that neurons in the CG70 group showed a cell body average area smaller than that observed in EG70, the differences in this parameter between the two groups were not significant ($P > 0.05$) (Table 2).

DISCUSSION

After autoclaving, the quality of the protein was altered in the diet sterilization procedure, reducing the usable protein content and indicating that animals in the EG21 and EG70 groups received feed with a lower protein quality than those in their respective control groups

(CG21 and CG70).

Regardless of whether the diet was autoclaved, animals gained body weight during the experiments because of the natural growth and development from birth to adulthood. Although not statistically significant ($P > 0.05$), animals in the EG21 and EG70 groups had weight gain 0.85% and 6.3% higher than their respective controls (CG21 and CG70). In contrast, studies that examined rats of various ages and during different periods of protein malnutrition reported a decrease in body weight[13-17,22,23]. These differences are justifiable since the autoclaving temperature of the feed used in this study does not significantly alter the performance of rats in pre- or post-weaning periods. The compromise in animal performance is seen after autoclaving feed at temperatures higher than those used in our study[9]. Thus, the change in protein quality of the autoclaved ration given to animals in this study was not sufficient to influence a significant variation in animals' body weight.

The reactive NADH-diaphorase myenteric neurons of the jejunum were organized predominantly in a dispersed ganglion distributed parallel to the direction of muscles fibers of circular layers of the muscular coat, as described for the myenteric plexus of rats[24].

In the quantitative analysis, although animals from the experimental groups (EG21 and EG70) had respective increases of 9.2% and 9% in the quantity of neurons compared to their controls (CG21 and CG70), the number of myenteric neurons present in 8.96 mm^2 of jejunum in animals that were fed with an autoclaved diet did not change, since the average amount of neurons observed did not differ ($P > 0.05$) among animals from the CG21 (1061 ± 50.72) and EG21 (881.4 ± 38.96) groups, and even among animals from CG70 (881.4 ± 38.96) and EG70 (969.4 ± 82.03).

On the other hand, the number of myenteric neurons present in 8.96 mm^2 of jejunum differed ($P < 0.05$) among 21- and 70-day-old animals, with lower values seen in 70-day-old animals. The decrease of 16.9% among the 21-day-old animals and 17% among 70-day-old animals indicates that the quantity of neurons was not influenced by the provided diet. It is believed that the numbers of neurons does not decrease, but increases during the animals' growth period, and the observed decreases are only related to the greater dispersion of neurons in the organ[24,25].

In the morphometric analysis, we verified that the average area of cell body of reactive NADH-diaphorase myenteric neurons varied and differed ($P < 0.05$) during nursing and post-weaning periods. In general, the cell body area of neurons increased in animals from the CG70 (347.4 ± 13.4 μm^2) and EG70 (377.4 ± 22 μm^2) groups when compared to animals from the CG21 (230 ± 10.9 μm^2) and EG21 (282.1 ± 7.0 μm^2) groups, respectively.

However, animals that received an autoclaved diet (EG21) during pregnancy and lactation had a significant increase in neuron cell body area (22.65%) ($P < 0.05$) during the suckling period. On the other hand, there was an increase of 8.65% in the cell body area of neurons for animals from the EG70 group compared to animals from the CG70 group, but this effect was not statistically significant. These data suggest an effect of autoclaved diet on the area of myenteric neurons during the suckling period, which is different from the effect shown in the other study[15], which observed a small reduction in the size of neurons in the myenteric plexus under severe protein restriction.

Thus, an increase in the area of the cell body of myenteric neurons could be a response to nutritional deficiency associated with exposure time or the level of this deficiency. The increase in cell body area was mainly seen in animals from the EG21 group, whose mothers received an autoclaved diet during the pregnancy and lactation period, and this could be a neuronal response in order to remedy possible deficiencies. Neurons can increase metabolic activities to compensate for the decrease in protein quality of the ration, but our results could indicate neurons' lower ability to achieve maximum expected development during the growth process.

The increase in neuronal size during animal growth is expected[24]. However, the fact that the neurons of animals that received an autoclaved diet during the post-weaning period had a growth level of 33.8% (lower than the 51.5% seen in CG70 animals) may suggest that an autoclaved diet causes a nutritional deficiency that inhibits the normal development of neurons during the post-weaning period. However, tests must be conducted to confirm this assumption.

Based on the solubility analysis of the protein in animal feed in KOH, it was found that the protein solubility level was 47.36% after autoclaving and 80.85% before autoclaving, indicating a decrease in the protein digestibility of 33.49%[8]. This means that the autoclaved diet had a usable protein level of 10.52% compared to the 17.97% of non-autoclaved diet, which falls below the 15% suggested by the National Research Council (NRC)[26] for rats undergoing pregnancy, lactation and growth.

Despite the low level of usable protein in the autoclaved diet (10.52%), it is still higher than the 8% used in some studies examining protein deficiency[14,16,17,22]. However, as the NRC[26] establishes a minimum protein level of 15% for rats during reproduction, pregnancy, lactation and growth periods and a level of 5% for maintenance phase, autoclaving the feed decreases the quality of the protein and may have interfered in neuronal development. This fact is corroborated by the size of neuronal cell bodies of animals that did not receive an autoclaved diet and those that did. Thus, although autoclaving the feed is more desirable, based on cost and safety, than other sterilization processes[4], its use must be considered when the objective of the research is to evaluate the nervous system.

In summary, the use of an autoclaved diet during pregnancy, lactation and post-weaning does not alter the quantity of reactive NADH-diaphorase myenteric neurons in the jejunum but does interfere with the increase

in cell body area of neurons, preventing the neuron cell body from reaching a size similar to that observed using a non-autoclaved diet, suggesting a lower metabolic activity for those neurons.

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COMMENTS

Background

Neurons morphological characteristics influence their functional performance. Most of these characteristics develop early in neuron development, and therefore may be influenced by external factors such as nutritional quality, which must be well known and controlled.

Research frontiers

The exposure to high temperature may compromise the components of the ration, destroying vitamins and proteins and affecting the nutritional value of the diet. It occurs during the autoclaving process, altering the expected performance of the provided diet.

Innovations and breakthroughs

Recent studies indicate that factors such as nutritional quality of the ration and animal age may interfere with the morphofunctional aspects of the myenteric plexus compromising digestive system function, and consequently the animal's performance.

Applications

Changes in the nutritional values of autoclaved diets may be considered to correct possible tissue loss during animal growth and development.

Terminology

The identification of myenteric neurons by the nicotinamide adenine dinucleotide (NADH)-diaphorase histochemical technique occurs through formation of formazan granules from an artificial electron acceptor (nitro blue tetrazolium), allowing the evaluation of respiratory activity of neurons, which provides evidence of their metabolic activity.

Peer review

This manuscript is an interesting article in an attempt to evaluate the effect of autoclaved diet on the jejunum neurons of the myenteric plexus of rats during their growth phase. The results suggest that changes similar to those observed for protein deficiency occur in rats that have been fed with an autoclaved diet, but that this occurs to a lesser degree than for true protein deficiency.

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BRIEF ARTICLE

De novo combination therapy with lamivudine and adefovir dipivoxil in chronic hepatitis B patients

Xiao-Hong Fan, Jian-Zhang Geng, Li-Fen Wang, Ying-Ying Zheng, Hai-Ying Lu, Jing Li, Xiao-Yuan Xu

Xiao-Hong Fan, Li-Fen Wang, Ying-Ying Zheng, Hai-Ying Lu, Xiao-Yuan Xu, Department of Infectious Diseases, Peking University First Hospital, Beijing 100034, China
Jian-Zhang Geng, Jing Li, Department of Infectious Diseases, Shijiazhuang Fifth Hospital, Shijiazhuang 050021, Hebei Province, China
Author contributions: Xu XY, Lu HY, Fan XH and Geng JZ designed the research; Fan XH, Geng JZ, Wang LF, Zheng YY and Li J performed the research; Fan XH analyzed the data and wrote the manuscript.
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Correspondence to: Xiao-Yuan Xu, Professor, Department of Infectious Diseases, Peking University First Hospital, No. 1, Dahongluochang Street, Xicheng District, Beijing 100034, China. yangpin@public3.bta.net.cn
Telephone: +86-10-83575019 Fax: +86-10-66551799
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Abstract

AIM: To investigate the appropriate time for combination therapy in HBeAg positive chronic hepatitis B (CHB) patients with decompensated cirrhosis.

METHODS: Thirty HBeAg positive CHB patients with decompensated cirrhosis were enrolled in the study. All of the patients were given 48 wk combination therapy with lamivudine (LAM) and adefovir dipivoxil (ADV). Briefly, 10 patients were given the *de novo* combination therapy with LAM and ADV, whereas the other 20 patients received ADV in addition to LAM after hepatitis B virus (HBV) genetic mutation.

RESULTS: Serum alanine aminotransferase and total bilirubin were both improved in the two groups at 4, 12, 24 and 48 wk after treatment. Serum albumin was also improved at 24 and 48 wk after combination therapy in both groups. The serum HBV DNA level was still detectable in every patient in the two groups at 4 and 12 wk after combination treatment. However, in the *de novo* combination group, serum HBV DNA levels in 4 (40%) and 9 (90%) patients was decreased to below 1×10^3 copies/mL at 24 and 48 wk after the combination treatment, respectively. In parallel, serum HBV DNA levels in 2 (20%) and 8 (40%) patients in the add-on combination group became undetectable at 24 and 48 wk after combination treatment, respectively. Furthermore, 6 (60%) patients in the *de novo* combination group achieved HBeAg seroconversion after 48 wk treatment, whereas only 4 (20%) patients in the add-on combination group achieved seroconversion. Child-Pugh score of patients in the *de novo* combination group was better than that of patients in the add-on combination group after 48 wk treatment. Moreover, patients in the *de novo* combination group had a significantly decreased serum creatinine level and elevated red blood cell counts.

CONCLUSION: *De novo* combination therapy with LAM and ADV was better than add-on combination therapy in terms of Child-Pugh score, virus inhibition and renal function.

Key words: Hepatitis B; Chronic; Cirrhosis; Decompensated; *De novo* combination; Lamivudine; Adefovir dipivoxil

Peer reviewer: Shashi Bala, PhD, Postdoctoral Associate, Department of Medicine, LRB 270L, 364 Plantation Street, UMass Medical School, Worcester, MA 01605, United States

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INTRODUCTION

Hepatitis B virus (HBV) is prevalent world-widely. World Health Origination estimates that approximately 400 million people are chronic HBV carriers around the world[1]. Chronic hepatitis B (CHB) is a leading cause of hepatic cirrhosis and hepatocellular carcinoma (HCC)[1]. Once cirrhosis develops, mortality is high in untreated patients. The 5 years mortality rate of CHB patients with decompensated cirrhosis is 86%[2]. Therefore, it is necessary to develop an effective therapy for those decompensated cirrhosis patients infected with chronic HBV.

The treatment for decompensated cirrhotic patients aims to delay the occurrence of HCC. Recommended therapy options are nucleos(t)ide analogues, such as lamivudine (LAM), adefovir dipivoxil (ADV) and entecavir (ETV). Previous studies showed that LAM is effective in cirrhotic patients infected with chronic HBV[3,4]. However, the clinical benefit of LAM is limited by the emergence of resistant mutant strains[5,6]. Recently, ADV has been strongly considered as a rescue therapeutic agent to resistant mutants[7,8]. Several studies showed that combination therapy with LAM and ADV is better than ADV monotherapy in LAM-resistant patients infected with HBV[7,9,10]. However, it remains unclear how to start the combination therapy in HBeAg positive patients with decompensated cirrhosis secondary to hepatitis B. In the present study, we aimed to evaluate the better combination therapy in HBeAg positive patients with decompensated cirrhosis secondary to hepatitis B.

MATERIALS AND METHODS

Patients

Adult patients who had CHB with decompensated cirrhosis and HBeAg positive were enrolled in the study from January 2008 to June 2010 in Peking University First Hospital and Shijiazhuang Fifth Hospital. The criteria for diagnosis of hepatitis were those which appear in the guidelines for prevention and treatment of CHB in China[11]. The diagnosis of decompensated cirrhosis was based on clinical, laboratory, previous histological, ultrasonographic and radiological signs of cirrhosis with at least one sign of liver decompensation (ascites, variceal bleeding, hepatic encephalopathy, non-obstructive jaundice). Patients co-infected with hepatitis A virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, or human immunodeficiency virus and with alcoholic cirrhosis, autoimmune hepatitis, hepatorenal syndrome, HCC or severe heart, brain, renal diseases were excluded from the study. All of the patients were both HBV DNA and HBeAg positive. A total of 30 patients were enrolled in the study. Child-Pugh score was used to assess the clinical status of every patient[12]. Sixteen patients were at B stage (mean score: 8.4), and the other 14 patients were at C stage (mean score: 11.1).

Treatment of patients

All of the patients were given LAM (100 mg/d QO, GSK, Suzhou, China) at the beginning of treatment. Ten of them were then given ADV 10 mg/d (QO, GSK, Suzhou, China) over the following 2 wk (*de novo* combination arm), while the other 20 patients received ADV 10 mg/d in addition to LAM after HBV genetic YMDD mutation (add-on combination arm). The duration of combination treatment was 48 wk for both arms.

Biochemical and virological analysis

Peripheral blood was taken from all of the patients in the morning with fasting for at least 8 h. HBsAg, HBsAg antibody (anti-HBs), HBeAg, HBeAg antibody (anti-HBe) and HBcAg antibody (anti-HBc) were detected by AxSYM MEI kits (Abbott Laboratories, United States). Serum HBV DNA level was measured by quantitative polymerase chain reaction (PCR) (Daan Gene Co., LTD of Sun Yan-sen University, Guangzhou, China) at certain time points (week 0, 4, 12, 24 and 48) during treatment. The detection limit of HBV DNA was 1×10^3 copies/mL.

Routine biochemical and hematological tests were performed at the participating centers using automated techniques at certain time points (week 0, 4, 12, 24 and 48) during treatment. Child-Pugh score was also assessed simultaneously.

Statistical analysis

Data were expressed as arithmetic mean ± SD, median (range), or frequency and percentage when appropriate. Student *t* test was used to analyze the normally distributed quantitative variables between the two study groups. Mann-Whitney rank sum test was used to analyze the skewed data. All tests were two tailed, and *P* value less than 0.05 was considered statistically significant. All statistical calculations were done using the SPSS 16.0 (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, United States) statistical software.

RESULTS

Baseline characteristics

A total of 30 patients were enrolled in the study, including 17 males (56.7%) with a median age of 42 years (40-49 years). Data showed that serum alanine aminotransferase (ALT), total bilirubin (TBIL) and albumin (ALB) levels at baseline in the *de novo* combination patients were significantly lower than those in the add-on combination patients. However, mean serum HBV DNA level and Child-Pugh score were not different between the two groups. Table 1 shows the baseline characteristics of the study population.

Virological response

The percentage of patients with undetectable HBV DNA in the *de novo* combination group was 0, 0, 40% and 90% at 4, 12, 24 and 48 wk after the treatment, respectively. However, it was 0, 0, 20% and 40% at each time point in the add-on combination group, respectively. Figure 1 shows that the percentage of patients

Table 1 Characteristics of patients at baseline, median (min-max)

Characteristic	*De novo* combination group (n = 10)	Add-on combination group (n = 20)	P value
Age (yr)	45.6 ± 8.2	45.2 ± 8.2	0.89
Gender (males,%)	6 (60%)	11 (55%)	1
Height (m)	1.7 ± 0.7	1.7 ± 0.9	0.58
Weight (kg)	68.9 ± 9.7	67.3 ± 10.5	0.69
BMI (kg/m^2)	23.8 ± 1.8	23.7 ± 1.6	0.88
HBV DNA levels (log_{10}, copies/mL)	6.23 ± 0.91	6.43 ± 0.95	0.58
Serum ALT (IU/L)	124 (82-156)	283 (187-321)	0
Serum TBIL (μmol/L)	49.5 (23-72)	73.5 (49.0-99.6)	0.001
Serum ALB (g/L)	29.75 (25.40-34.00)	26.65 (23.00-32.00)	0.021
Child-Pugh score	11 (9-13)	11 (9-13)	0.809
Red blood cell counts (× 10^{12}/L)	3.6 (3.0-4.5)	3.3 (1.9-5.0)	0.234
HB (g/L)	106 (90-120)	97 (75-115)	0.059
White blood cell counts (× 10^9/L)	3.85 (2.70-6.80)	3.75 (1.90-6.00)	0.657
Blood PLT count (× 10^9/L)	96 (76-150)	96 (50-112)	0.657
Serum BUN (mmol/L)	6.05 (3.70-8.10)	4.35 (1.90-8.50)	0.139
Serum Cr (μmol/L)	99.5 (77.0-145.0)	77.0 (40.0-156.0)	0.024

BMI: Body mass index; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; TBIL: Total bilirubin; ALB: Albumin; HB: Hemoglobin; PLT: Platelet; BUN: Blood urine nitrogen; Cr: Creatinine.

with undetectable HBV DNA was significantly different between the two groups after the 48 wk treatment (P = 0.017). Moreover, no patient in the two groups showed detectable virological resistance during the 48 wk combination treatment.

Biochemical response

We showed that ALT, TBIL and ALB of patients were decreased in the two groups after treatment. Furthermore, 6 (60%) patients in the *de novo* combination group achieved HBeAg seroconversion, whereas only 4 (20%) patients in the add-on combination group achieved seroconversion (P = 0.045).

Child-Pugh score at baseline was not statistically different in the two groups. Table 2 shows that Child-Pugh score in the *de novo* combination group was significantly lower than that in the add-on combination group after the 48 wk treatment.

Hematological changes and renal function

Red blood cell counts and hemoglobin levels in the 2 groups showed no difference at baseline. However, red blood cell counts and hemoglobin in the *de novo* combination group were higher than those in the add-on combination group at 24 and 48 wk after treatment. White blood cell counts and platelet (PLT) level showed no difference between the two groups at baseline and during the treatment period.

Moreover, serum blood urine nitrogen (BUN) level was not different between the two groups at baseline and during treatment. Serum creatinine (Cr) at baseline in the *de novo* combination group was significantly higher than

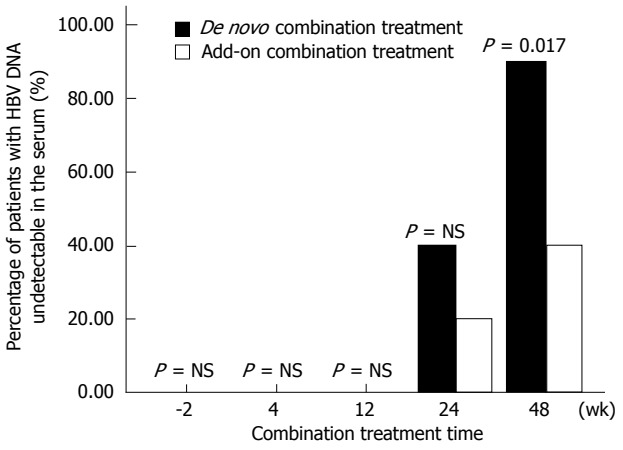

Figure 1 The percentage of decompensated cirrhotic chronic hepatitis B patients with undetectable hepatitis B virus (HBV) DNA in the serum of the two groups during the treatment period. NS: Not signifcant.

that in the add-on combination group. However, serum Cr level in the 2 groups was not significantly different after treatment (Table 3).

Side effects

Both *de novo* combination treatment and add-on combination treatment were well tolerated. No patient in either group discontinued the drug during the period.

In the *de novo* combination group, serum BUN level of two patients was slightly increased, two patients had slight diarrhea, and one patient had nausea. In parallel, in the add-on combination group, serum BUN level of two patients was slightly increased, two patients had nausea, one patient had slight diarrhea, and one patient had upper gastrointestinal hemorrhage. All these symptoms disappeared after relevant management.

DISCUSSION

In this retrospective study, we compared *de novo* combination therapy with add-on combination therapy in HBeAg positive and decompensated cirrhosis patients infected with HBV. The percentage of patients with undetectable HBV DNA was 40% (4/10) and 20% (4/20) in the *de novo* combination and add-on combination groups at 24 wk after treatment, respectively. However, this difference between two groups was not statistically different. The percentage of patients with undetectable HBV DNA in the *de novo* combination group (9/10, 90%) was significantly higher than that in the add-on combination group (8/20, 40%) at 48 wk after treatment. We, for the first time, compared *de novo* combination treatment with add-on combination treatment in cirrhotic decompensated patients secondary to hepatitis B. Some researchers compared LAM and ADV combination therapy with ADV monotherapy after LAM-induced viral genetic resistance. They showed that LAM and ADV combination therapy is a more effective treatment to get a virological response and has lower genetic resistance than ADV monothera-

Table 2 Biochemical responses of the patients, median (min-max)

	De novo combination group (n = 10)					Add-on combination group (n = 20)				
	2 wk before combination treatment	4 wk after combination treatment	12 wk after combination treatment	24 wk after combination treatment	48 wk after combination treatment	2 wk before combination treatment	4 wk after combination treatment	12 wk after combination treatment	24 wk after combination treatment	48 wk after combination treatment
Child-Pugh score	11 (9-13)	10 (8-12)	10 (8-12)	9 (7-11)[c]	7 (6-9)[c,a]	11 (9-13)	10 (9-12)	10 (9-12)	10 (7-11)[c]	9 (7-10)[c]
Serum ALT (IU/L)	124 (82-156)[a]	111 (80-141)[a]	109 (69-125)[a]	89 (59-112)[c,a]	49 (28-67)[c,a]	283 (187-321)	251 (171-302)[c]	218 (146-277)[c]	173 (105-239)[c]	105 (65-186)[c]
Serum TBIL (μmol/L)	49.5 (23-72)[a]	47.3 (25.0-70.1)[a]	41.3 (25.5-63.0)[a]	37.8 (22.5-57.4)[c,a]	31.0 (22.0-41.0)[c,a]	73.5 (49.0-99.6)	68.3 (44.5-88.7)	63.5 (35.8-79.0)[c]	56.7 (32.0-72.3)[c]	41.2 (29.0-58.1)[c]
Serum ALB (g/L)	29.75 (25.40-34.00)[a]	30.15 (27.20-34.10)[a]	30.55 (27.60-33.00)[a]	32.80 (29.00-35.00)[c,a]	34.00 (30.00-37.00)[c,a]	26.65 (23.00-32.00)	28.20 (25.60-32.90)	27.85 (25.00-31.90)	28.75 (25.50-33.00)[c]	30.5 (28.0-34.8)[c]

[a]$P < 0.05$ *vs* add-on combination group; [c]$P < 0.05$ *vs* 2 wk before combination treatment in the same group. ALT: Alanine aminotransferase; TBIL: Total bilirubin; ALB: Albumin.

Table 3 Blood cell counts and renal function of the patients, median (min-max)

	De novo combination group (n = 10)					Add-on combination group (n = 20)				
	2 wk before combination treatment	4 wk after combination treatment	12 wk after combination treatment	24 wk after combination treatment	48 wk after combination treatment	2 wk before combination treatment	4 wk after combination treatment	12 wk after combination treatment	24 wk after combination treatment	48 wk after combination treatment
Red blood cell counts ($\times 10^{12}$/L)	3.6 (3.0-4.5)	3.4 (3.0-4.3)	3.5 (2.9-4.1)	3.6 (3.1-4.0)[a]	3.8 (3.0-5.1)[a]	3.3 (1.9-5.0)	3.2 (2.3-4.1)	3.1 (2.5-4.0)	3.1 (2.5-3.9)	3.1 (2.7-3.4)[c]
HB (g/L)	106 (90-120)	104 (92-113)[a]	102 (99-115)	107 (94-114)[a]	105 (85-121)[a]	97 (75-115)	95 (80-114)	95 (85-111)	99 (88-112)	97 (84-105)
White blood cell counts ($\times 10^9$/L)	3.85 (2.70-6.80)	3.90 (2.90-5.70)	3.80 (3.00-5.20)	3.95 (3.20-4.70)	4.00 (3.00-4.50)	3.75 (1.90-6.00)	3.75 (2.50-5.60)	3.70 (2.90-5.00)	3.75 (2.90-4.70)	3.85 (3.00-5.10)
Blood PLT counts ($\times 10^9$/L)	96 (76-150)	90 (72-100)	94 (70-116)	95 (68-108)	98 (72-103)	96 (50-112)	94 (63-111)	92 (76-110)	90 (69-107)	93 (73-112)
Serum BUN (mmol/L)	6.05 (3.70-8.10)	5.90 (3.00-7.40)	5.65 (3.60-7.10)	5.50 (2.50-6.80)	5.45 (3.30-7.00)	4.35 (1.90-8.50)	5.25 (2.20-8.70)	5.00 (2.20-8.00)	5.00 (2.90-7.50)	4.95 (2.30-7.10)
Serum Cr (μmol/L)	99.5 (77.0-145.0)[a]	96.5 (76.0-141.0)	96.0 (69.0-112.0)	78.0 (56.0-99.0)[c]	78.5 (61.0-107.0)[c]	77.0 (40.0-156.0)	95.5 (61.0-124.0)	86.0 (68.0-131.0)	73.5 (42.0-132.0)	79.0 (41.0-132.0)

[a]$P < 0.05$ *vs* add-on combination group;[c]$P < 0.05$ *vs* 2 wk before combination treatment in the same group. HB: Hemoglobin; PLT: Platelet; BUN: Blood urine nitrogen; Cr: Creatinine.

py[7-9]. However, the efficiency of LAM and ADV combination therapy in treatment naïve patients with decompensated cirrhosis remains unclear. In the present study, we showed that *de novo* combination therapy was more effective than add-on combination therapy in terms of virological response and HBeAg seroconversion. No patient achieved virological resistance during combination treatment in both two groups.

It is known that HBeAg seroconversion is accompanied by biochemical and histological regression of liver disease[13,14]. Our data showed that the percentage of HBeAg seroconversion in the *de novo* combination group (6/10, 60%) was significantly higher than that in the add-on combination group (4/20, 20%).

Due to its simplicity in clinical practice, Child-Pugh score has been widely applied as the prognostic marker in patients with decompensated cirrhosis[15,16]. Child-Pugh score is one of the risk factors for assessing patients with decompensated cirrhosis[17]. In this study, Child-Pugh score of all patients was significantly decreased in the two groups after 24 and 48 wk combination treatment. Child-Pugh score at baseline was not different between the two groups. However, the score in the *de novo* combination group was superior to that in the add-on combination group after 48 wk treatment. LAM and ADV combination therapy could improve clinical symptoms in patients with decompensated cirrhosis. But, *de novo* combination therapy was more effective in improving hepatic function than LAM and ADV combination therapy after genetic resistance.

ALT, TBIL and ALB of the patients were different at baseline between the two groups. However, these three parameters were all significantly decreased in the two groups during the treatment period. LAM and ADV combination therapy was an effective way to produce a biochemical response in CHB patients with decompensated cirrhosis.

It has been reported that ADV decreases renal function[18]. In our study, serum Cr and BUN levels of all patients were not increased during the treatment period. However, serum Cr level in the *de novo* combination group was significantly higher than that in the add-on combination group (104.0 ± 23.5 *vs* 83.0 ± 29.4, $P < 0.05$) at baseline. But serum Cr in the two groups was not dif-

ferent after the 48 wk treatment. Our data showed LAM and ADV combination therapy did not affect renal function during the treatment period. The *de novo* combination therapy could improve serum Cr level in the decompensated cirrhosis patients infected with chronic HBV.

Taken together, a higher percentage of patients with undetectable HBV DNA and HBeAg seroconversion was obtained from the *de novo* combination group than from the add-on combination group. Moreover, Child-Pugh score in patients with the *de novo* combination therapy was better than that in patients with the add-on combination therapy after 48 wk treatment. Therefore, HBeAg positive decompensated cirrhotic patients infected with chronic HBV should receive LAM and ADV combination therapy at the beginning of antiviral treatment.

In the present study, we have several shortcomings as follows. First, the number of subjects was small because the number of patients treated with *de novo* combination therapy was limited in China. Second, the period of the combination treatment was not long enough. Therefore, a larger number of subjects and longer treatment duration are required in our future study.

COMMENTS

Background

The mortality rate of chronic hepatitis B patients with decompensated cirrhosis is very high. Recommended therapy options are nucleos(t)ide analogues. But, the combination treatment option with nucleos(t)ide analogues for HBeAg positive patients with decompensated cirrhosis secondary to hepatitis B is not very clear.

Research frontiers

Combination therapy with lamivudine (LAM) and adefovir dipivoxil (ADV) is better than ADV monotherapy in LAM-resistant patients infected with hepatitis B virus. But in HBeAg positive patients with decompensated cirrhosis secondary to hepatitis B, it remains unclear whether combination treatment at the beginning of therapy is better than add-on combination therapy. In this study, the authors demonstrate that *de novo* combination therapy is more effective than add-on combination therapy with LAM and ADV in HBeAg positive patients with decompensated cirrhosis secondary to hepatitis B.

Innovations and breakthroughs

Some research showed that LAM and ADV combination therapy is more effective as a means to get a virological response and has lower genetic resistance than ADV monotherapy after LAM-induced viral genetic resistance. However, the efficiency of LAM and ADV combination therapy in naïve patients with decompensated cirrhosis is unclear. For the first time, this study compared *de novo* combination treatment with add-on combination treatment in the cirrhotic decompensated patients secondary to hepatitis B.

Applications

By understanding that *de novo* combination therapy is more effective than add-on combination therapy, this study may represent a future strategy in the treatment of HBeAg positive patients with decompensated cirrhosis secondary to chronic hepatitis B.

Terminology

De novo combination treatment means combination treatment with two or more drugs from the beginning of the treatment. Child-Pugh score is a system to assess the disease stage for decompensated cirrhotic patients.

Peer review

In this study, the authors have made an attempt to determine the effective time frame for combined therapy used in HBeAg positive chronic hepatitis B patients with decompensated cirrhosis. Though the study is limited to only 30 patients, it is novel and contributes a substantial amount of knowledge to the field.

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BRIEF ARTICLE

Schistosoma japonicum ova maintains epithelial barrier function during experimental colitis

Chen-Mei Xia, Yuan Zhao, Li Jiang, Jie Jiang, Shun-Cai Zhang

Chen-Mei Xia, Yuan Zhao, Li Jiang, Jie Jiang, Shun-Cai Zhang, Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai 200032, China
Author contributions: Xia CM and Zhao Y performed majority of the experiments and contributes equality to this work; Jiang L and Jiang J were responsible for the pathology and bacterial translocation parts; Zhang SC designed the study and provided financial support.
Correspondence to: Shun-Cai Zhang, Professor, MD, PHD, Department of Gastroenterology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China. doctorzhangsc@163.com
Telephone: +86-21-64041990-2424 Fax: +86-21-64035399
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Abstract

AIM: To evaluate the impacts of *Schistosoma japonicum* (*S. japonicum*) ova on the tight junction barriers in a trinitrobenzenesulfonic acid (TNBS)-induced colitis model.

METHODS: Balb/c mice were randomly divided into three groups: control group; $TNBS^+ova^-$ group and $TNBS^+ova^+$ group. TNBS was used intracolonic to induce colitis and mice of the $TNBS^+ova^+$ group were pre-exposed to *S. japonicum* ova as a prophylactic intervention. Colon inflammation was quantified using following variables: mouse mortality, weight loss, colon extent and microscopic inflammation score. Serum expression of tumor necrosis factor-α and interferon-γ were assessed to evaluate the systemic inflammatory response. NOD2 and its mRNA were also tested. Bacterial translocations were tested by culturing blood and several tissues. ZO-1 and occludin were chosen as the representations of tight junction proteins. Both the proteins and mRNA were assessed.

RESULTS: Ova pre-treatment contributed to the relief of colitis and decreased the mortality of the models. NOD2 expression was significantly downregulated when pretreated with the ova. The TNBS injection caused a significant downregulation of ZO-1 and occludin mRNA together with their proteins in the colon; ova pre-exposure reversed these alterations. Treatment with *S. japonicum* ova in the colitis model caused lower intestinal bacterial translocation frequency.

CONCLUSION: *S. japonicum* ova can maintain epithelial barrier function through increasing tight junction proteins, thus causing less exposure of NOD2 to the luminal antigens which may activate a series of inflammatory factors and induce colitis.

Key words: Crohn's disease; *Schistosoma japonicum* ova; Tight junction protein; ZO-1; Occludin

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Although the etiology is multifactorial and remains incompletely understood, inflammation, immunity, genetic and environmental factors have been suggested to predispose to CD[1]. Despite the multiple mechanisms that have been investigated

hitherto, proinflammatory cytokines play the most important role since they are the ultimate pathway that leads to the colitis. Therapies targeting these proinflammatory cytokines have been extensively investigated and shown to be effective both in animal experiments and clinical trials[2-3].

In addition, CD patients demonstrate increased intestinal bacterial translocation (IBT), which may be due to the increased intestinal pericellular permeability, reflecting the decreased epithelial barrier function[4]. The tight junctions (TJs), which form the pericellular barrier, are thought to be the primary determinant of mucosal permeability in the presence of an intact epithelium[5]. Impaired TJs lead to an increase of exposure of intestinal bacteria to submucosal pattern pathogen recognition receptors (PPRRs), such as toll-like receptors (TLRs) and nucleotide-binding-oligomerization domains (NODs). NOD2 has been found to exert antibacterial activity limiting survival of enteric bacteria after invasion[6-7]. As one of the main responsible genes, NOD2 and its protein have been found to be upregulated in CD patients[8].

It is well established that helminthes can protect mice from experimental colitis[9-11]. We have also demonstrated in our former study that freeze-killed *Schistosoma japonicum* (*S. japonicum*) ova could relieve the colon inflammation and prevent IBT in a trinitrobenzenesulfonic acid (TNBS)-induced model by upregulating Th2-type cytokine and downregulating TLR4 mRNA expression[12]. In the present study, we further investigate the influence of *S. japonicum* ova on TNBS-induced colitis in mice and whether *S. japonicum* ova prevent IBT by upregulating tight junction proteins. Since TLR4, one of the main extracellular receptors of enteric antigens, has been found to be upregulated in TNBS-induced colitis which was blocked by the pretreatment of *S. japonicum* ova[12], we further discuss here whether NOD2, one of the main intracellular receptors, has the same ability.

MATERIALS AND METHODS

Animals

Ninety Balb/c mice (Animal Center of Shanghai Laboratory, Chinese Academy of Science) were randomly divided into 3 groups. Twenty mice from the control group received an intra-colonic injection of 0.5 mL saline on day 15. Forty mice from the $TNBS^+ova^-$ group received an intra-colonic injection of 0.5 mL TNBS (Sigma) on day 15. Thirty mice from the $TNBS^+ova^+$ group received intra-peritoneal injections of 10 000 freeze-killed *S. japonicum* ova (Zhejiang Academy of Medical Science) on day 1 and day 11, and were challenged with TNBS on day 15. The surviving mice were sacrificed on day 22. Serum expression of tumor necrosis factor (TNF)-α and interferon (IFN)-γ were detected. Ten animals from each group were randomly selected and the full-length colon from these animals was isolated and evaluated.

Another 30 mice were grouped according to the former methods and were sacrificed on day 16. Blood, liver, spleen and mesenteric lymph nodes (MLN) were cultured and the identification of bacteria was completed using VITEK-32 Auto Microbiotic System (bioMérieux).

Table 1 Primers for polymerase chain reaction

Primer	Sequence	Product (bp)
ZO-1 sense	5′-AGCCAGTCCATTCTCAGG-3′	151
ZO-1 antisense	5′-TGTACTGTGAGGGCAACG -3′	
Occludin sense	5′-TACAGACCCAAGAGCAGC -3′	177
Occludin antisense	5′-GTGGCAATAAACACCATG -3′	
NOD2 sense	5′-CCGAGGAGTCGTGATGGTT-3′	150
NOD2 antisense	5′-GTGTCACCCACATGCAGTG-3′	
GAPDH sense	5′-GGTGAAGGTCGGTGTGAACG -3′	233
GAPDH antisense	5′-CTCGCTCCTGGAAGATGGTG-3′	

GAPDH: Glyceraldehyde phosphate dehydrogenase.

Evaluations of the colonic inflammation

Mice were weighed daily and the numbers of surviving mice were recorded. The extents of colon were assessed in the sacrificed mice. The proximal 1.0 cm of the colonic segment was used for histology. The segment was fixed in 4% formaldehyde and embedded in paraffin. Morphometric analysis was performed on haematoxylin-eosin-stained 4 μm transverse sections. The microscopic slides were reviewed by two histologists blinded to the groups. The extent of damage and colonic inflammation was assessed using a modification of the histopathological grading system of Macpherson and Pfeiffer[13].

Enzyme linked immunosorbent assay for serum IFN-γ and TNF-α

Mouse serum was assembled for enzyme linked immunosorbent assay (ELISA). ELISA was performed according to the manufacturer of the kits instructions. TNF-α ELISA kit was a product of Sigma Co. IFN-γ ELISA kits were purchased from Jingmei biotech.

Realtime-PCR analysis

Colons were removed, washed in phosphate buffered solution, and then flash frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (TAKARA) according to the manufacturer's protocol. RNA was treated with DNase (TAKARA) and was converted to cDNA using a reverse transcription kit as described by the manufacturer (INVITROGEN). Primers for PCR (INVITROGEN) were listed in Table 1. The parameters for PCR amplification were as follows: 95 ℃: 10" × 1 cycle, 95 ℃: 5"→60 ℃ 20"→55 ℃ 20" × 40 cycles. The concentration of each sample was calculated from the threshold cycle (Ct). The fluorescent products were detected by LightCycle system (BIO-RAD IQ5) before the completion of each cycle.

Western blotting analysis

The colons were lysed and homogenized in ice-cold RIPA lysis buffer (Beyotime, Jiangsu, China). Proteins were loaded onto each well of SDS-polyacrylamide Ready

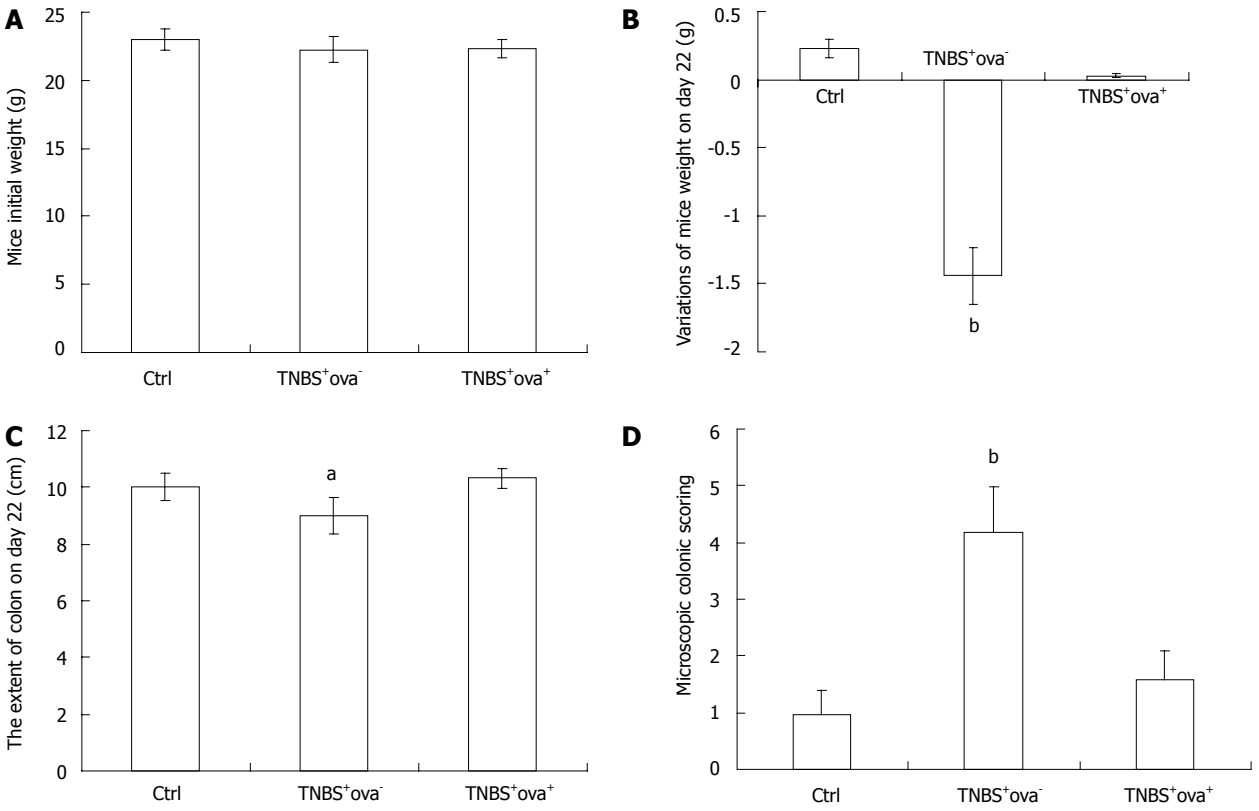

Figure 1 The evaluations of colitis. The initial weight of each group (A) and variations of weight (B), colon extent (C) and microscopic colonic scoring (D) on day 22. Data are presented as mean ± SD. $n = 10$, $^{a}P < 0.05$ *vs* the other two groups; $^{b}P < 0.01$ *vs* the other two groups. TNBS: Trinitrobenzenesulfonic acid.

Gels (BioRad, Hercules) for electrophoresis. Proteins were transferred onto a nitrocellulose membrane (BioRad, Hercules) by electroblotting. The membrane was washed and blocked with 5% milk in Tris-buffered saline with 0.05% Tween-20, and incubated overnight with specific primary antibody at 4 ℃. Rabbit anti-mouse-Occludin-mAb (2 μg/mL, ZYMED), rabbit anti-mouse-ZO-1-mAb (3 μg/mL, Abcam) and rat anti-mouse-NOD2-mAb (eBioscience) were used as the primary antibodies. HRP-conjugated goat anti-rabbit-IgG (1:1000, Santa Cruz) was employed as the secondary antibody. Membranes were washed and the assessed proteins were detected using an enhanced chemiluminescence reagent (Amersham Biosciences). Relative intensity of the bands was quantified using Leica image analysis software (Alphaln notech).

Assessment of bacterial translocation

Blood, liver, spleen and MLN were removed under sterile conditions. Blood was plated onto sheep's blood agar and tissues were slit and cultured with sterile broth at 36 ℃ for 24 h. The limpid medium was then incubated for another 6 d. Each turbid medium was considered as positive regardless of its incubation time. The positive medium was plated onto sheep's blood agar and eosin-methylene blue (EMB) agar. The blood agar and EMB agar were purchased from Shanghai Reagent Providing and Research Center for Diarrhea Disease Control. Cultures were incubated at 37 ℃ for 3 d. Any bacterial growth in the broth or on the agar plates was identified with standard microbiological methods on VITEK-32 Auto Microbic System.

Data analysis

All data were analyzed using SPSS 14.0 and are presented as means ± SD. Nonparametric data were analyzed by Kruskal-Wallis test with Mann-Whitney *U* post-hoc test. Parametric data were analyzed by 2-way ANOVA with SNK post-hoc test. The *P* value was considered significant if less than 0.05.

RESULTS

Mice common appearance and mortality

Exposure to schistosome eggs reproducibly ameliorated TNBS colitis in treated mice. Mice of the TNBS^{+}ova^{+} group presented with inapparent bloody stools, weight loss and activity reduction. The extent of the colon was shortened in TNBS-induced models and this was reversed in the ova-treated group (Figure 1). Nothing abnormal was found in the control group. During the first week after intra-colonic injection of TNBS, the mortality of each group was as follows: control group 0/20 (0%), TNBS^{+}ova^{-} group 20/40 (50%), TNBS^{+}ova^{+} group 6/30 (20%) ($P < 0.05$ between each group).

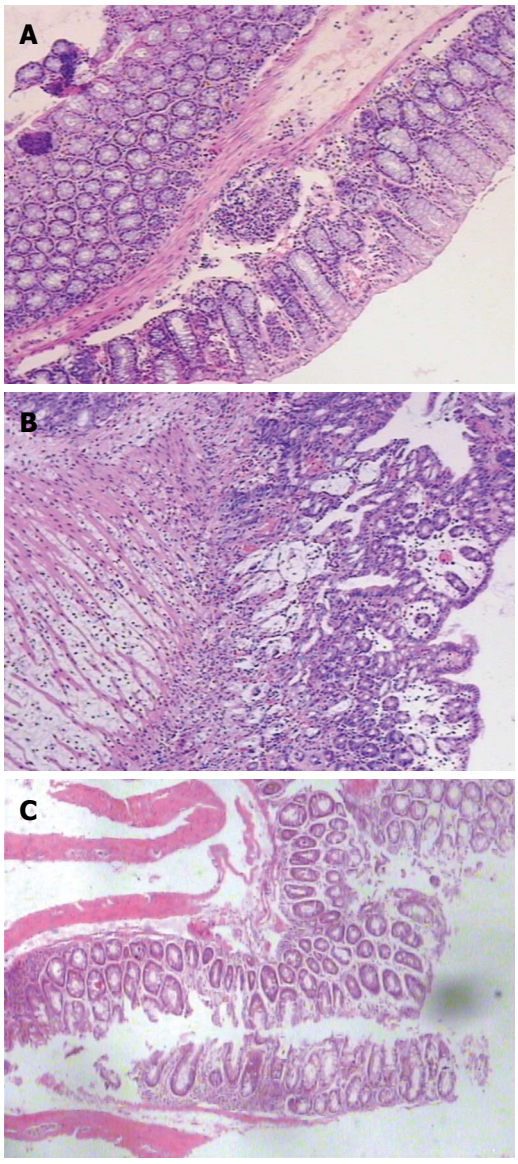

Figure 2 The histology of the colon in different group of mice (HE, × 10). Rare inflammation was found in controls (A). Transmural chronic inflammation was observed in TNBS-induced colitis (B). The inflammation was significantly relieved in schistosome ova treated mice (C). HE: Hematoxylin and eosin; TNBS: Trinitrobenzenesulfonic acid.

Microscopic inflammation score

No microscopic damage was found in the colon of control animals (Figure 2A). Compared with the colon of control mice, TNBS induced a distortion of colonic mucosal architecture and an inflammatory infiltration throughout the whole colonic wall. The inflammation was characterized by hyperaemia and edema of mucosa which led to thickening of the colonic wall, ulcer formation, folliculus lymphatics hyperplasia, and transmural chronic inflammatory cell infiltration (Figure 2B). Treatment with *S japonicum* ova tended to relieve the inflammation with infiltration of only a few inflammatory cells (Figure 2C). The histology score also showed a significant relief of colon inflammation in $TNBS^+ova^+$ group compared with the $TNBS^+ova^-$ group (Figure 1).

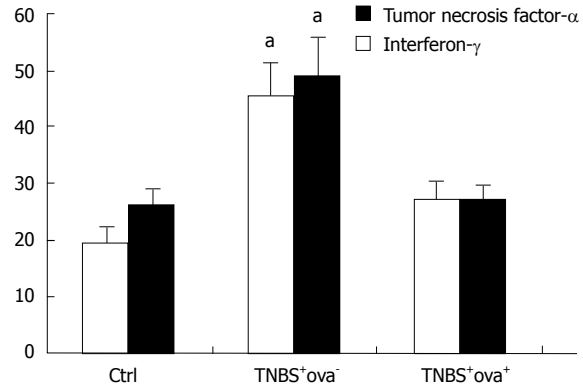

Figure 3 The serum levels of tumor necrosis factor-α (white bar) and interferon-γ (black bar). Data are presented as mean ± SD. There were 20 animals in the control group and the $TNBS^+ova^-$ group and 24 in the $TNBS^+ova^+$ group. $^aP < 0.05$ *vs* the other two groups. TNBS: Trinitrobenzenesulfonic acid.

Measurement of inflammatory factors in serum

We also surveyed the expression of inflammatory factors in the serum to further evaluate the inflammatory reaction in the different groups. As shown in Figure 3, a highly increased expression of TNF-α and IFN-γ was detected in the $TNBS^+ova^-$ group. In the $TNBS^+ova^-$ group, the expression of TNF-α was significantly higher than that of the control group and the $TNBS^+ova^-$ group. IFN-γ expression was downregulated in the $TNBS^+ova^+$ group compared with $TNBS^+ova^-$ group ($P < 0.05$).

The expression of NOD2 and its mRNA in the colon

Since NOD2 is one of the most important pattern pathogen recognition receptors which could induce a large quantity of inflammatory factors such as TNF-α and IFN-γ when inappropriately activated, we further studied the variations of NOD2 and its mRNA in the colon to verifying whether the NOD2 pathway was influenced during TNBS-induced colitis and whether it can be reversed by *S. japonicum* ova.

As shown in Figure 4, TNBS injection caused a distinct increase of NOD2 in the colon as compared with control mice. However, when mice were pretreated with *S. japonicum* ova, NOD2 expression was much lower in spite of the TNBS effect. Similar effects were also found in NOD2 mRNA expression among the three groups (Figure 5).

The evaluation of the intestinal barrier function

To further understand the cause of the over-activation of NOD2, we used IBT frequency to evaluate the intestinal barrier function. Bacterial identification showed the translocated bacteria were predominantly opportunistic pathogens especially *Escherichia coli*, *Bacillus proteus*, *Klebsiellas*, *Pseudomonas fluorescens*, *Pseudomonas pyocyanea* and enterococci were also found in several mice. IBT was more common in the $TNBS^+ova^-$ group. After exposure to *S japonicum* ova, the frequency of IBT to MLN, blood, liver and spleen was obviously reduced (Table 2).

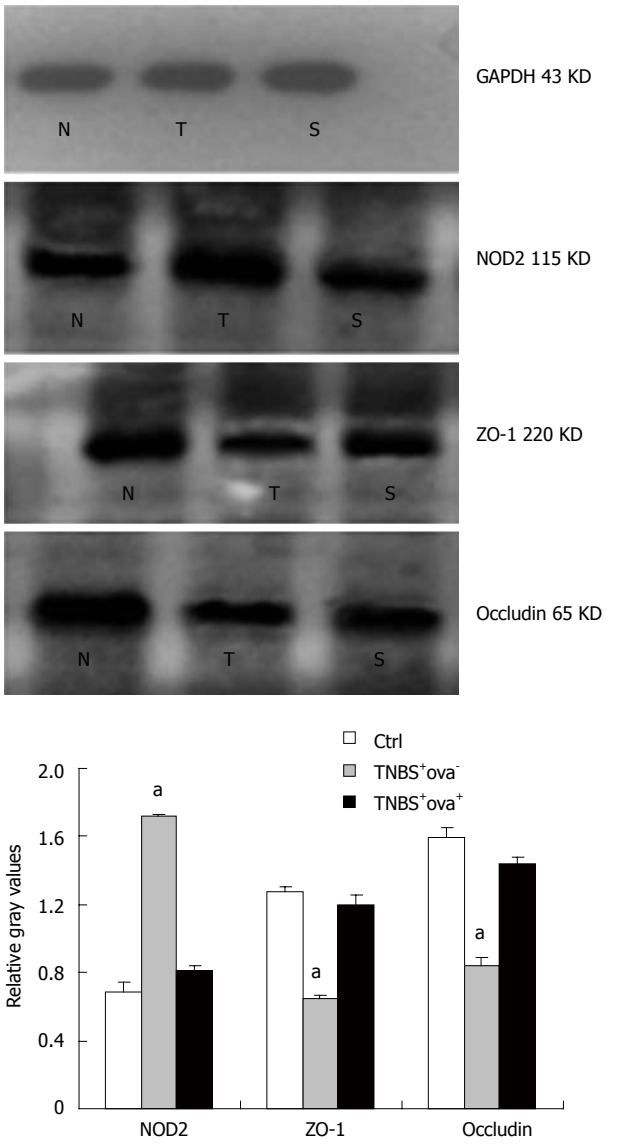

Figure 4 Effect of *Schistosoma japonicum* ova on the expression of NOD2, ZO-1 and occludin (Western blotting). White bar represents the control group (N), grey bar represents the TNBS$^+$ova$^-$ group (T), black bar represents the TNBS$^+$ova$^+$ group (S). Data are presented as mean ± SE. n = 10. $^aP < 0.05$ *vs* the other two groups. NOD2: Nucleotide-binding-oligomerization domain 2; TNBS: Trinitrobenzenesulfonic acid; GAPDH: Glyceraldehyde phosphate dehydrogenase.

The expressions of ZO-1, occludin and their mRNAs in the colon

In order to gain further insight into the mechanism of intestinal barrier function alteration, we detected the tight junction proteins that constitute the main frame of the barrier. The *ZO-1* and occludin expression levels were reduced in the TNBS$^+$ova$^-$ group compared with the normal controls. Egg pre-exposure produced a significant elevation in the expression of these tight junction proteins in the experimental induced models (Figure 4). *ZO-1* and occludin mRNA was examined using realtime-PCR. Similar to the study of the proteins, ZO-1 and occludin mRNA expression was obviously downregulated in the TNBS$^+$ova$^-$ group compared with the controls. Egg pretreatment led to a dramatic upregulation of these genes (Figure 6).

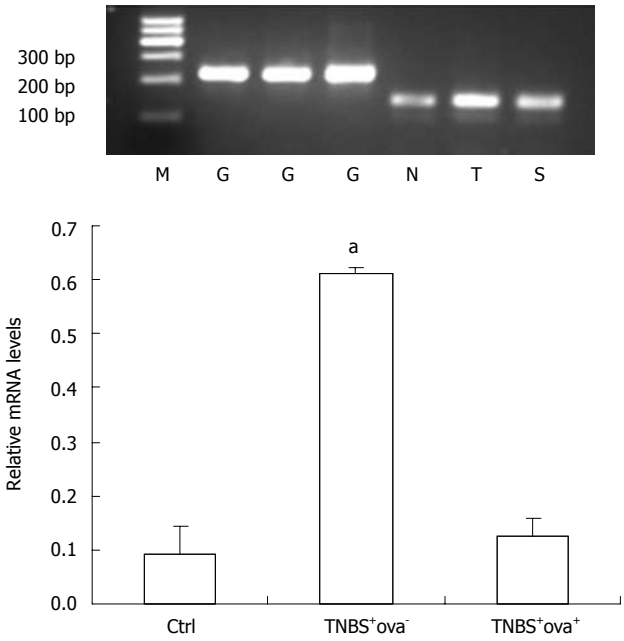

Figure 5 Effect of *Schistosoma japonicum* ova on the expression of *NOD2* mRNA (Realtime-PCR). M: Marker; G: GAPDH; N: Control group; T: TNBS$^+$ova$^-$ group; S: TNBS$^+$ova$^+$ group. Data are presented as mean ± SE. n = 10. $^aP < 0.05$ *vs* the other two groups. GAPDH: Glyceraldehyde phosphate dehydrogenase; TNBS: Trinitrobenzenesulfonic acid.

Table 2 Culture of blood, mesenteric lymph nodes, liver and spleen

	No. of mice	MLN	Blood	Liver	Spleen
Control	10	1/10	0/10	0/10	0/10
TNBS$^+$ova$^-$	10	8/10	10/10	9/10	5/10
TNBS$^+$ova$^+$	10	1/10^a	3/10^a	2/10^a	1/10^a

$^aP < 0.05$ compared with the TNBS group. MLN: Mesenteric lymph nodes; TNBS: Trinitrobenzenesulfonic acid.

DISCUSSION

CD is a disease more prevalent in developed countries where the sanitary conditions are better. Scientists believe that intestinal hygiene might be a risk factor of CD and helminthes have been thought to be beneficial to CD[9-11]. Elliot *et al*[9] found that pretreating mice with *Schistosoma mansoni* ova could relieve TNBS-induce colitis. We have also demonstrated in our previous study that *S. japonicum* ova pretreatment could prevent experimental colitis in mice by a mechanism of Th2-polarizing stimuli[12]. Summers *et al*[9] reported a safe and effective result from their short-term study of live *Trichuris suis* ova therapy in 29 patients with CD. Croese *et al*[11] also established a potential role of *Necator americanus* in the remission of autoimmune disease, including CD.

In the present study, we also found an obvious inflammatory reaction both in the colon and the blood.

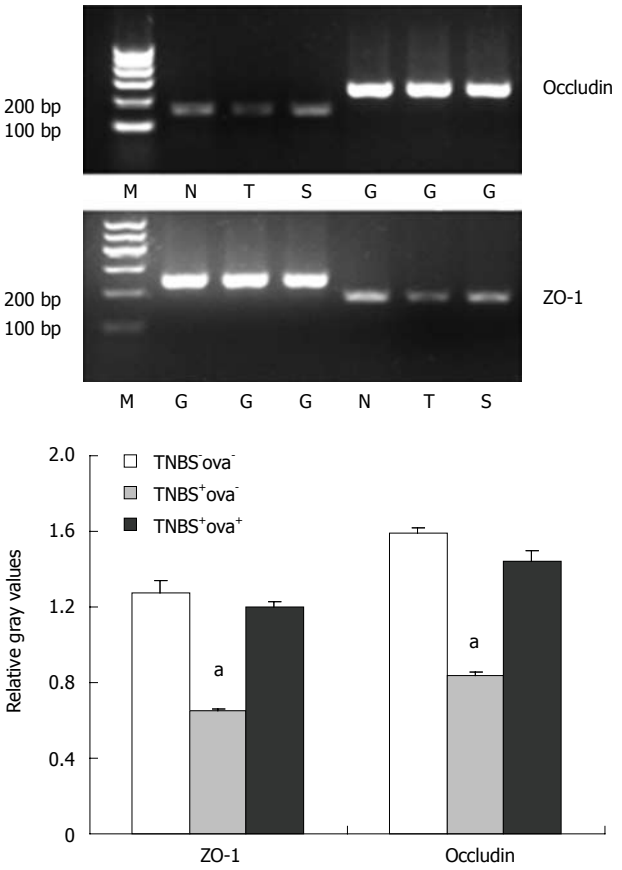

Figure 6 Effect of *Schistosoma japonicum* ova on the expression of *ZO-1* and occludin mRNA (Realtime-PCR). White bar represents the control group (N), grey bar represents the TNBS$^+$ova$^-$ group (T), black bar represents the TNBS$^+$ova$^+$ group (S). M: Marker; G: GAPDH. Data are presented as mean ± SE. n = 10. $^aP < 0.05$ *vs* the other two groups. GAPDH: Glyceraldehyde phosphate dehydrogenase. TNBS: Trinitrobenzenesulfonic acid.

The innate immune system plays an important role in the production of these inflammatory factors. Among varieties of the PPRRs, TLRs and NODs are the most important in antigen recognition. In our former study we demonstrated that TLR4 expression was elevated after the mice were injected with TNBS and was downregulated in the ova group[12]. *NOD2/CARD15*, famous as the first predisposing gene of CD, codes a cytosolic protein which provides a second line of innate immune defense within IECs and adjacent immune cells, expanding immune surveillance beyond membrane-associated TLRs[14]. Thus we assume that the ova might also decrease the production of the inflammatory cytokines through downregulating NOD2 expression in a direct or indirect pattern. We detected the expression of *NOD2* mRNA using realtime-PCR. The results showed that in the TNBS$^+$ova$^-$ group, the *NOD2* mRNA was increased and the ova pre-exposure significantly downregulated *NOD2* mRNA expression. We suppose that the ova relieved colitis and suppressed the inflammatory reaction partially because of the downregulation of PPRRs which might be inappropriately activated and thus produced large quantities of inflammatory cytokines.

The intestinal epithelial barrier also plays an important role in the onset and progression of CD. Increased IBT to the MLN, liver, blood and spleen was found in the present study. We supposed it might be due to the increased intestinal pericellular permeability, which was usually caused by impaired intestinal barrier function. The intestinal barrier function was maintained by a series of epithelial TJs, protecting the body from pathogens and other toxic luminal substances. Transmembrane proteins (such as occludin, tricellulin, claudins and junctional adhesion molecule) interact with cytoplasmic peripheral membrane proteins (such as ZO-1, ZO-2, ZO-3 and cingulin), and thus constitute the frames of TJs[15].

An obvious downregulation of ZO-1 and occludin was found in the present study using Western blotting, which could be prevented by pre-exposure to the *S. japonicum* ova. We further tested the expression of ZO-1 and occludin mRNA and the outcome was consistent with the protein study.

Inflammatory mediators such as cytokines, toxins and reactive oxygen species contributed to the impairment of TJs[16-20]. The best-studied cytokine that causes barrier dysfunction due to epithelial tight junction regulation is TNF-α. Poritz *et al*[19] incubated MDCK cells with different concentrations of TNF-α and found the fragmentation staining of ZO-1, suggesting a disruption of the TJs. They also found that claudin-1 expression was decreased along with an increasing concentration of TNF-α. It was demonstrated that the TNF-α-induced TJs impairment was mediated by NF-κB activation in Caco-2 cells[19]. Treatment of T84 cells with IFN-γ also caused a dose- and time-dependent increase in monolayer permeability. Examination of specific proteins associated with TJs by immunoblotting and confocal microscopy revealed changes in the localization and expression levels of the tight junction proteins after exposing the cells to IFN-γ. Specifically, they found that IFN-γ treatment resulted in an almost total loss of ZO-1[20].

Impaired barrier function leads to the exposure of submucosal immunocytes to luminal antigens and initiates the innate immune reaction. TLRs and NODs are PPRRs that are expressed by intestinal epithelial cells and submucosal immunocytes. The inappropriate activation of TLRs and NODs may cause the over-reaction of the immune system and overproduction of cytokines (such as IFN-γ and TNF-α), leading to the formation of CD. We assume that treating mice with *S. japonicum* ova might cut down the impairment of the intestinal epithelial barrier through protecting the TJs, thus preventing the activation of PPRRs and downregulating inflammatory mediators like TNF-α and IFN-γ, leading to the remission of colitis.

Despite various therapeutic measures against CD, even the most experienced clinician might have problems with treatment of many cases, especially those with complications. Helminthes might be beneficial in CD. The clarification of this mechanism contributes to finding out the key effective component, which may be a novel potential way of treating CD.

COMMENTS

Background

Crohn's disease (CD) is more prevalent in developed countries where sanitary conditions are better. Scientists have found that helminthes can protect mice from experimental colitis. The intestinal epithelial barrier plays an important role in the onset and progress of CD. Tight junctions (TJs), which form the pericellular barrier, are thought to be the primary determinant of mucosal permeability in the presence of an intact epithelium. The impaired TJs lead to an increase of exposure of intestine bacteria to submucosal proteins, like nucleotide-binding-oligomerization domains (NODs). Nucleotide-binding-oligomerization domain 2 (NOD2) has been found to exert antibacterial activity limiting survival of enteric bacteria after invasion. As one of the main responsible genes, NOD2 and its protein have been found to be upregulated in CD patients.

Research frontiers

TJs could be affected by a large amount of factors, such as cytokines, toxins and reactive oxygens. Many proteins and signal transduction systems participate in the regulation of TJs such as Rho/Ras-GTPases. Recently, it was reported that Notch-1 plays an important role in maintaining epithelial barrier function and promoting tight junction protein expression.

Innovations and breakthroughs

Till now various studies have proved that helminthes and their eggs do favor the relief of colitis both in experimental models and in some small scale clinical trials, but the rationale of how this works remains unknown. It was usually thought that helminthes might relieve colonic inflammation by a T cell immunity balance adjustment. Zhang and his co-workers investigated the impact of *S. japonicum* ova on trinitrobenzenesulfonic acid (TNBS)-induced colitis from a new point of view. They found that helminthes and their eggs might protect the intestine from inflammation by increasing the TJs and protecting the intestinal barrier function.

Applications

Despite various therapeutic measures against CD, even the most experienced clinician might have problems with treatment of many cases, especially those with complications. Helminthes and their eggs might be beneficial to treatment of CD. The clarification of this mechanism contributes to finding out the key effective component, which may be a novel potential method for treating CD.

Terminology

Intestinal bacterial translocation: The bacteria in the intestine migrate to the peripheral circulation and other organs (liver, spleen, *etc.*) through the portal vein due to the increased intestinal permeability; NOD2: One of the pattern pathogen recognition receptors which exists in the cytoplasm and recognize muramyl dipeptide of the bacteria. NOD2 is also the first predisposing gene of CD; Tight junctions: tight junctions form a network of close contacts between membranes of adjacent cells. They control the pericellular transport of ions, water, and solutes, and in addition they constitute a fence separating apical and basolateral membrane proteins.

Peer review

The paper is an intriguing and well designed paper which addresses a relevant issue.

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BRIEF ARTICLE

A special recurrent pattern in small hepatocellular carcinoma after treatment: Bile duct tumor thrombus formation

Qing-Yu Liu, Dong-Ming Lai, Chao Liu, Lei Zhang, Wei-Dong Zhang, Hai-Gang Li, Ming Gao

Qing-Yu Liu, Ming Gao, Department of Radiology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China
Dong-Ming Lai, Chao Liu, Lei Zhang, Department of General Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China
Wei-Dong Zhang, Department of Radiology, Cancer center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China
Hai-Gang Li, Department of Pathology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China
Author contributions: Liu QY and Lai DM contributed equally to this work; Liu QY and Lai DM study concept and design, drafting of the manuscript; Liu C, Zhang L and Li HG: analysis and interpretation of data; Zhang WD and Gao M acquisition of data.
Correspondence to: Qing-Yu Liu, PhD, Department of Radiology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107 Yan Jiang Xi Road, Guangzhou 510120, Guangdong Province, China. liu.qingyu@163.com
Telephone: +86-20-81332243 Fax: +86-20-81332702
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Abstract

AIM: To investigate the clinicopathologic features of bile duct tumor thrombus (BDTT) occurrence after treatment of primary small hepatocellular carcinoma (sHCC).

METHODS: A total of 423 patients with primary sHCC admitted to our hospital underwent surgical resection or local ablation. During follow-up, only six patients were hospitalized due to obstructive jaundice, which occurred 5-76 mo after initial treatment. The clinicopathologic features of these six patients were reviewed.

RESULTS: Six patients underwent hepatic resection (*n* = 5) or radio-frequency ablation (*n* = 1) due to primary sHCC. Five cases had an R1 resection margin, and one case had an ablative margin less than 5.0 mm. No vascular infiltration, microsatellites or bile duct/canaliculus affection was noted in the initial resected specimens. During the follow-up, imaging studies revealed a macroscopic BDTT extending to the common bile duct in all six patients. Four patients had a concomitant intrahepatic recurrent tumor. Surgical re-resection of intrahepatic recurrent tumors and removal of BDTTs (*n* = 4), BDTT removal through choledochotomy (*n* = 1), and conservative treatment (*n* = 1) was performed. Microscopic portal vein invasion was noted in three of the four resected specimens. All six patients died, with a mean survival of 11 mo after BDTT removal or conservative treatment.

CONCLUSION: BDTT occurrence is a rare, special recurrent pattern of primary sHCC. Patients with BDTTs extending to the common bile duct usually have an unfavorable prognosis even following aggressive surgery. Insufficient resection or ablative margins against primary sHCC may be a risk factor for BDTT development.

Key words: Small hepatocellular carcinoma; Recurrence; Bile ducts; Jaundice; Diagnosis

Peer reviewer: Clifford S Cho, MD, Assistant Professor, Department of Surgery, Section of Surgical Oncology, University of Wisconsin School of Medicine and Public Health, H4/724 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792-7375, United States

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies, especially in Asian countries[1]. With advanced imaging techniques, small HCC (sHCC) (≤ 3.0 cm) can be detected with increasing ease during screening in patients with chronic hepatitis or cirrhosis. Surgical resection and local ablation therapy (including percutaneous ethanol injection, percutaneous microwave coagulation, and percutaneous radio-frequency ablation) are effective against sHCC[2-4]. Although major progress has been made in the detection and treatment of sHCC, the efficacy of sHCC treatments remains undesirable: the 3- and 5-year disease-free survival rates are only 49% and 30%, respectively[5]. A major cause of the unfavorable prognosis of sHCC is the high incidence of postoperative recurrence. Kumada *et al*[6] reported that the cumulative 3- and 5-year recurrence rates were up to 64.5% and 76.1%, respectively. HCC recurrence is a leading cause of death that affects patients' long-term survival.

Extrahepatic and intrahepatic recurrences are two different sHCC recurrent patterns. Extrahepatic recurrence may extend to lymph nodes, peritoneum and extra-abdominal organs, while intrahepatic recurrence includes local recurrence, intrahepatic metastasis and multicentric carcinogenesis in the remnant liver[6-10]. However, bile duct tumor thrombus (BDTT) occurrence is rarely reported as a recurrent pattern of primary sHCC. Herein we present the clinicopathologic features of six patients with macroscopic BDTT occurrence after primary sHCC resection or local ablation. To the best of our knowledge, there is no such report in the English-language literature.

MATERIALS AND METHODS

Between March 2002 and June 2010, 423 patients with primary sHCC admitted to our hospital underwent surgical resection or local ablation. Patients were followed up every 1 or 3 mo after initial treatment. During follow-up, only six patients were hospitalized due to obstructive jaundice, which occurred at 5-76 mo (median, 8.5 mo) after the initial treatment. We retrospectively analyzed the clinicopathologic features of the six sHCC patients who developed a BDTT after treatment. The surgical margin was classified by an experienced pathologist (HG Li) as follows. R0 resection indicated complete removal of all tumors without microscopic tumor cells in the surgical margin. R1 resection indicated that the edges of the resection specimen showed microscopic tumor cells. R2 resection indicated that portions of tumor visible to the naked eye were not removed.

RESULTS

The sex, age, chief complaint, hepatitis markers, presence of cirrhosis, α-fetoprotein (AFP) level, location and size of sHCC, treatment, and pathological diagnosis at the initial hospital visit are summarized in Table 1 for the six patients with primary sHCC. The patients were all male, with a median age of 44 years. Three patients had epigastric pain, while the others were asymptomatic. Five patients were hepatitis B surface antigen (HBsAg)-positive, and one was HBsAg-negative. The serum AFP level was elevated in each patient, ranging from 25.0 to 725.3 ng/mL (normal, ≤ 10.0 ng/mL). The primary sHCCs were 1.5-2.7 cm in diameter. Five patients underwent hepatic resection, and pathological examination showed poorly differentiated (n = 3) or moderately differentiated HCC (n = 2). One patient (case 3) underwent a biopsy before radiofrequency ablation (RFA), and the tumor was moderately differentiated HCC. Five cases had an R1 resection margin, and one case had an ablative margin less than 5.0 mm. No vascular infiltration, microsatellites or bile duct/canaliculus invasion was noted in the initial resected specimens.

The clinicopathologic features of macroscopic BDTT occurrence after therapy are summarized in Table 2. Imaging examination [computed tomography (CT) or magnetic resonance imaging (MRI)] revealed macroscopic BDTT and concomitant intrahepatic recurrence in all six patients. Total bilirubin (TBil) and direct bilirubin (DBil) increased in all six patients (normal ranges, ≤ 24.0 μmol/L and ≤ 11.0 μmol/L, respectively). Serum AFP was increased in four patients and was normal in two patients. Four patients showed concomitant intrahepatic recurrent HCC lesions and BDTTs, while the other two revealed no intrahepatic recurrence. Surgical re-resection of the intrahepatic recurrent tumor and removal of the BDTT was performed in four patients, and BDTT removal *via* choledochotomy was performed in one patient. The other patient (case 6) underwent conservative treatment including percutaneous transhepatic cholangial drainage (PTCD) and transarterial chemoembolization (TACE) because of poor liver function. Histologic examination showed the recurrent tumor or BDTT to be poorly (n = 4) or moderately differentiated HCC (n = 1). All patients died with a mean survival of 11 mo after BDTT removal or conservative treatment.

Clinical course of each case is briefly presented below

Case 1: A 35-year-old male underwent partial resection of segments V and Ⅷ (Couinaud's nomenclature) due to a moderately differentiated sHCC measuring 2.7 cm in diameter (Figure 1A). At follow-up 6 mo after the operation, the patient was hospitalized due to jaundice. Serum AFP was normal, and CA19-9 was 350.2 U/mL (normal range, ≤ 35 U/mL). Abdominal sonography and dynamic contrast-enhanced MRI examination showed a recurrent tumor of 2.0 cm in diameter at the edge of the residual cavity (segment Ⅷ) after resection of the primary sHCC, and a BDTT extending to the proximal segment of the common bile duct was depicted simultaneously (Figures 1B and C). The BDTT and intrahepatic recurrence were directly connected, and

Table 1 Clinicopathologic characteristics of six cases of small hepatocellular carcinoma at the initial diagnosis

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Sex/age (yr)	M/35	M/57	M/45	M/50	M/38	M/41
Chief complaint	Epigastric pain	Asymptomatic	Asymptomatic	Epigastric pain	Asymptomatic	Epigastric pain
HBsAg/HCV-Ab	+/-	+/-	+/-	+/-	+/-	-/-
Liver cirrhosis	+	+	+	+	+	-
AFP (ng/mL)	203.5	25	283	226.1	578.3	725.3
sHCC location/size	Ⅷ/2.7 cm	Ⅴ/2.0 cm	Ⅵ/1.5 cm	Ⅳ/2.0 cm	Ⅴ/1.6 cm	Ⅵ/2.6 cm
Treatment	Partial segments Ⅴ and Ⅷ resection	Partial segment Ⅴ resection	RF	Left lobectomy	Partial segment Ⅴ resection	Segments Ⅵ and Ⅶ resection
Resection state	R1	R1	Ablative margin less than 5 mm	R1	R1	R1
Histologic differentiation	Moderate	Moderate	Moderate	Poor	Poor	Poor
Vascular infiltration	-	-		-	-	+
Tumor microsatellites	-	-		-	-	-
Bile duct or canaliculus invasion	-	-		-	-	-
Capsule presence	-	+		+	+	-

+: Positive; -: Negative. sHCC: Small hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; HCV-Ab: Hepatitis C antibody.

Table 2 Summary of clinical characteristics of bile duct tumor thrombus occurrence after treatment of small hepatocellular carcinoma

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Interval[1]	6 mo	47 mo	76 mo	5 mo	8 mo	9 mo
Chief complaint	Jaundice	Jaundice	Jaundice	Jaundice and epigastric pain	Jaundice	Jaundice
TBil/DBil (μmol/L)	47.1/28.8	365.0/283.3	286.0/206.5	35.7/27.3	146.0/87.2	480.0/360.3
AFP (ng/mL)	1.3	223.5	6.7	251.6	148.5	546
Recurrent tumor location/size	Ⅷ/2.0 cm	-	Ⅵ/3.2 cm	Ⅴ, Ⅷ/5.2 cm	Ⅵ/2.8 cm	-
BDTT location	RIHBD-CBD	CHD-CBD	RIHBD-CBD	RHD-CBD	RIHBD-CBD	RIHBD-CBD
Treatment	Hepatic resection and BDTT removal	BDTT removal	Hepatic resection and BDTT removal	Hepatic resection and BDTT removal	Hepatic resection and BDTT removal	PTCD and TACE
Resection state	R1		R1	R1	R0	
Histologic differentiation	Moderate	Poor	Poor	Poor	Poor	
Vascular infiltration	-		+	+	+	
Microsatellites	-		+	+	-	
Capsule presence	-		-	-	-	
Survival/prognosis	13 mo/dead	4 mo/dead	19 mo/dead	14 mo/dead	10 mo/dead	6 mo/dead

[1]Interval between BDTT occurrence and initial treatment. AFP: Alpha-fetoprotein; TBil: Total bilirubin; DBil: Direct bilirubin; RIHBD: Right intrahepatic bile duct; CHD: Common hepatic duct; CBD: Common bile duct; RHD: Right hepatic duct; +: Positive; -: Negative; BDTT: Bile duct tumor thrombus.

both exhibited typical HCC enhancement characteristics, such as early enhancement in the hepatic arterial phase with rapid wash-out of contrast agent in the portal vein phase on dynamic contrast-enhanced MRI. The patient underwent partial resection of hepatic segments (Ⅳ, Ⅷ) and removal of the BDTT *via* choledochotomy. Pathological examination showed the intrahepatic recurrent tumor and the BDTT were moderately differentiated HCC (Figure 1D). The patient died 13 mo after surgery due to recurrence.

Case 2: A 57-year-old male underwent partial resection of segment Ⅴ due to a moderately differentiated sHCC measuring 2.0 cm in diameter (Figure 2A). Forty-three months after the initial operation, the patient underwent resection of segment Ⅴ again for a recurrent tumor of 1.8 cm in diameter that was detected on dynamic contrast-enhanced CT, and the recurrent tumor was a moderately differentiated HCC with a microscopic BDTT observed pathologically. Four months after the second operation, the patient developed jaundice with increased AFP, and serum CA125 was 122.0 U/mL (normal range, ≤ 35 U/mL). Dynamic contrast-enhanced CT showed no intrahepatic recurrence; however, a BDTT extending through the common hepatic duct to the common bile duct was noted. The BDTT depicted a typical HCC enhancement pattern (Figure 2B). The patient underwent PTCD to relieve jaundice prior to the third operation (Figure 2C). During the third operation, the BDTT was tightly adherent to the common bile duct, so the BDTT was removed through resection of the common bile duct and bilio-enteric reconstruction was performed. The BDTT specimen was positive for hepatocyte on immunohistochemical staining, and cancer nest invasion into the bile duct wall was noted (Figure 2D). The patient died 4 mo after surgery due to hepatic failure.

Case 3: A 45-year-old male underwent RFA therapy for

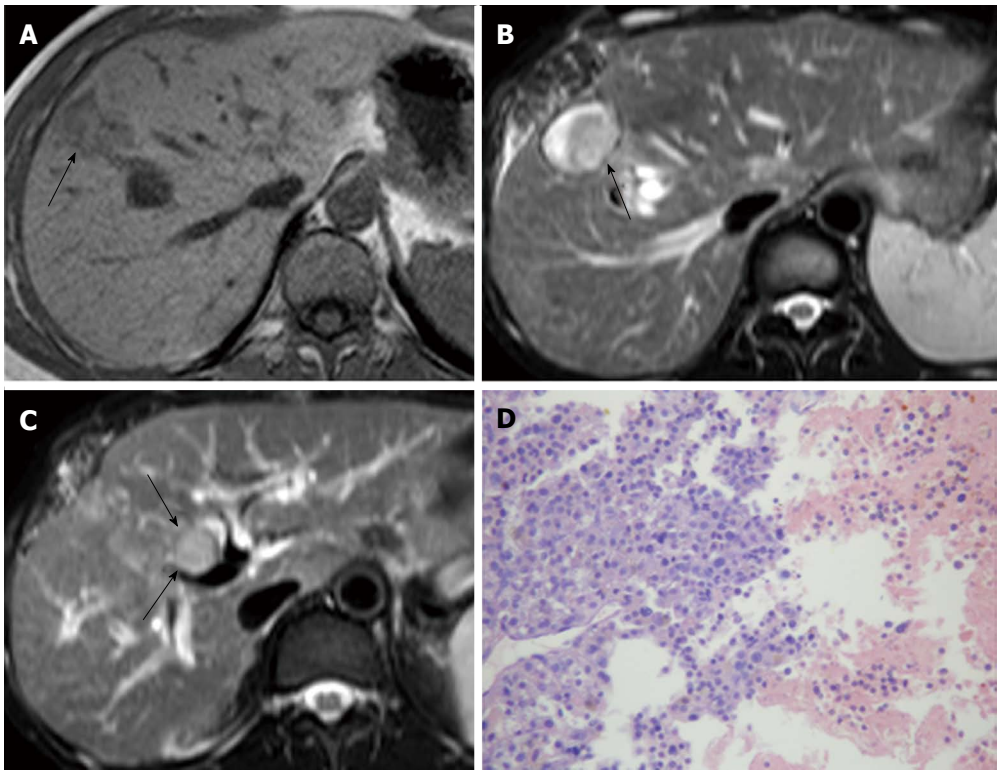

Figure 1 Small hepatocellular carcinoma in a 35-year-old male. A: T1-weighted magnetic resonance imaging (MRI) shows primary sHCC (arrow) in segment Ⅷ; B: T2-weighted MRI shows an intrahepatic recurrent tumor (arrow) at the margin of the residual cavity following resection of primary sHCC; C: A BDTT (arrows) extending through the intrahepatic bile duct into the common bile duct is depicted in a T2-weighted image; D: Histologically, intrahepatic recurrent tumor and BDTT are moderately differentiated HCC [hematoxylin and eosin (HE), × 100]. sHCC: Small hepatocellular carcinoma; BDTT: Bile duct tumor thrombus; HCC: Hepatocellular carcinoma.

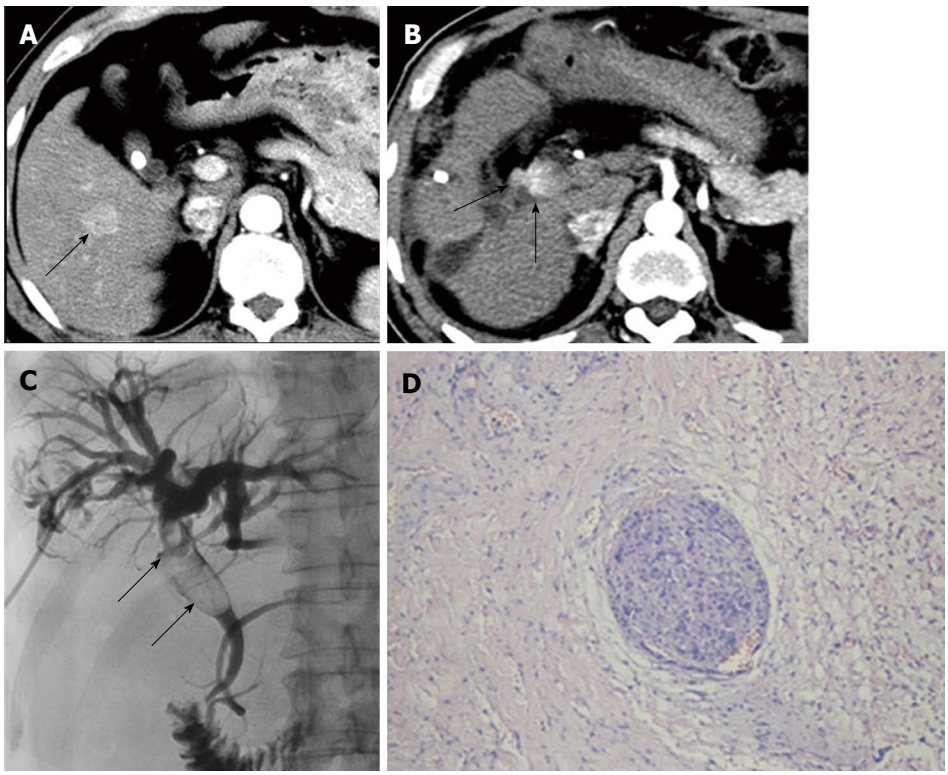

Figure 2 Small hepatocellular carcinoma in a 57-year-old male. A: Computed tomography (CT) shows primary sHCC (arrow) in segment Ⅴ at the hepatic arterial phase; B: Forty-seven months after the initial operation, CT reveals a BDTT (arrows) with characteristics of early enhancement at the hepatic arterial phase; C: Tumor thrombus (arrows) extending through the common hepatic duct into the common bile duct is demonstrated during PTCD; D: Cancer nest invasion into the bile duct wall is observed histologically (HE, × 100). sHCC: Small hepatocellular carcinoma; BDTT: Bile duct tumor thrombus; HE: Hematoxylin and eosin; PTCD: Percutaneous transhepatic cholangial drainage.

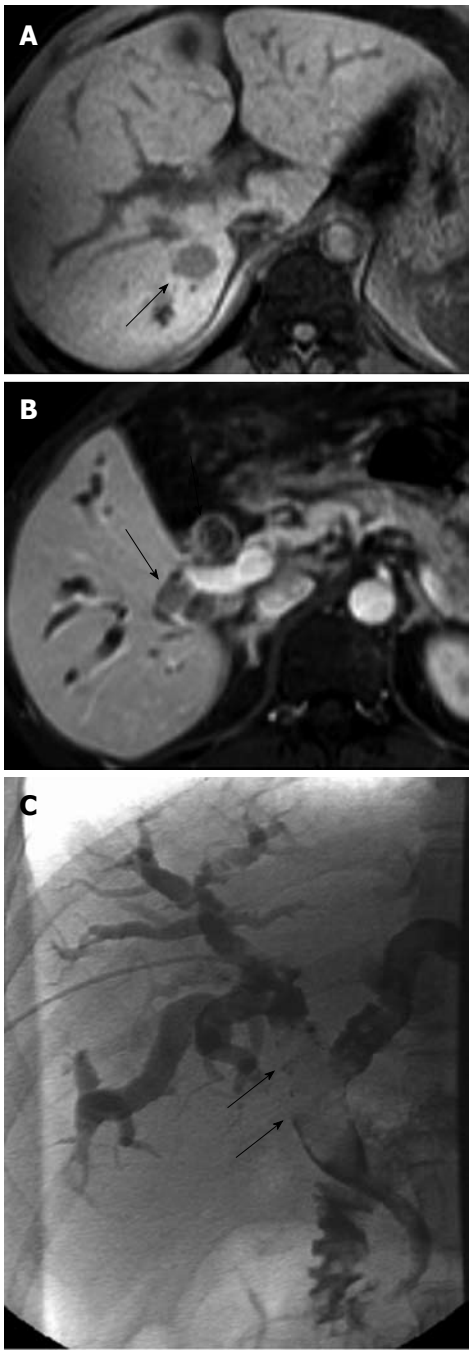

Figure 3 Small hepatocellular carcinoma in a 41-year-old male. A: T1-weighted MRI shows primary sHCC (arrow) in segment Ⅵ; B: A BDTT (arrows) in the right intrahepatic bile duct and the common bile duct is noted on contrast-enhanced T1-weighted MRI; C: Filling defect (arrows) in the biliary tree is depicted during percutaneous transhepatic cholangial drainage. MRI: Magnetic resonance imaging; sHCC: Small hepatocellular carcinoma; BDTT: Bile duct tumor thrombus.

a sHCC measuring 1.5 cm in diameter in segment Ⅵ. A recurrent tumor of 2.5 cm in diameter was found at the margin of the RFA site 17 mo after the initial RFA. He was again treated with RFA (ablative margin less than 5.0 mm). A recurrent tumor of 2.0 cm in diameter was again found at the margin of the RFA site 18 mo after second RFA therapy. Then TACE was performed. He developed jaundice 41 mo after TACE. Dynamic contrast-enhanced MR showed a BDTT extending through the right intrahepatic bile duct to the common bile duct, with typical HCC enhancement characteristics, and a recurrent tumor of 3.2 cm in diameter was found in segment Ⅵ. A plastic stent was placed for biliary decompression and drainage. He underwent resection of segment Ⅵ and removal of the BDTT *via* choledochotomy 2 mo after stent placement. Pathological examination showed that the intrahepatic recurrent tumor and the BDTT was poorly differentiated HCC. The patient died 19 mo after surgery due to intrahepatic recurrences and BDTT recurrence.

Case 4: A 50-year-old male underwent left lobectomy due to a poorly differentiated sHCC of 2.0 cm in diameter in segment Ⅳ. The patient had epigastric pain and jaundice 5 mo after the operation. Serum AFP was 251.6 ng/mL, and CA19-9 was 399.2 U/mL. MRI revealed a recurrent tumor of 5.2 cm in diameter near the resected hepatic stump (segments Ⅴ and Ⅷ) and a BDTT extending through the right hepatic duct to the common bile duct. He underwent resection of segments Ⅴ and Ⅷ and removal of the BDTT through resection of the common bile duct, followed by Roux-en-Y biliary-enteric anastomosis. The intrahepatic recurrence and the BDTT were diagnosed histologically as poorly differentiated HCC, and no bile duct wall invasion was noted. The patient died 14 mo after surgery due to HCC recurrence and distant metastasis.

Case 5: A 38-year-old male underwent partial resection of segment Ⅴ due to a poorly differentiated sHCC of 1.6 cm in diameter. The patient developed obstructive jaundice 8 mo after the operation. CT showed a recurrent tumor of 2.8 cm in diameter in segment Ⅵ and a BDTT extending through the right intrahepatic bile duct to the common bile duct. The patient received re-resection of segment Ⅵ and removal of the BDTT *via* choledochotomy. Histologically, the intrahepatic recurrence and the BDTT were diagnosed as poorly differentiated HCC. The patient died 10 mo after surgery due to distant metastasis.

Case 6: A 41-year-old male underwent resection of segment Ⅵ due to a poorly differentiated sHCC of 2.6 cm in diameter (Figure 3A). He developed jaundice 9 mo after the operation. MRI revealed a BDTT extending through the right intrahepatic bile duct to the common bile duct (Figure 3B); however, no intrahepatic recurrence was detected. A filling defect in the bile duct was noted during PTCD (Figure 3C). Because the patient had poor liver reserve and was classified in the Child-Pugh C subgroup, he underwent PTCD and TACE instead of surgery. The patient died 6 mo after PTCD and TACE due to multiple abscesses and hepatic failure.

DISCUSSION

HCC has a high frequency of portal vein or/and hepatic

vein invasion; however, bile duct invasion has been reported in only 0.79%-3% patients with primary HCC[11-13]. The size of primary HCC is not correlated with the occurrence of BDTT, and BDTTs may exist in patients with primary sHCC (≤ 3.0cm) and even in patients without detectable primary HCC[11,14,15]. However, BDTT occurrence after treatment of primary sHCC, which may be a new sHCC recurrence pattern, is very rare; only 1.4% (6/423) of patients with primary sHCC showed BDTTs recurrence in our study. Schmelzle *et al*[16] reported a case of extrahepatic growth of BDTT 3 mo after resection of multilocular HCC; however, the size of the primary multilocular HCC was not mentioned. The development of BDTT after primary sHCC treatment has not been reported in the English-language literature.

The mechanism for the emergence of HCC in the biliary system is still unknown, although the originally formed portal vein tumor thrombus may invade the bile duct *via* the peribiliary abundant vascular plexus or by direct invasion, or the primary HCC may rupture into the bile duct[13,17,18]. A wide ablative margin (more than 5 mm) or resection margin (2 cm) is the most important factor for local control of HCC[19,20]. In our cases, insufficient resection or ablative margins against sHCC seemed to be a risk factor for developing a BDTT after sHCC treatment because five cases had an R1 resection margin and one case had an ablative margin less than 5.0 mm. Residual microscopic tumor cells in resection margins may grow into the intrahepatic bile duct through injured bile duct walls due to hepatic resection or RFA and may extend distally to the left or right bile duct, or even the common bile duct. Interestingly, extrahepatic BDTT was reported by Schmelzle *et al*[16] in a patient with bile leakage after the resection of primary HCC, and BDTT development was supposed to be due to migration of cancer cells into the intrahepatic bile duct. Tumor thrombus in the portal vein was seen microscopically in the resected specimens in three cases (case 3, case 4 and case 5) in our group. A BDTT may develop *via* portal vein tumor thrombus invasion to the bile duct since the portal vein and bile duct are located in the same Galisson sheath[13,17,18].

Obstructive jaundice due to BDTT following sHCC treatment should be differentiated from jaundice that results from diffuse tumor recurrence infiltration, progressive hepatic failure, or hepatic hilum invasion due to intrahepatic recurrence, because the latter is often not curable, while the former may benefit from surgical resection[13]. Dynamic contrast-enhanced CT or MRI is valuable to detect BDTT and possible concomitant intrahepatic recurrence. As the BDTT directly connects to the intrahepatic tumor and is vascularized by the same blood supply with the intrahepatic tumor, they both show typical HCC enhancement characteristics in dynamic contrast-enhanced CT or MRI, including early enhancement in the hepatic arterial phase and rapid wash-out of contrast agent in the portal vein phase[14,21,22]. Magnetic resonance cholangiopancreatography is valuable for identifying the location and extent of a BDTT[23]. It is not necessary to diagnose a BDTT using invasive methods in most cases, such as endoscopic retrograde cholangio-pancreatography (ERCP) and PTCD because the obstruction site or extent depicted on ERCP or PTCD is not a specific finding for the diagnosis of BDTT, and cholangitis or pancreatitis may be easily induced by these invasive methods. Obstructive jaundice in a patient with a BDTT is marked by a predominant increase in DBil, making it distinct from jaundice that results from cirrhosis and active hepatitis[13]. The serum AFP level is of limited value in the diagnosis of BDTT occurrence because AFP reflects not only tumor cell proliferation but also the activity and severity of hepatitis[24].

Obstructive jaundice would not be regarded as a contraindication of surgery[13]. The goal of therapy is removal of the BDTT and intrahepatic recurrence. A BDTT is often easy to remove, as it is not tightly adhesive to the bile duct wall. Removal of BDTT *via* choledochotomy and re-resection of intrahepatic recurrence may be considered in patients with adequate hepatic function[13,25]. Removal of a BDTT through resection of the extrahepatic bile duct followed by biliary-enteric anastomosis should be adopted for cases with a tumor thrombus that is tightly adhesive to the extrahepatic bile duct wall, as it indicates that the tumor thrombus has invaded the bile duct[13]. Bile duct drainage, such as endoscopic nasobiliary drainage, plastic stent placement, PTCD, or PTCD plus stent placement, should be practiced to relieve jaundice in inoperable patients, such as patients with intrahepatic recurrence at unresectable sites, multiple recurrent lesions or inadequate hepatic function. After bile duct drainage is performed, TACE may be performed to inhibit the vascularization of the recurrent tumor and the BDTT and thereby control tumor growth or even prevent fatal bile duct bleeding caused by the BDTT[13,26]. However, TACE without prior bile duct drainage is highly risky, as it may induce progressive hepatic failure[13]. Some researchers have made efforts in using liver transplant therapy to treat primary HCC associated with BDTT, but in patients with BDTT occurrence after sHCC treatment this technique has not been reported[13,27].

Many recent studies have shown that there is no significant difference in survival between HCC patients with BDTT and those without BDTT following appropriate preoperative management and aggressive surgery. The 1-, 3-, and 5-year survival rates for such patients have reached 73.3%-93.2%, 40%-56.0% and 24%-28%, respectively[13,25,28,29]. As BDTT development could lead to further deterioration of liver function, early detection and early relief of biliary system obstruction caused by tumor thrombus is an important factor to improve survival[30]. During admission, BDTTs extended to the common bile duct in all six patients in our study, which may be a negative factor leading to poor prognosis (with a mean survival time of only 11 mo).

In conclusion, BDTT occurrence is a rare, special

recurrent pattern after treatment of primary sHCC. Insufficient resection or ablative margins against primary sHCC may be a risk factor for BDTT development. Patients with BDTTs extending to the common bile duct usually have unfavorable prognosis even following aggressive surgery. Early removal of BDTTs and possible concomitant intrahepatic recurrences is crucial to prolong the survival of patients.

COMMENTS

Background

Recurrence is an important unfavorable prognostic factor in small hepatocellular carcinoma (sHCC) after surgical resection or local ablation therapy. Two different sHCC recurrent patterns have been reported. Extrahepatic recurrence may extend to lymph nodes, peritoneum and extra-abdominal organs, while intrahepatic recurrence includes local recurrence, intrahepatic metastasis and multicentric carcinogenesis in the remnant liver. However, bile duct tumor thrombus (BDTT) occurrence is rarely reported as a recurrent pattern of primary sHCC.

Research frontiers

Bile duct invasion by primary HCC is rare, and its pathogenesis is still not completely understood. A BDTT may exist in patients with primary sHCC ($\leqslant$ 3.0cm) and even in patients without detectable primary HCC. The clinicopathologic features of primary HCC with BDTT have been clarified in the literature. However, BDTT occurrence after sHCC treatment has not been reported in the English-language literature, and its clinicopathologic features have not been specified.

Innovations and breakthroughs

Six patients with BDTT occurrence after primary sHCC treatment were reported in this study, and their clinicopathologic features were reviewed. This study showed that BDTT occurrence is a rare recurrent pattern of sHCC (1.4%), and insufficient resection or ablative margins against sHCC may be a risk factor for BDTT development. Patients with BDTTs extending to the common bile duct usually have unfavorable prognosis even following aggressive surgery.

Applications

With a better understanding of the clinicopathologic features of BDTT occurrence after sHCC treatment, sufficient wide resection or ablative margins against sHCC should be performed to avoid BDTT recurrence. Early detection and removal of BDTT may be crucial to prolong the survival of patients. Further studies are needed to clarify the mechanism of BDTT recurrence.

Terminology

BDTT formation can occur in HCC, combined hepatocellular and cholangiocellular carcinoma and liver metastasis. The mechanism for the emergence of a tumor thrombus in the biliary system is still uncertain. Patients with BDTT development are usually admitted due to obstructive jaundice, and the prognosis is generally dismal.

Peer review

In this report, Liu and colleagues provide a brief report on six patients who developed intrabiliary tumor thrombi after previous therapy for hepatocellular carcinoma. This is a useful series that demonstrates that the onset of intrabiliary recurrences portends a very poor prognostic outlook. In fact, based on the survival outcomes described following treatment interventions, it could be argued that aggressive surgical intervention should not be undertaken for patients presenting with this unique pattern of recurrent disease.

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BRIEF ARTICLE

Effects of CpG-ODNs on phenotype and function of monocyte-derived dendritic cells in chronic hepatitis B

Xiao-Xing Xiang, Xia-Qiu Zhou, Jun-Xue Wang, Qing Xie, Xiong Cai, Hong Yu, Hui-Juan Zhou

Xiao-Xing Xiang, Department of Gastroenterology, Northern Jiangsu People's Hospital, Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Xia-Qiu Zhou, Qing Xie, Hong Yu, Hui-Juan Zhou, Department of Infectious Disease, Rui jin Hospital, Medical College of Shanghai Jiao Tong University, Shanghai 200000, China
Jun-Xue Wang, Xiong Cai, Department of Infectious Disease, Changzheng Hospital, Second Military Medical University, Shanghai 200000, China
Author contributions: Xiang XX designed and performed the majority of the research, and wrote the paper; Zhou XQ, Xie Q, and Cai X contributed to the design, analysis, and financial support of the research; Wang JX, Yu H, and Zhou HJ performed some of the experiments, provided materials and reagents, and were involved in editing the manuscript.
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Correspondence to: Xiao-Xing Xiang, MD, Associate Professor of medicine, Department of Gastroenterology, Northern Jiangsu People's Hospital, Medical College of Yangzhou University, 98 West Nantong Road of Yangzhou city, Yangzhou 225001, Jiangsu Province, China. xiangxx1965@yahoo.com.cn
Telephone: +86-514-87370315 Fax: +86-514-87937406
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Abstract

AIM: To study the effects of synthetic nonmethylated CpG-containing oligodeoxynucleotides (CpG-ODNs), either alone or combined with recombinant Hepatitis B surface antigen (HBsAg) polypeptide, on the phenotype, function, and intracellular signaling pathways of monocyte-derived dendritic cells (DCs) in patients with chronic hepatitis B (CHB).

METHODS: Peripheral blood monocytes isolated from CHB patients and healthy volunteers were induced to be dendritic cells by recombinant human granulocyte-monocyte colony stimulating factor and interleukin-4. The DCs were then treated with CpG-ODNs, CpG-ODNs/HBsAg, or tumor necrosis factor (TNF)-α for 18 h. The expression of surface molecules including HLA-DR, CD86, and CD1a in DCs were detected by flow cytometry, and the expression of signal transducers and activators of transcription (STAT1, 3, 4, 5, 6) and suppressors of cell signaling (SOCS1, 3) were determined by Western blotting assay. In addition, the capacity of DCs to stimulate allogeneic T lymphocytes and the amount of IL-12p70 released from DCs were measured.

RESULTS: In the DCs derived from patients with CHB, treatment with TNF-α, CpG-ODNs, or CpG-ODNs/HBsAg, as compared to the vector control, significantly increased the expression of HLA-DR, stimulated the release of IL-12p70, and enhanced the capacity of DCs to stimulate allogenic T lymphocytes. The expressions of STAT1/4/6 and SOCS1/3, but not STAT3/5, were upregulated by TNF-α, CpG-ODNs, and CpG-ODNs/HBsAg. In addition, the expression of CD1a was upregulated only in the presence of both CpG-ODNs and HBsAg.

CONCLUSION: The treatment with CpG-ODNs, either alone or combined with HBsAg, has a remarkable stimulatory effect on the impaired phenotype and function of DCs in CHB, possibly by regulating the expression of STAT1, 4, 6 and SOCS1, 3.

Key words: Chronic hepatitis B; Dendritic cell; CpG oligodeoxynucleotides; Hepatitis B surface antigen; Signal transduction

Peer reviewers: Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico Ⅱ University Medical School, Via S Pansini, 5, Naples 80131, Italy; Mihaela Petrova, MD, PhD, Dr., Clinic of Gastroenterology, Medical Institute, Ministry of Interior, Sofia 1606, Bulgaria

Xiang XX, Zhou XQ, Wang JX, Xie Q, Cai X, Yu H, Zhou HJ. Effects of CpG-ODNs on phenotype and function of monocyte-derived dendritic cells in chronic hepatitis B. *World J Gastroenterol* 2011; 17(43): 4825-4830 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i43/4825.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i43.4825

INTRODUCTION

Peripheral blood monocyte-derived dendritic cells (MoDCs) from patients with chronic hepatitis B (CHB) are abnormal in morphology and function[1,2]. However, the molecular mechanism of the MoDCs' dysfunction in CHB remains unclear. This study aimed to evaluate the effect of synthetic nonmethylated CpG-containing oligodeoxynucleotides (CpG-ODNs), combined with recombinant hepatitis B surface antigen (HBsAg) polypeptide, on the phenotype and function of monocyte-derived dendritic cells (DCs) in patients with chronic hepatitis B. In addition, we attempted to shed light on the involvement of intracellular signaling molecules, including signal transducers and activators of transcription (STAT1, 3, 4, 5, 6) and suppressors of cell signaling (SOCS1, 3) in the MoDCs dysfunction under the condition of CHB[3,4,5].

MATERIALS AND METHODS

Patients

Twenty-four patients with CHB [23 males and 1 females, mean age (33.4 ± 11.8) years] were enrolled in this study. Diagnosis of CHB was made by clinical findings: elevated serum alanine aminotransferase (ALT) levels for more than six months and the presence of HBsAg, HBeAg, anti-HBc, and HBV DNA (> 1 × 106 copies/mL). Among all patients, other hepatitis-related virus infection such as hepatitis A virus, hepatitis C virus, hepatitis E virus, and transfusion transmitted virus were excluded. None of the enrolled patients had received treatment with interferon, thymosin, thymus extracts, or lamivudine. Thirteen healthy volunteers were selected as control subjects with the average age of (31.7 ± 8.7) years.

Reagents

The recombinant human granulocyte-macrophage colony-stimulating factor (hGM-CSF), recombinant human interleukin-4 (hIL-4), and recombinant human tumor necrosis factor-α (hTNF-α) were purchased from Peprotech EC Ltd. (London, United Kingdom). The recombinant HBsAg was purchased from ViroStat (Portland, United States). Fluorescent isothiocyanate (FITC) anti-human CD1a, Phycoerythrocin (PE)-Cy5 anti-human CD86, Phycoerythrocin (PE), and anti-human HLA-DR were purchased from Amersham Pharmacia Biotech Inc (United States). MACS CD14 MicroBeads and MS Separation Columns were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Human T cell enrichment columns were purchased from R and D Systems, Inc. The human IL-12p70 ELISA kit was purchased from Jingmei Biotech (Shenzhen, China). The mouse monoclonal antibody against beta actin was purchased from Abcam Ltd (Cambridge, United Kingdom). The mouse monoclonal antibody against phosphorylated STAT1/3 and the rabbit polyclonal antibody against phosphorylated STAT4/5/6 were purchased from Cell Signaling Technology (Beverly, MA, United States). The rabbit polyclonal antibodies for SOCS1/3 were purchased from Assay Designs Ltd (Michigan, United States). NE-PER nuclear and cytoplasmic extraction reagents were purchased from Pierce Biotechnology (Rockford, United States). 3H-TdR (2 mci/mL) was purchased from Isotope Institute of Atomic Energy Academy of China. Mitomycin-C and HISTOPAQUE-1077 were purchased from SIGMA (ST. LOUIS, MO, United States). The CpG-containing phosphorothioated oligodeoxynucleotides (CpG-ODNs) was custom synthesized by Shanghai Sangon Biological Engineering Technology and Service Ltd, based on the report by Merad *et al*[4].

Cell preparation and culture

The DCs were prepared following a method described by Romani *et al*[6], with a few modifications. Briefly, peripheral blood mononuclear cells (PBMC) were separated from the peripheral blood of patients and volunteers by HISTOPAQUE-1.077 density gradient centrifugation, and the monocytes were further selected from PBMCs using CD14 MicroBeads. The monocytes were induced to differentiate into DCs by combined treatment with hGM-CSF (1000 U/mL), hIL-4 (500 U/mL), and hTNF-α (1000 U/mL) for five or seven days according to requirement. Dendritic cells were identified by inverted phase contrast microscopy, transmission electron microscopy and flow cytometry. The DCs were then collected for subsequent experiments.

Treatment of dendritic cells with different immune adjuvants

After 5-d culture, the immature DCs were collected and dispensed into a culture plate with 24 wells (1×10^6 cells/well). CpG-ODNs (10 μg/mL), CpG-ODNs (10 μg/mL) + HBsAg (10 μg/mL) or TNF-α (1000 U/mL) in phosphate buffer solution (PBS, 50 μL/mL) were added[6,7]. The DCs derived from volunteers were only treated with CpG-ODNs (10 μg/mL). All cells were cultured at 37 ℃ in a 5% CO_2 incubator for 18 h. The cells and culture supernatant were then separately collected for further assays.

Detection of surface molecules and cytokine release of dendritic cells

The expression rates of surface molecules, including HLA-DR-PE, CD86-(PE)-Cy5, and CD1a-FITC in DCs were analyzed by flow cytometry (FCM), and isotope control was performed as previously described[2]. The IL-12p70 content of the culture supernatant was detected by an ELISA kit according to the manufacture's

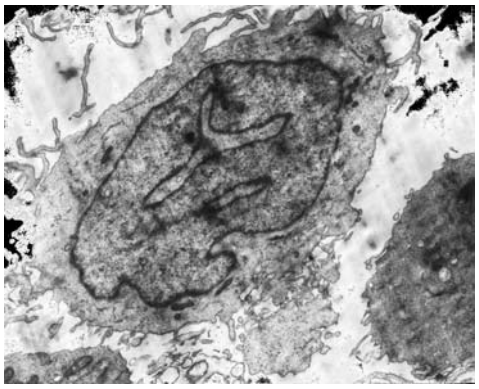

Figure 1 A representative image of monocyte-derive dendritic cells under transmission electron microscope after 7-d induction with recombinant granulocyte-macrophage colony-stimulating factor and interleukin-4 (magnification × 7500). Dendritic cells were irregular in form, and abundant in extended beard-like prick and mitochondria in cytoplasm.

instructions. The average optical density (OD) of three replicates was calculated.

Effect of dendritic cells treated with different immune adjuvants on proliferation of allogeneic T lymphocytes

The effect stimulation of DCs on T-cell proliferation was determined using a method described by Li *et al*[2]. T lymphocytes, separated by T cell separation columns, were used as response cells. The DCs exposed to different immune adjuvants for five days were further treated with mitomycin C (25 μg/mL) for 45 min and used as stimulating cells. The response cells and stimulating cells were co-cultured for 96 h at the ratio of 20:1, and 3H-TdR was added 18 h before the end of culture. The radioactivity (counts per minute, cpm), reflecting T-cell proliferation, was detected by a liquid scintillation counter and expressed as the mean value of five replicates.

Western blotting assay

Cytosolic proteins were extracted from DCs treated with different immune adjuvants, separated by 10% SDS-PAGE gel electrophoresis (30 μg protein per lane), and transferred onto a nitrocellulose membrane. The membrane was incubated in blocking buffer for 2 h at room temperature, a probed with a specific primary antibody (1:1000, 4 ℃, overnight), followed by the secondary antibody conjugated with horseradish peroxidase, and then developed by enhanced chemiluminescence (ECL) staining. All experiments were repeated three times and the protein signal was quantified by densitometry with a PrecisionScanLTX scanning apparatus, and normalized to β-actin as an internal reference.

Statistical analysis

The experimental data in this study were expressed as mean ± SD and analyzed by SAS6.12 statistical software. One-way ANOVA, followed by the Student-Newman-Keuls test, was used to compare the numerical data between multiple groups. A P value of < 0.05 was considered to be statistically significant.

Table 1 Effects of different immunologic adjuvants on expression levels of surface molecules (%) on DCs derived from blood monocytes of patients with chronic hepatitis B

Surface molecules	CpG	CpG + HBsAg	TNF-α	HBsAg	PBS
HLA-DR	93.8 ± 3.7[a]	95.1 ± 4.5[a]	94.8 ± 3.1	87.6 ± 4.3[a]	70.4 ± 3.9
CD86	83.7 ± 5.3	81.5 ± 3.2	82.3 ± 2.7	83.2 ± 3.6	80.2 ± 4.2
CD1a	12.0 ± 0.9	44.5 ± 1.2[a]	12.7 ± 1.9	15.0 ± 2.5	13.6 ± 0.8

DCs: Dendritic cells; CHB: Chronic hepatitis B; CpG-ODNs: Synthetic nonmethylated CpG-containing oligodeoxynucleotide; HBsAg: Hepatitis B surface antigen; PBS: Phosphate buffer solution; TNF: Tumor necrosis factor. HLA-DR, CD86 and CD1a are the specific surface molecules of DCs; CpG, CpG + HBsAg, TNF-α and HBsAg are the different adjuvants on DCs. [a]$P < 0.05$ using Student's t test, represents the stimulating effects of different immunologic adjuvants in comparison to control group (PBS).

RESULTS

In vitro development and identification of dendritic cells

DCs were developed from $CD14^+$ blood monocytes. Monocytes of high purity were separated from the peripheral blood using CD14 MicroBeads. After treatment with recombinant GM-CSF 1000 U/mL and IL-4500 U/mL, a large number of cell clusters appeared on day three, and most cells were suspended on day six to seven in irregular form. Abundant extended beard-like pricks and mitochondria were observed under inverted phase contrast microscope and transmission electron microscope (Figure 1). The purity of DCs was more than 85% according to the numbers of cells positive for HLA-DR, CD86 and CD1a detected by FCM. DCs treated with TNF-α (1000 U/mL) showed strong ability to stimulate the proliferation of allogeneic T lymphocytes (Figure 1).

Effect of CpG-ODN on the phenotype of dendritic cells

The number of DCs positive for HLA-DR, CD86, and CD1a after induction with GM-CSF and IL-4 were 70.4%, 81.3%, and 13.67% for CHB ($n = 6$), and were 83.2%, 80.6%, and 22.4% for healthy volunteers ($n = 3$), respectively. The number of cells positive for HLA-DR and CD1a were significantly lower in CHB than those in healthy volunteers ($P < 0.05$). Treatment with TNF-α, CpG-ODNs, or CpG-ODNs plus HBsAg, beginning at day 5 and continuing for 18 h, increased the numbers of cells positive for HLA-DR and CD86 by about 10%, and the rate of CD1a by about 20% in DCs from healthy volunteers ($n = 3$). By contrast, in DCs from the patients with CHB ($n = 6$), only the rates of HLA-DR were increased by those treatments (94.7%, 93.8%, and 95.14% for TNF-α, CpG-ODNs, and CpG-ODNs plus HBsAg, respectively), except that CpG-ODNs plus HBsAg significantly raised the positive rate of CD1a from 13.67% to 44.52% (Table 1).

Effects of CpG-ODNs on the amount of IL-12p70 secreted by dendritic cells

In DCs derived from CHB patients, treatment with CpG-ODNs and TNF-α, either alone or combined with HBsAg, resulted in significant increases of supernatant IL-12p70

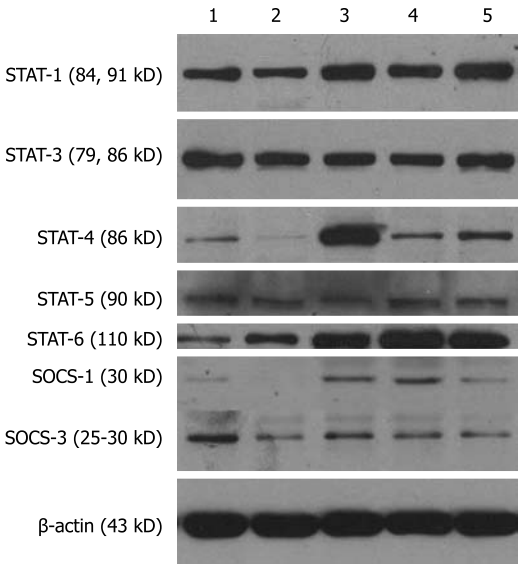

Figure 2 Expression levels of cytoplasmic signal transducers and activators of transcription and suppressors of cell signaling in dendritic cells stimulated with different immunologic adjuvants. 1: Dendritic cells (DCs) from healthy subjects stimulated with synthetic nonmethylated CpG-containing oligodeoxynucleotides (CpG-ODNs); 2-5: DCs from patients with chronic hepatitis B (CHB) stimulated respectively with PBS, CpG-ODNs, TNF-α and CpG-ODNs plus HBsAg. CpG, CpG + HBsAg, TNF-α, and HBsAg are the different adjuvants on DCs. STAT1, 3, 4, 5 and SOCS1, 3 respectively represents the expressing levels of the intracellular signaling molecules including signal transducers and activators of transcription-1, 3, 4, 5 and suppressors of cell signaling-1, 3 in CHB-derived DCs. HBsAg: Hepatitis B surface antigen; TNF: Tumor necrosis factor.

level, suggesting enhanced release of IL-12p70. However, the supernatant levels of IL-12p70 did not increase upon the treatment with HBsAg or PBS (Table 2).

Effects of CpG-ODN-induced dendritic cells on the proliferating response of allogeneic T lymphocyte

The radioactive counts (cpm) representing allogeneic T lymphocyte proliferation in a mixed lymphocyte reaction (MLR) for normal DCs was 2290 ± 1861.1. In contrast, the cpm for CHB-derived DCs was 487.4 ± 216.3, significantly lower than that for the normal DCs ($P < 0.05$). Treatment with CpG-ODNs, CpG-ODNs plus HBsAg, or TNF-α greatly increased the cpm for CHB-derived DCs to 5407 ± 2359, 5831 ± 2815.4, and 9947 ± 3395.2, respectively ($P < 0.05$). This suggested that CpG-ODNs, either alone or combined with HBsAg, enhanced the ability of CHB-derived DCs to present antigen, similar to the effect of TNF-α.

Effects of CpG-ODN on the expression of cytosolic STAT and SOCS proteins in DCs

As shown by the Western blotting experiments (Figure 2), the phosphorylation signal of STAT1, STAT3, and STAT6 in mature DC cytoplasm was relatively strong, while the expression intensities of STAT4, STAT5, SOCS1, and SOCS3 were relatively weak. Compared with mature DCs, the expression intensities of STAT1, STAT3, and STAT5 in immature DC cytoplasm (Lane 2, PBS group) were weak, the expression intensity of STAT6 was strong, the expressions of STAT4 and SOCS1 was almost absent, and the expression of SOCS3 was also weak. The immune adjuvants, CpG-ODNs, CpG-ODNs plus HBsAg and TNF-α, enhanced the expression of cytosolic STAT1, STAT4, STAT6, and SOCS1 in DCs derived from CHB. In particular, the protein band intensities of STAT4 and SOCS1 in CHB-derived DCs stimulated with CpG-ODNs were increased by 12 times and five times, respectively. However, the expression of STAT3, STAT5, and SOCS3 did not change between mature DCs and immature DCs (Lane 2, PBS group). In addition, the addition of HBsAg to CpG-ODNs did not alter the effects of CpG-ODNs on the expression of the above signal proteins (Figure 2).

Table 2 Supernatant IL-12p70 levels (ng/L) before and after the treatment with different immunologic adjuvants in dendritic cells derived from blood monocytes of patients with chronic hepatitis B

	CpG-ODN	TNF-α	CpG-ODN + HBsAg	TNF-α + HBsAg	HBsAg	PBS
Before treatment	43.1 ± 40.9[a]	58.5 ± 52.2[a]	31.8 ± 65.4	20.4 ± 8.3	21.5 ± 14.2	36.3 ± 27.6
After treatment	103.6 ± 106.6[a]	98.70 ± 76.5[a]	81.4 ± 70.9	83.9 ± 76.5	13.9 ± 9.0	35.5 ± 31.8

[a]$P < 0.05$ *vs* PBS. DCs: Dendritic cells; HBsAg: Hepatitis B surface antigen; CpG-ODN: Synthetic nonmethylated CpG-containing oligodeoxynucleotide; PBS: Phosphate buffer solution; TNF: Tumor necrosis factor. CpG, CpG + HBsAg, TNF-α, HBsAg and TNF-α + HBsAg are the different adjuvants on DCs. [a]$P < 0.05$ using Student's *t* test, representing supernatant IL-12p70 levels of DCs from CHB before and after the treatment with different immunologic adjuvants in comparison to control group (PBS).

DISCUSSION

Recent studies indicate that DCs derived from the peripheral blood of patients with CHB have abnormal phenotypes and functions, such as downregulated expression of surface molecules (HLA-DR, CD86, CD1a, CD11c and ICAM-1), reduced ability to stimulate the proliferation of allogeneic T lymphocytes, reduced secretion of IL-12p70 and IFNγ, and disorder in the proportion of DC1/DC2[1,2]. However, the molecular immunological mechanism of DC dysfunction in CHB is unclear, and immunotherapy aimed to improve the dysfunction of DC is in the initial stages. Bacterial lipopolysaccharide (LPS), TNF-α, and phytohemagglutinin (PHA) are able to stimulate maturation of DCs; however, as inflammatory compounds, their application is limited in *in vitro* research and animal experiments. As reported recently, bacterial genomic DNA containing a non-methylated CpG motif is able to activate antigen-presenting cells (APC), and preferentially induce a Th1-dominating immune response[7]. An artificially synthesized non-methylated CpG-containing oligodeoxynucleotides (CpG-ODNs), similar to LPS and TNF-α, is capable of inducing maturation of DCs derived from mouse bone marrow and human peripheral blood monocyte and en-

hances their antigen-presenting ability[6,8-11]. CpG-ODNs administered together with a hemopoietic growth factor or an immunomodulator was shown to improve the function of DCs in experimental tumor models and reinforce the immune ability of scavenging tumor[4,5]. As a highly effective Th1-type inducing adjuvant, a phase I clinical trial of CpG-ODNs has been carried out, but little research data exists to define the effects and mechanisms of CpG-ODNs on the proliferation and maturation of peripheral blood DCs in CHB.

It is reported that the flanking sequences of non-methylated CpG-containing CpG-ODNs with highest stimulating activity differ between mice and humans. The following CpG-ODNs sequence was adopted in this study, TCCATGTCGTTCCTGTCGTT, which has the highest immune stimulating activity in humans. Our data showed that CpG-ODNs, at a dose of 10 μg/mL, significantly induced the proliferation and maturation of peripheral blood DCs from CHB over a 5-d culture period. FCM detection showed that the HLA-DR expression on DCs treated with CpG-ODNs was significantly upregulated, the amount of IL-12p70 secreted by DC was also increased, and the ability of DCs to stimulate the proliferation of allogeneic T lymphocytes was enhanced. The effect of CpG-ODNs on the proliferation of DCs was similar to that of TNF-α. However, neither TNF-α nor CpG-ODNs increased the expression of CD1a, a specific marker of mature DCs. Interestingly, the combination of CpG-ODN and the specific antigen HBsAg of HBV significantly increased the number of cells positive for CD1a among DCs from CHB. The above data indicated that combined stimulation with a Th1-type immune adjuvant and a specific antigen such as HBsAg is essential for DCs to obtain ideal antigen-presenting ability against HBV.

The STAT and SOCS signaling pathway are an important pair of positive and negative feedback systems for the intracellular signal transduction of cytokines[12,13], and play crucial roles in the development of chronic hepatitis B[14-17]. The present study showed that, under the induction with GM-CSF and IL-4, the expression levels of STAT4, SOCS1, and SOCS3 in CHB-derived DCs were very low, the expression levels of STAT3 and STAT5 were similar to those of normal mature DCs, the expression of STAT1 was somewhat lower than that of normal control DCs, and the expression of STAT6 was more enhanced than that of control mature DCs. It is reported that a variety of cytokines such as IFNα/β/γ, IL-4, IL-12, IL-10, IL-6, and GM-CSF are dependent on the STAT and SOCS pathways to initiate a cascade of phosphorylation events that lead to changed expression of target genes[18,19]. These data suggest the complexity of cytokine signaling network in DCs from CHB, which might be an important molecular immunological mechanism for the differences between individuals in terms of the short-term and long-term therapeutic effects of antiviral drugs, such as interferon and lamivudine. Our data suggested that CpG-ODNs, TNF-α, and CpG-ODNs plus HBsAg increased, to some extent, the expression levels of STAT1, STAT4, STAT6, SOCS1, and SOCS3 in the cytoplasm of DCs from CHB. The enhancement effect of CpG-ODNs on the expression of STAT4 was particularly dramatic, but the expression of STAT3 and STAT5 showed no differences between immature and mature DCs, indicating that the actions of CpG-ODNs on the STAT and SOCS pathways in DCs might be a crucial mechanism of CpG-ODNs to promote proliferation and maturation and enhance the antigen-presenting function of DCs. It is CpG-ODNs, not other nucleoside analogs, that functions as a Th1-type immune adjuvant, which has been clarified many studies[3-5,7-8,20]. Thus, it is not unexpected that CpG-ODNs improved the partial function of DCs derived from peripheral blood monocytes of CHB in vitro. What is the exact mechanism of the above effects, besides those on the STAT and SOCS pathways, and whether there are expected effects on DCs from CHB *in vivo* in view of complicated immunological pathogenesis of CHB, remain important questions. In the subsequent research, our study group will further investigate the clinical/therapeutic consequences of this study and will uncover the exact mechanisms of DC dysfunction in CHB, with a view to developing novel immunological treatments for CHB[16,21,22].

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We are heartily thankful to Mr. Da-Kai Xiao, Mr. Dong Li and Mrs. Yi Zhang of Shanghai Institute of Hematology and Digestive Surgery for their selfless help with the experiments.

COMMENTS

Background

It has been demonstrated that the dendritic cells from patients with chronic hepatitis B (CHB) are abnormal in morphology and function, which is closely related to the immunologic pathogenesis of CHB. However, the molecular mechanism of dendritic cell (DC) dysfunction in CHB and how to regulate it remains unclear.

Research frontiers

As it is known, artificially synthesized non-methylated CpG containing oligodeoxynucleotides (CpG-ODNs), similar to lipopolysaccharide and tumor necrosis factor-α, is capable of inducing DCs derived from mouse bone marrow and human peripheral blood monocyte to mature and enhancing its antigen-presenting ability. However, few research data exist to define the effects and mechanisms of CpG-ODNs on the proliferation and maturation of peripheral blood DCs in CHB.

Innovations and breakthroughs

The treatment of CpG-ODNs either alone or combined with Hepatitis B surface antigen (HBsAg) has a remarkable stimulatory effect on the impaired phenotype and function of DCs in CHB possibly by regulating the expression of signal transducer and activator of transcription (STAT) 1,4,6 and suppressor of cytokine signaling (SOCS) 1,3, which might help to develop novel immunological treatments for CHB.

Applications

In the following research, the study group will try to deepen the clinical/therapeutic consequences of this study and further study is also needed to uncover the exact mechanisms of DC dysfunction in CHB, and to develop novel immunological treatments for CHB.

Terminology

Dendritic cell, Considered as a kind of professional antigen-presenting cell, is capable of initiating strong primary cellular immune response of host and determine its direction so as to play an important role in the immunologic response

of anti-infection. Artificially synthesized non-methylated CpG motif containing oligodeoxynucleotides (CpG-ODNs) might directly activate antigen-presenting cells, superiorly induce Th1 type immune reaction and have wide application prospect. However, there are few research data reported about the effects of CpG-ODNs on some important cell signal transducers in cytoplasm or nucleus of DC such as STAT, SOCS.

Peer review

The study is interesting, well done. The study improved the existing knowledge for DC dysfunction in chronic hepatitis B. It would be very well appreciated. Deepen the clinical/therapeutic consequences of this study possibly in the future research.

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CASE REPORT

Simultaneous large cell neuroendocrine carcinoma and adenocarcinoma of the stomach

Tadashi Terada, Hirotoshi Maruo

Tadashi Terada, Department of Pathology, Shizuoka City Shimizu Hospital, 424-8636 Shizuoka, Japan
Hirotoshi Maruo, Department of Surgery, Shizuoka City Shimizu Hospital, 424-8636 Shizuoka, Japan
Author contributions: Terada T performed the pathological studies and wrote the manuscript; Maruo H performed the clinical studies and biopsies.
Correspondence to: Tadashi Terada, MD, PhD, Department of Pathology, Shizuoka City Shimizu Hospital, Miyakami 1231 Shimizu-Ku, 424-8636 Shizuoka, Japan. piyo0111jp@yahoo.co.jp
Telephone: +81-54-3361111 Fax: +81-54-3361315
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Abstract

A large cell neuroendocrine carcinoma (LCNEC) of the stomach is very rare. A 76-year-old Japanese man was admitted to our hospital because of epigastralgia and nausea. Endoscopy revealed 2 large tumors in the stomach. He did not have multiple endocrine neoplasia type I or Zollinger-Ellison syndrome. Imaging modalities, including computed tomography and magnetic resonance imaging, revealed no other tumors. Gastrectomy, cholecystectomy, and lymph node dissection were performed. The resected stomach had 2 tumors: one was an antral ulcerated type 3 tumor measuring 5 cm x 5 cm, and the other was a polypoid type 1 tumor measuring 6 cm x 6 cm x 3 cm in the fundus. Microscopically, the antral ulcerated tumor was a well differentiated adenocarcinoma with deep invasion. The fundus polypoid tumor was a LCNEC, being composed of malignant large cells arranged in trabecular and nested patterns. The tumor cells were large and the nuclei were vesicular. Nucleoli were frequently present, and there were many mitotic figures, apoptotic bodies, and necrotic areas. Much lymphovascular permeation was seen. Seven out of 29 dissected lymph nodes showed metastatic foci; 6 were from the LCNEC and 1 from the adenocarcinoma. Many intravascular tumor emboli of LCNEC were seen in the peritoneum around the lymph nodes. Mucins were present in the adenocarcinoma but not in the LCNEC. Immunohistochemically, the LCNEC tumor cells were positive for pancytokeratins, synaptophysin (50% positive), chromogranin A (10% positive), Ki-67 (90% labeled), and platelet-derived growth factor-α (80% positive). They were negative for KIT, p53, CD56, and neuron-specific enolase. The non-cancerous stomach showed a normal number of endocrine cells. The patient is now treated with adjuvant chemotherapy.

Key words: Stomach; Carcinomas

Peer reviewer: Jian-Zhong Zhang, Professor, Department of Pathology and Laboratory Medicine, Beijing 306 Hospital, 9 North Anxiang Road, PO Box 9720, Beijing 100101, China

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INTRODUCTION

Large cell neuroendocrine carcinoma (LCNEC) of the stomach is very rare, and has been reported mainly in Japan[1-4]. In the World Health Organization (WHO) Blue Book[5], gastric neuroendocrine tumors were classified into well differentiated endocrine neoplasms (carcinoid) and poorly differentiated endocrine neoplasms (small cell carcinoma). In the latter section of the Blue Book, LCNEC of the stomach was defined as follows: LCNEC is a malignant neoplasm composed of large cells having organoid, nesting, trabecular, rosette-like and palisading

patterns that suggest endocrine differentiation, and in which the last can be confirmed by immunohistochemistry and electron microscopy[5]. In contrast to small cell carcinoma, LCNEC has abundant cytoplasm, more vesicular nuclei, and prominent nucleoli[5]. Although single LCNEC[1-4] and composite LCNEC and adenocarcinoma[6] of the stomach have been reported, there has been no English literature of simultaneous and distinct LCNEC and adenocarcinoma of the stomach, to the best of the author's knowledge.

CASE REPORT

A 76-year-old Japanese man presented with epigastralgia and nausea, and attended our hospital. Upper gastric endoscopy revealed 2 large tumors in the stomach, and a biopsy from one tumor showed well differentiated tubular adenocarcinoma. He did not have multiple endocrine neoplasia type I or Zollinger-Ellison syndrome. Preoperative imaging techniques, including computed tomography and magnetic resonance imaging, revealed no other tumors. Gastrectomy, cholecystectomy, and lymph node dissection were performed. The resected stomach showed 2 large tumors (Figure 1). One was an antral ulcerated type 3 tumor measuring 5 cm × 5 cm, and the other was a polypoid type 1 tumor measuring 6 cm × 6 cm × 3 cm located in the fundus (Figure 1). Microscopically, the antral ulcerated tumor was a well differentiated adenocarcinoma invading the subserosa (Figure 2). The fundus polypoid tumor was composed of malignant large cells arranged in trabecular and nested patterns (Figure 3A). No tubular formations were recognized. The tumor cells were large and the nuclei were vesicular (Figure 3B). Nucleoli were frequently identified, and there were many mitotic figures, apoptotic bodies, and small necrotic areas (Figure 3B). The tumor was located in the mucosa and submucosa, and invasion into proper muscle and subserosa was not noted. Much lymphovascular invasion was seen. Seven out of 29 dissected lymph nodes showed metastatic foci; 6 were from the LCNEC and 1 was from the adenocarcinoma. Many intravascular tumor emboli of the LCNEC were seen in the peritoneum around the lymph nodes. The non-cancerous stomach showed chronic atrophic gastritis.

The adenocarcinoma was positive for mucins, while the LCNEC was negative for mucins, as revealed by mucin stains including d-periodic acid-Schiff, Alcian blue, and mucicarmine.

An immunohistochemical study was performed with the Dako Envision method (Dako Corp., Glostrup, Denmark) as previously described[7,8]. The tumor cells of the polypoid tumor were positive for pancytokeratins (AE1/3 and CAM 5.2) (Figure 4A), synaptophysin (50% positive) (Figure 4B), chromogranin A (10% positive), Ki-67 (90% labeled) (Figure 4C), and platelet-derived growth factor-α (PDGFRA) (80% positive) (Figure 4D). The cells were negative for KIT, p53, CD56, and neuron-specific enolase. The non-cancerous stomach showed a normal number of endocrine cells.

Figure 1 Gross features of the 2 tumors of the stomach. One is an ulcerated tumor in the antrum (arrow) and the other is a polypoid tumor in the gastric body (double arrows). The ulcerated tumor is an adenocarcinoma, and the polypoid tumor is a large cell neuroendocrine carcinoma. The organ on the right is the gallbladder.

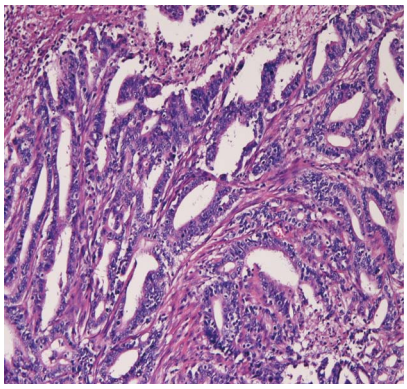

Figure 2 Microscopic features of adenocarcinoma. Well defined tubules are seen (× 200).

The gallbladder showed no significant changes. No peritoneal dissemination was recognized. The patient is now treated with adjuvant chemotherapy.

DISCUSSION

LCNEC of the stomach is not a well recognized entity. In the WHO Blue Book[5], neuroendocrine tumors of the stomach are classified into well differentiated tumors (carcinoids) and poorly differentiated neuroendocrine carcinoma (small cell carcinoma), and LCNEC is not included as a clinicopathological entity. However, as in the present case, a non-carcinoid and non-small neuroendocrine carcinoma may be present[1-6]. Therefore, LCNEC of the stomach should be listed as a neuroendocrine carcinoma in the WHO Blue Book.

The presentation of the current case is different from a carcinoid tumor because it showed significant atypia, mitosis, apoptotic cells, and necrosis, all of which are not seen in carcinoids. The present case is apparently different from small cell carcinoma because of the atypical features of the tumor cells.

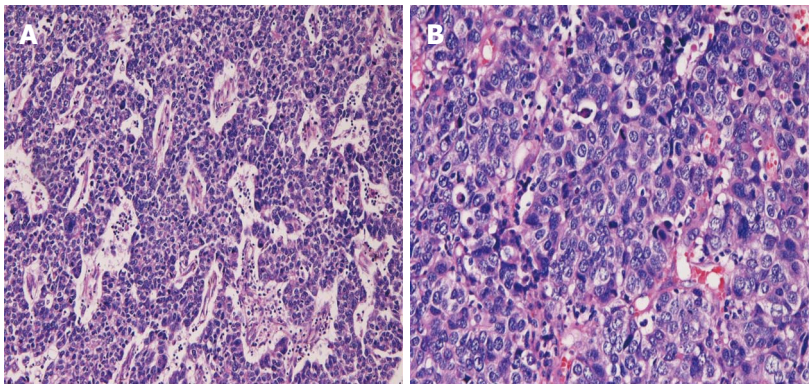

Figure 3 Polypoid tumor. A: The polypoid tumor consists of malignant cells with hyperchromatic nuclei arranged in a trabecular pattern (HE, × 100); B: Higher power view shows large cells, vesicular nuclei, nucleoli, mitotic figures and apoptotic bodies (HE, × 200). HE: Hematoxylin and eosin.

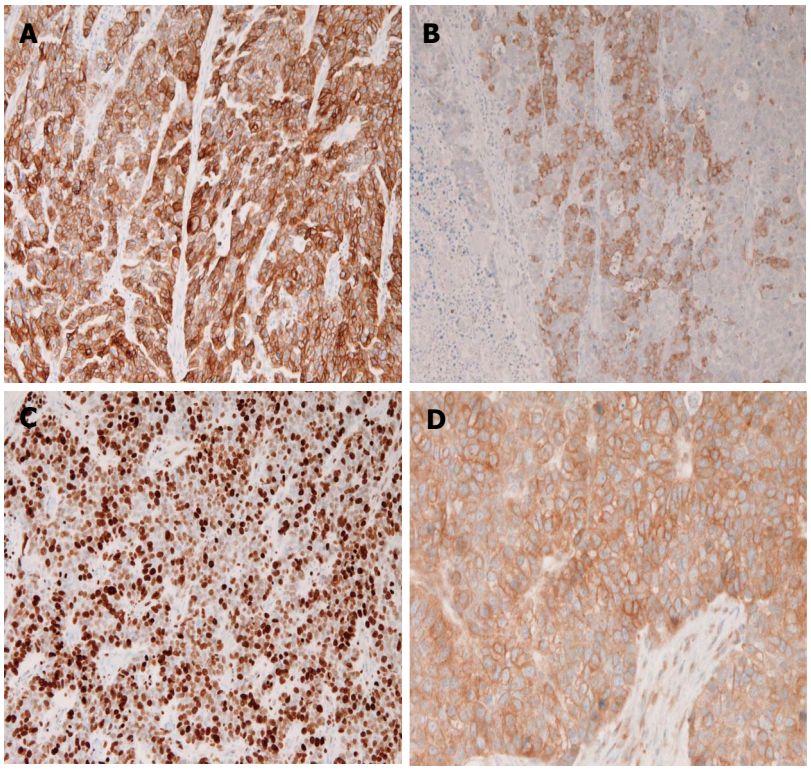

Figure 4 Immunohistochemical findings. Tumor cells are positive for pancytokeratin AE1/3 (A), synaptophysin (B), Ki67 (C), and PDGFRA (D) (× 100). PDGFRA: Platelet-derived growth factor-α.

The present case is not an adenocarcinoma with endocrine differentiation. Endocrine cells of the latter are a very minor component, accounting for less than 20%[2]. In the present case, the neuroendocrine component accounted for 50% of LCNEC cells, thus confirming that the current case is not an adenocarcinoma with neuroendocrine differentiation but a LCNEC.

The frequency of gastric LCNEC is unclear. Jiang *et al*[2] examined 2835 cases of gastric carcinoma and found 42 cases (1.5%) of LCNEC and 44 cases (1.6%) of adenocarcinoma with endocrine differentiation. The frequency is relatively high. In general, pathologists do not perform an immunohistochemical study of surgical specimens of gastric cancers. It is recommended that pathologists examine at least the immunohistochemistry of neuroendocrine markers in poorly differentiated carcinoma showing neuroendocrinoid histology.

The biological behavior of gastric LCNEC is significantly more aggressive than that of gastric adenocarcinoma[2]. Adenocarcinoma with an endocrine component is more aggressive than ordinary adenocarcinoma[2]. The authors think that the prognosis of the present case is poor because the adenocarcinoma is advanced and lymph node metastasis was found in 6 of 29 lymph nodes. In addition, there were many intravascular tumor emboli of LCNEC in the perilymphatic peritoneum.

The present tumor is not a composite or combined tumor, but a separate tumor. There is one report of com-

bined or collision LCNEC and adenocarcinoma[6]. In the present case, the LCNEC and adenocarcinoma were remote from each other, thus were simultaneous. Such a simultaneous LCNEC and adenocarcinoma of the stomach has not been reported in the English literature, to the best of the author's knowledge. It is very interesting that the present LCNEC expressed PDGFRA, but not KIT. Tumors expressing PDGFRA have been not examined. The PDGFRA may be related to neuroendocrine features of a LCNEC, as in gastrointestinal stromal tumors. Thus, a stomach LCNEC can be included in a group of tumors expressing PDGFRA.

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CASE REPORT

Lipomatous hemangiopericytoma of the stomach: A case report and a review of literature

Hong-Biao Jing, Qing-Da Meng, Yan-Hong Tai

Hong-Biao Jing, Department of Pathology, The General Hospital, Jinan Military Command, Jinan 250031, Shandong Province, China
Qing-Da Meng, Department of Pathology, The Zhangqiu City Hospital, Zhangqiu 250200, Shandong Province, China
Yan-Hong Tai, Department of Pathology, The PLA General Hospital, Beijing 100853, China
Author contributions: Jing HB designed the study; Meng QD and Tai YH performed data collection and analysis; Jing HB and Tai YH wrote the paper.
Correspondence to: Hong-Biao Jing, MD, Department of Pathology, The General Hospital, Jinan Military Command, Shifan Road 25, Jinan 250031, Shandong Province, China. jinghong.biao@hotmail.com
Telephone: +86-0531-51666857 Fax: +86-0531-51666284
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Abstract

Lipomatous hemangiopericytomas (LHPCs) are rare soft-tissue tumors that are histologically characterized by hemangiopericytomatous vasculature and the presence of mature adipocytes. We present the clinicopathological features of a case of gastric LHPC in a 56-year-old female, along with a literature review. Endoscopy and endoscopic ultrasound showed a submucosal tumor 0.8 cm across in the greatest dimension in the lesser curvature side of the gastric antrum. Grossly, the well-defined mass had a solid and tan-white cut surface admixed with myxoid regions and yellowish areas. Histological examination revealed a submucosal well-circumscribed lesion composed of cellular nodules with the classic appearance of an hemangiopericytoma admixed with clusters and lobules of mature adipocytes. The ill-defined tumor cells had weakly eosinophilic cytoplasm and contained spindled nuclei with occasional small nucleoli. Nuclei atypia and mitoses were absent, and no cellular atypia, necrosis or vascular invasion was observed. Immunohistochemistry showed that the tumor cells were diffusely positive for CD34, CD99, and vimentin and were focally reactive for bcl-2. This is the first known report of an LHPC in the stomach. The patient was followed for 12 mo without any evidence of metastasis or recurrence.

Key words: Lipomatous hemangiopericytoma; Hemangiopericytoma; Solitary fibrous tumor; Stomach; Immunohistochemistry

Peer reviewer: Dr. Francesco Franceschi, Department of Internal Medicine, Catholic University of Rome, Largo A Gemelli, 8, Rome 00168, Italy

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INTRODUCTION

Lipomatous hemangiopericytoma (LHPC) is a recently recognized rare hemangiopericytoma (HPC) variant that is histologically composed of an admixture of benign hemangiopericytomatous and mature lipomatous components[1]. To date, 49 cases of LHPC have been documented in published English language reports (Table 1)[1-19], but tumors developing in visceral organs such as the stomach have not been reported. Herein, we report the clinicopathological and immunohistochemical features of what is, to our knowledge, the first case of an LHPC occurring in the stomach and present a review of the literature.

CASE REPORT

A 56-year-old female presented with slight left epigastric pain that had been present for two months. Her past medical history and family history were unremarkable.

Table 1 Clinicopathologic data for reported cases of lipomatous hemangiopericytoma

Ref.	Sex/age (yr)	Location	Size (cm)	Follow-up (mo)
Liu *et al*[19]	M/61	Mediastinum	9.5	NED/28
Aftab *et al*[18]	M/38	Spine	NA	NED/6
Dozois *et al*[17]	M/21	Pelvic cavity	18	NA
Kim *et al*[16]	M/43	Perineum	5.5	NA
Pitchamuthu *et al*[15]	F/49	Orbit	3.5	NA
Yamazaki *et al*[14]	M/54	Lung	4	NA
Farah-Klibi *et al*[13] (Abstract)	NA	Orbit	NA	NA
Shaia *et al*[12]	F/36	Skull base	5.5	NED/12
Amonkar *et al*[11]	M/39	Mediastinum	6.5	NED/12
Verfaillie *et al*[10]	F/79	Axilla	9	NA
Yamaguchi *et al*[9]	F/51	Kidney	10	NA
Alrawi *et al*[8]	F/55	Occipital area and upper neck	9	NED/12
Domanski[7]	F/51	Thigh	5	NED/24
	M/56	Abdominal wall	3	NED/12
Cameselle-Teijeiro *et al*[6]	M/36	Thyroid	6	NED/25
Davies *et al*[5]	M/65	Orbit	3.5	NED/48
Guillou *et al*[4]	M/54	Retroperitoneum	7.5	NED/72
	F/42	Mediastinum	2.5	NED/36
	F/68	Hip	1.7	NA
	M/33	Hip	4.5	NED/14
	M/48	Elbow	5	NA
	F/39	Thigh	10	NED/22
	F/27	Neck	5.5	NED/12
	F/43	Right iliac fossa	5.5	Recent case
	F/75	Epicardium	4.5	NED/60
	F/67	Poplitea fossa	9	NED/6
	M/46	Retroperitoneum	19	NED/6, then lost to follow-up
	M/53	Orbit	2	NED/77
	M/51	Retroperitoneum	18	NED/6
Folpe *et al*[3]	M/51	Calf	3	NED/36
	M/74	Pelvic cavity	9	NA
	M/53	Retroperitoneum	NA	NED/84
	M/33	Retroperitoneum	18	NA
	M/72	Supraclavicular	NA	NED/60
	M/61	Pelvic cavity	12	NA
	F/49	Submandibular	5	NED/60
	M/33	Thigh	6	NA
	F/49	Thigh	13	NA
	M/60	Thigh	21	NED/48
	F/39	Calf	4	NED/36
	M/60	Para-spinous, T7-10	NA	NA
	M/51	Thigh	17	NED/24
	M/64	Chest wall	6	Recent case
	M/63	Inguinal	NA	Recent case
	F/74	Hip	8	Recent case
Ceballos *et al*[2]	F/41	Thigh	9	NED/13
Nielsen *et al*[1]	F/44	Sinonasal area	7	NED/48
	M/56	Shoulder	4	NED/4
	M/72	Retroperitoneum	10	Recent case
Present case	F/56	Stomach	0.8	NED/12

M: Male; F: Female; NED: No evidence of disease; NA: Not available.

The laboratory data were within normal limits. Endoscopy showed a submucosal tumor of 0.8 cm in the greatest dimension in the lesser curvature side of the gastric antrum. The broad-base mass with a smooth external surface protruded into the lumen. The overlying mucosa was normal in texture and color. On endoscopic ultrasound, an oval, well-defined, hyperechoic mass was detected lying beneath the mucosa but protruding into the lumen. Given the clinical suspicion of a gastrointestinal stromal tumor (GIST), endoscopic resection was performed with a diathermy snare after ligation with a detachable snare. The neoplasm was completely excised. Her postoperative course was uneventful. She was well, without evidence of recurrence or metastasis, 12 mo after resection.

On gross pathology, the specimen consisted of a nodular mass measuring 0.8 cm × 0.6 cm × 0.5 cm with an attached portion of gastric mucosa. Sectioning revealed a well-demarcated, nonencapsulated mass with a solid and tan-white appearance admixed with myxoid regions and yellowish areas. The tumor was soft and rubbery in consistency without involving the overlying mucosa.

Histological examination revealed a submucosal well-circumscribed lesion composed of cellular nodules with the classic appearance of an HPC admixed with clusters and lobules of mature adipocytes. HPC-like areas showed a patternless architecture of oval to spindle cells surrounding the ectatic thin-walled branching vessels (staghorn configuration) (Figure 1A). The ill-defined tumor cells had weakly eosinophilic cytoplasm and contained spindled nuclei with occasional small nucleoli. Nuclei atypia and mitoses were absent, and no cellular atypia, necrosis, or vascular invasion was observed (Figure 1B). Mature, nonatypical adipocytes were identified throughout the lesion in varying proportions in various regions of the tumor, such as in lobules and foci of fat cells (Figure 1C). Focal myxoid changes were present.

Immunohistochemical staining demonstrated that the neoplastic oval and spindle (nonadipocytic) cells were strongly and diffusely positive for vimentin (Figure 2A), CD34 (Figure 2B), and CD99 and were focally reactive for bcl-2, whereas staining for smooth muscle actin (SMA), pankeratin, CD117, platelet-derived growth factor receptor α (PDGFRA), HMB45, melan-A, and S-100 protein was negative. MIB-1 stained only approximately 1% of the tumor cells (1000 cells counted).

DISCUSSION

An LHPC, which was first formally described as a new entity in the 2002 edition of the WHO classification of soft-tissue tumors, is a rare hemangiopericytoma variant composed of mature adipocytes and hemangiopericytoma-like areas[20]. LHPC was first described as a unique hemangiopericytoma variant by Nielsen *et al*[1] and was named "LHPC" in the English language literature in 1995. In 2000, Guillou *et al*[4] noted that LHPCs and solitary fibrous tumors (SFTs) share similar clinical, pathological, immunohistochemical, and ultrastructural features, except for the presence of mature adipocytes in LHPC, and suggested that LHPC does not correspond to a well-defined entity but, rather, represents a fat-containing variant of SFT.

To date, a total of 50 pathologically confirmed cases of LHPC have been reported, including our patient[1-19]. Most lesions presented clinically as long-standing, deep-seated, indolent tumors, discovered fortuitously in some patients, whereas others had symptoms of compression

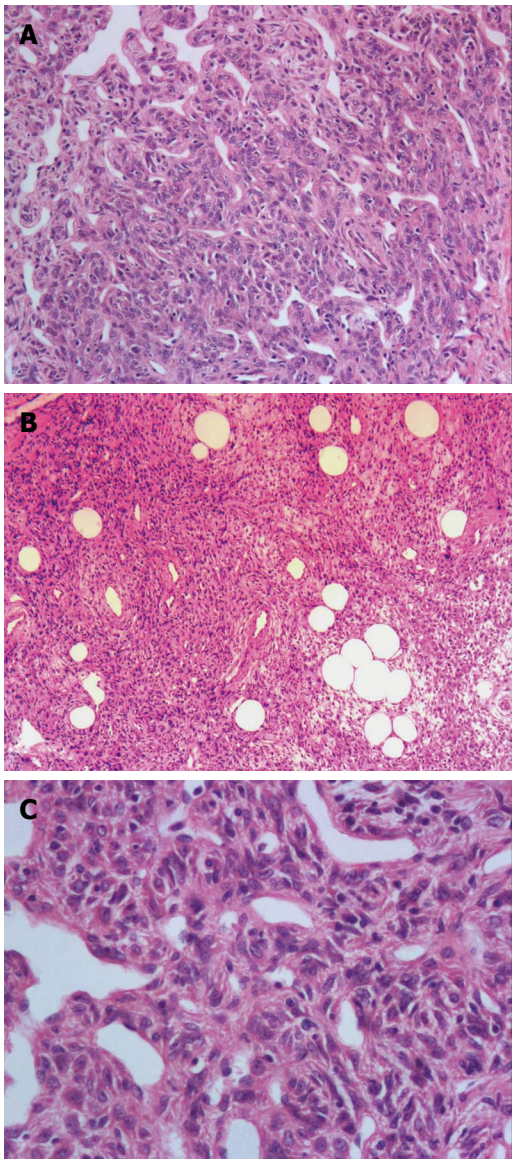

Figure 1 Histological findings of gastric lipomatous hemangiopericytoma by hematoxylin and eosin stains. A: Hemangiopericytoma-like areas in the lipomatous hemangiopericytoma (LHPC) showing a patternless architecture of oval to spindle cells surrounding the ectatic thin-walled branching vessels (HE, × 100); B: The clusters of mature adipocytes in the LHPC (HE, × 40); C: The nonatypical, ill-defined tumor cells had a weakly eosinophilic cytoplasm and contained spindled nuclei (HE, × 200). HE: Hematoxylin and eosin.

related to tumor enlargement[4]. As documented in Table 1, LHPC seems to occur in middle-aged adult patients (range, 21-79 years; mean, 51.7 years), similar to the mean age of 55 years reported by Folpe *et al*[3]. The sex distribution for the LHPC cases for which this information is known is 20 females and 29 males, demonstrating a slight male predilection of LHPC. The size of the lesions ranged from 0.8 cm to 21 cm (mean, 7.9 cm). LHPCs have a wide anatomical distribution, but the most commonly affected sites are the lower extremities (14/50), including the thigh, hip, calf, popliteal fossa, and inguina. Other sites (72%) included the retroperitoneum, orbit, mediastinum, and pelvic cavity. The thigh, retroperitoneum and the orbit were the most commonly affected sites. Most of the tumors were located in the deep soft tissue[4].

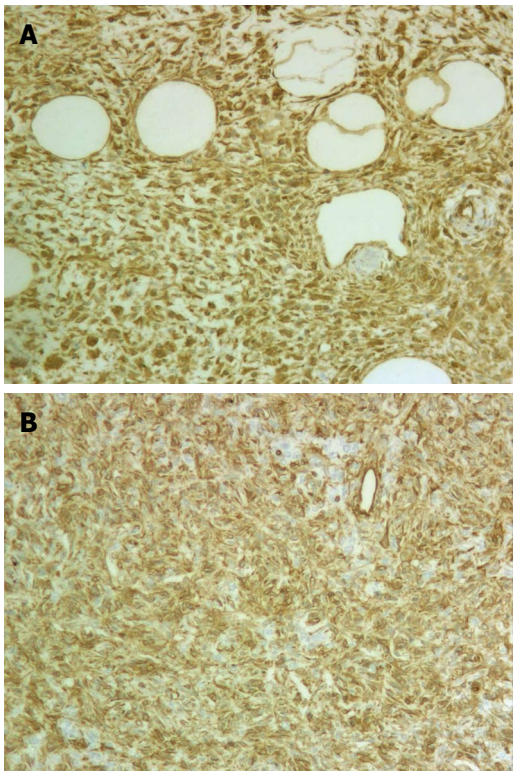

Figure 2 Immunohistochemical findings for vimentin and CD34. The tumor cells immunohistochemically expressed vimentin (A) and CD34 (B) (immunoperoxidase technique with hematoxylin counterstain, × 100).

Given that no LHPC patients developed recurrence during the follow-up interval and that all were without disease, some authors have concluded that LHPCs are likely to be benign tumors[3,4]. However, in our opinion, their biological behavior has yet to be determined because only 30 cases have been followed up in the English language literature to date, and the mean follow-up length was only 30.5 mo, not long enough to draw definitive conclusions about the prognosis of LHPCs. Davies *et al*[5] suggested that patients with LHPC should be followed up for more than 10 years.

Grossly, LHPC generally presents as a well-demarcated, variably encapsulated, medium-sized mesenchymal tumor. Cut sections show alternating areas of whitish and yellowish tumor tissue, in most cases with a lobular or fascicular appearance. Microscopically, the tissue characteristically shows a varying combination of cellular areas composed of round to spindle cells, collagenous or myxoid stroma with focal sclerotic areas, hemangiopericytoma-like vasculature made of small- to medium-sized thick-walled branched vessels, and lipomatous areas made up of mature adipocytes[4]. LHPCs may become large and occasionally show nuclear atypia within sclerotic zones, a phenomenon probably akin to that seen in degenerated schwannomas. LHPCs may also rarely show foci of high cellularity but do not approach the level seen in malignant LHPCs or in other malignancies that commonly show a hemangiopericytomatous pattern, such as synovial sarcoma or mesenchymal chondrosarcoma[3]. Immunohistochemistry showed that the nonadipocytic tumor cells are strongly and diffusely positive for

vimentin and CD34, and they can also be positive for CD99 and bcl-2[21]. Ultrastructurally, the nonadipocytic tumor cells show features consistent with fibroblastic, myofibroblastic or pericytic differentiation[21].

Considering the location and histological appearance of this type of tumor, the main differential diagnoses considered should include GIST, angiomyolipoma, liposarcoma, and spindle cell lipoma (SCL). The stomach is the most common location for GIST, with 60%-70% of tumors occurring in this location[22]. In fact, in our study, the initial clinical diagnosis was GIST. Briefly, GISTs contain bundles of spindle cells and do not contain the mature adipocytes characteristic of LHPC. LHPCs are positive for CD34 but are negative for CD117. In contrast, GISTs are positive for CD117, CD34, and PDGFRA[23]. There was consistent negative staining for CD117 and PDGFRA. Hence, the tumor was not a GIST but, judging by its histological and immunohistochemical features, an LHPC. Differentiating an angiomyolipoma, especially one with a prominent smooth muscle component, from an LHPC is based on the former's distinctive arrangement of the spindle cells around thick-walled vessels and their immunoreactivity for SMA and melanocytic markers (HMB45 and melan-A)[4,9]. Liposarcoma, especially the myxoid variant, can have a prominent vasculature. The vascular configuration should serve as a useful clue in distinguishing between liposarcoma and LHPC. The vessels in myxoid liposarcoma form a delicate plexiform capillary vascular network, unlike the staghorn pattern of LHPC. Furthermore, LHPCs are devoid of lipoblasts[1]. SCL usually develops in the subcutaneous tissue of the neck and upper back of male patients and contains short bundles of wiry collagen admixed with the spindle cells. SCLs do not contain the hemangiopericytoma-like vasculature of LHPCs[21].

In conclusion, the clinicopathological features of our case were entirely compatible with those of LHPC, and to our knowledge, this is the first reported case affecting the stomach. More cases of LHPC of the stomach must be studied to draw definitive conclusions about its distinct behavior and management.

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Asmaa Gaber Abdou, Assistant Professor, Department of Pathology, Faculty of Medicine, Menofiya University, Shebein Elkom 002-048, Egypt

Bruno Annibale, Professor, Digestive and Liver Disease Unit, University "La Sapienza" II School of Medicine, Via di Grottarossa 1035, Roma 00189, Italy

Dean Bogoevski, Dr., Senior Resident, Department of General, Visceral and Thoracic Surgery, University Clinic Hamburg, Eppendorf, Hagenbeckstrasse 167, 22527 Hamburg, Germany

Robert S Bresalier, MD, Professor, Department of Gastrointestinal Medicine and Nutrition, the University of Texas MD Anderson Cancer Center, Houston, TX 77030-4009, United States

Markus W Büchler, MD, Department of General Surgery, University of Heidelberg, Im Neuenheimer Feld 110, Heidelberg D-69120, Germany

Ronan A Cahill, Dr., Department of General Surgery, Waterford Regional Hospital, Waterford, Cork, Ireland

Adam S Cheifetz, MD, Assistant Professor, Clinical Director, Center for Inflammatory Bowel Disease, Beth Israel Deaconess Medical Center, 330 Brookline Ave, Boston, MA 02215, United States

Cho Chi-Hin, PhD, Professor of Pharmacology and Chairman, Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China

Fernando J Corrales, Associate Professor of Biochemistry, Division of Hepatology and Gene Therapy, Proteomics Laboratory, CIMA, University of Navarra, Avd Pío XII, 55, Pamplona 31008, Spain

David R Gretch, MD, PhD, Viral Hepatitis Laboratory, Room 706, UW Research and Training Building, Harborview Medical Center, PO Box 359690, 325 Ninth Avenue, Seattle, Washington, DC 98104, United States

Henrike Hamer, PhD, Department of Internal Medicine, Division of Gastroenterology (Box 46), Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands

James CH Hardwick, Dr., MD, PhD, Department of Gastroenterology, Leiden University Medical Center, Albinusdreef 2, 2300RC Leiden, The Netherlands

Kalpesh Jani, Dr., Consultant GI and Laparoscopic Surgeon, SIGMA Surgery, Baroda, Gujarat 394220, India

Jin-Hong Kim, Professor, Department of Gastroenterology, Ajou University Hospital, San 5, Wonchon-dong, Yeongtong-gu, Suwon 442-721, South Korea

Sang Kil Lee, MD, Assistant Professor, Department of Gastroenterology, Yonsei University College of Medicine, No. 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea

Huanbiao Mo, PhD, Associate Professor, Department of Nutrition and Food Sciences, Texas Woman's University, PO Box 425888, Denton, TX 76204, United States

Alan C Moss, MD, FACG, Assistant Professor of Medicine, Director of Translational Research, Center for Inflammatory Bowel Disease, Beth Israel Deaconess Medical Center, Harvard Medical School, Rose 1/East, 330 Brookline Ave, Boston, MA 02215, United States

Pete Muscarella, MD, Division of Gastrointestinal Surgery, The Ohio State University, N711 Doan Hall, 410 W 10th Ave, Columbus, OH 43210, United States

Satoshi Osawa, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan

George Papatheodoridis, MD, Assistant Professor in Medicine and Gastroenterology, 2nd Department of Internal Medicine, Athens University Medical School, Hippokration General Hospital of Athens, 114 Vas Sophias Ave, 115 27 Athens, Greece

Salih Sanlioglu, Professor, Dr., VMD, PhD, Department of Medical Genetics, Human Gene Therapy Division of Akdeniz University Faculty of Medicine, Dumlupinar Boulevard Campus, Antalya 07058, Turkey

Hon-Yi Shi, PhD, Associate Professor, Graduate Institute of Healthcare Administration, Kaohsiung Medical University, 100, Shih-Chuan 1st Road, San Ming District, Kaohsiung 807, Taiwan, China

Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Fang Yan, MD, PhD, Research Associate Professor, Division of Gastroenterology, Department of Pediatrics, Hepatology, and Nutrition, Vanderbilt University Medical Center, 2215 Garland Avenue, MRB IV, Room 1035J, Nashville, TN 37232, United States

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

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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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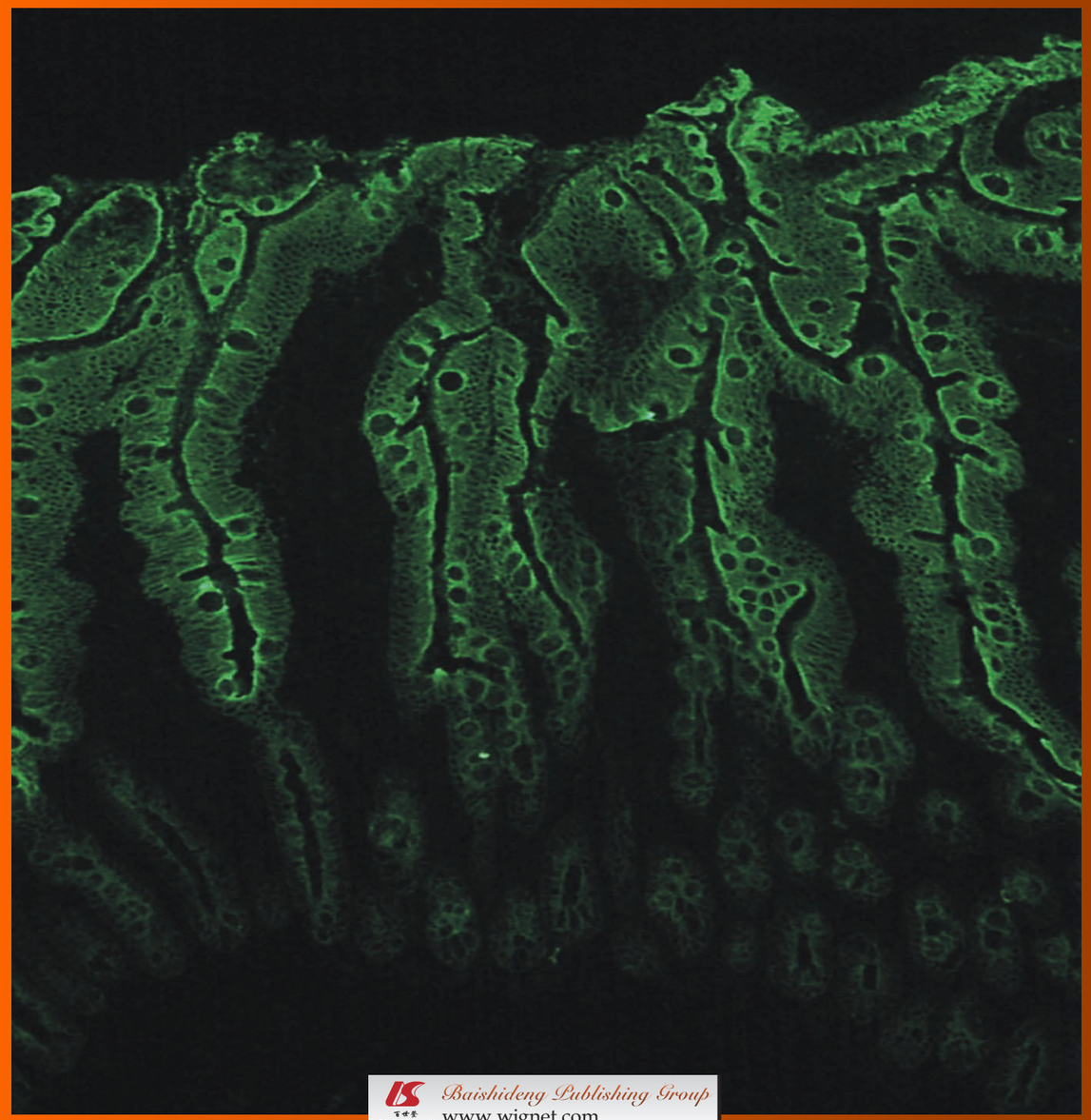

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EDITORIAL

Juvenile polyposis syndrome

Lodewijk AA Brosens, Danielle Langeveld, W Arnout van Hattem, Francis M Giardiello, G Johan A Offerhaus

Lodewijk AA Brosens, Danielle Langeveld, G Johan A Offerhaus, Department of Pathology, University Medical Centre Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands
Danielle Langeveld, W Arnout van Hattem, G Johan A Offerhaus, Department of Pathology, Academic Medical Centre, PO Box 22660, 1100 DD Amsterdam, The Netherlands
Francis M Giardiello, Division of Gastroenterology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, United States
Author contributions: Brosens LAA designed and wrote the paper; Langeveld D, van Hattem WA, Giardiello FM and Offerhaus GJA provided significant intellectual content and critically revised the manuscript.
Correspondence to: Dr. Lodewijk AA Brosens, Department of Pathology, University Medical Center Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands. l.a.a.brosens@umcutrecht.nl
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Abstract

Juvenile polyposis syndrome is a rare autosomal dominant syndrome characterized by multiple distinct juvenile polyps in the gastrointestinal tract and an increased risk of colorectal cancer. The cumulative life-time risk of colorectal cancer is 39% and the relative risk is 34. Juvenile polyps have a distinctive histology characterized by an abundance of edematous lamina propria with inflammatory cells and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes. Clinically, juvenile polyposis syndrome is defined by the presence of 5 or more juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract or any number of juvenile polyps and a positive family history of juvenile polyposis. In about 50%-60% of patients diagnosed with juvenile polyposis syndrome a germline mutation in the *SMAD4* or *BMPR1A* gene is found. Both genes play a role in the BMP/TGF-beta signalling pathway. It has been suggested that cancer in juvenile polyposis may develop through the so-called "landscaper mechanism" where an abnormal stromal environment leads to neoplastic transformation of the adjacent epithelium and in the end invasive carcinoma. Recognition of this rare disorder is important for patients and their families with regard to treatment, follow-up and screening of at risk individuals. Each clinician confronted with the diagnosis of a juvenile polyp should therefore consider the possibility of juvenile polyposis syndrome. In addition, juvenile polyposis syndrome provides a unique model to study colorectal cancer pathogenesis in general and gives insight in the molecular genetic basis of cancer. This review discusses clinical manifestations, genetics, pathogenesis and management of juvenile polyposis syndrome.

Key words: Juvenile polyposis syndrome; Hamartoma; Colorectal cancer; *SMAD4*; *BMPR1A*

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INTRODUCTION

Juvenile polyposis syndrome (JPS) is a rare autosomal dominant hereditary disorder characterized by multiple distinct juvenile polyps in the gastrointestinal tract and an increased risk of colorectal cancer. Sporadic solitary colorectal juvenile polyps occur in approximately 2% of the paediatric population but these polyps are not associated with an increased risk of gastrointestinal cancer[1,2]. Juvenile polyposis syndrome is defined by the presence of five or more juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract or any

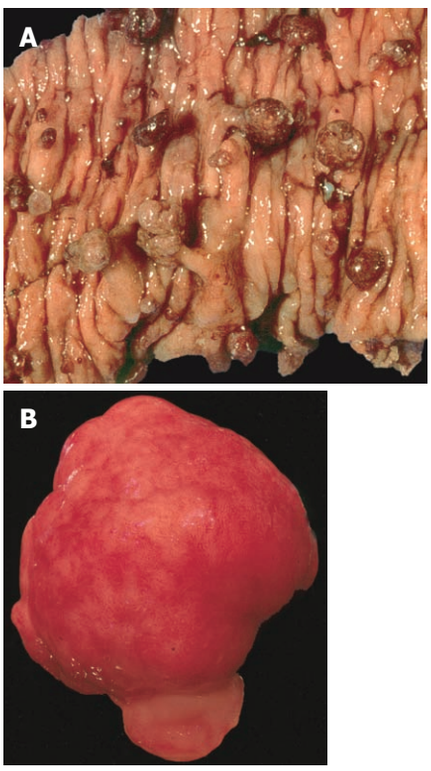

Figure 1 Macroscopic appearance of juvenile polyposis. A: Bowel resection of a patient with juvenile polyposis syndrome showing multiple spherical pedunculated polyps with a smooth surfaces; B: Gross appearance of a juvenile polyp from a patient with juvenile polyposis syndrome. Note the smooth surface, in contrast with a Peutz-Jeghers polyp.

number of juvenile polyps, and a positive family history of juvenile polyposis[1,3]. About 50%-60% of JPS patients have a germline mutation in the *SMAD4* or *BMPR1A* gene[4-6].

HISTOLOGY

The juvenile polyp is a histopathological entity first reported by Diamond[7] in 1939 and later described in more detail by Helwig[8]. Macroscopically, juvenile polyps vary in size from 5 mm to 50 mm, and typically have a spherical, lobulated and pedunculated appearance with surface erosion (Figure 1A and B). Microscopically, a juvenile polyp is characterized by an abundance of edematous lamina propria with inflammatory cells and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes (Figure 2A and B). The distinction between an inflammatory and a juvenile polyp is often difficult. In essence, juvenile polyps in juvenile polyposis syndrome appear similar to sporadic solitary juvenile polyps, although syndromic polyps often have a frond-like growth pattern with fewer stroma, fewer dilated glands and more proliferative smaller glands[9]. In addition, polyps in juvenile polyposis syndrome frequently show neoplastic changes to the epithelium not found in sporadic solitary juvenile polyps. Colorectal polyps from individuals with a *SMAD4* germline mutation often have a more proliferative epithelial phenotype and fewer stroma compared to those from patients with a *BMPR1A* germline mutation (Figure 2A and B)[10]. In addition, absence of the SMAD4 protein on immunohistochemistry of a juvenile polyp indicates that the patient carries a germline *SMAD4* mutation (Figure 2C)[11].

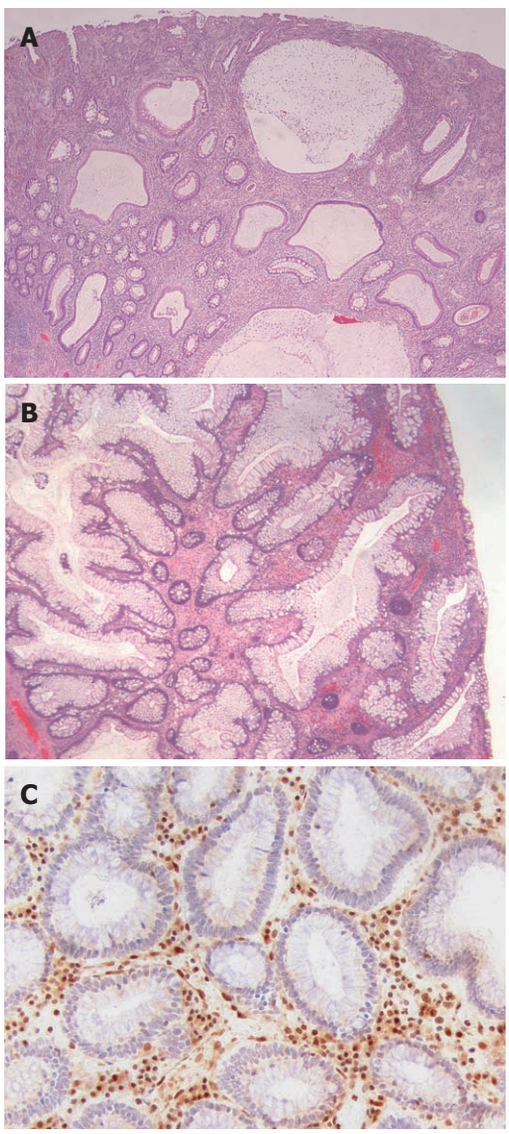

Figure 2 Histological appearance of juvenile polyposis. A: Histological section of a juvenile polyp from a juvenile polyposis patient with a germline mutation of *BMPR1A*. Typically, juvenile polyps are characterized by prominent lamina propria with edema and inflammatory cells, and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes; B: Histological section of a juvenile polyp from a juvenile polyposis patient with a germline mutation of *SMAD4*. This polyp shows relatively fewer stroma, fewer dilated glands and more proliferative smaller glands; C: SMAD4 immunohistochemistry on a juvenile polyp showing absent SMAD4 expression in the epithelium, indicating that this patient carries a germline *SMAD4* mutation.

Small intestinal polyps in JPS have been classified as juvenile[12,13], hyperplastic and/or inflammatory polyps[14-16], and as lymphoid hyperplasia[15,17]. The larger small intestinal polyps resemble juvenile polyps in the colon[17]. In addition, juvenile/hamartomatous polyps with dysplastic changes and adenomas have been found in the duodenum, jejunum, and ileum of patients with JPS[12,14,16]. Moreover,

we have seen a Brunner gland hamartoma in the duodenum of a juvenile polyposis patient with a *SMAD4* germline mutation. Most gastric polyps in JPS patients have been diagnosed as hyperplasic polyps[14] and are indistinguishable from gastric hyperplastic polyps[18].

GENETICS

A germline mutation in the *SMAD4* or *BMPR1A* gene is found in about 50%-60% of JPS patients[4-6]. Both genes are involved in the BMP/TGF-beta signalling pathway. Most germline defects are point mutations or small base pair deletions in the coding regions of *SMAD4* or *BMPR1A* that can be identified by conventional sequence analysis. About 15% of the germline genetic defects are deletions of one or more exons, or the entire *SMAD4* or *BMPR1A* coding sequence, which necessitates identification by techniques that analyze large genomic deletions, such as multiplex ligation-dependent probe amplification (MLPA)[4,6]. Recently, previously unknown mutations in the *BMPR1A* promoter region were found in about 10% of JPS patients[19].

About 30%-40% of JPS patients have no germline mutation; therefore, a number of candidate genes, mostly involved in the transforming growth factor β (TGF-β)/bone morphogenetic proteins (BMP) pathway, have been investigated for a role in JPS pathogenesis. Although not confirmed, and questioned by others, germline mutations of the TGB-β co-receptor Endoglin has been reported in two JPS patients[4,20]. In addition, *SMAD1*, *SMAD2*, *SMAD3*, *SMAD5*, *SMAD7*, *BMPR2*, *BMPR1B*, *ACVRL1, TGFBR II* and *CDX2* have been analyzed; however, no germline mutations have been found in these genes[20]. In addition, *PTEN*, the gene linked to Cowden (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS), has been suggested as a *JPS* gene. However, *PTEN* mutations in patients with juvenile polyps likely represent CS or BRRS patients that have not (yet) developed extraintestinal clinical features specific to these conditions[21]. A recent study involving a large number of *PTEN* germline-mutation positive Cowden syndrome patients substantiated this notion by showing that both upper- and lower gastrointestinal polyps are a common manifestation of this syndrome[22]. Patients afflicted by Cowden syndrome may develop colorectal juvenile polyps indistinguishable from those in juvenile polyposis syndrome. Therefore, although the exact gastrointestinal manifestations of Cowden syndrome remain to be clarified, particularly with respect to the upper gastrointestinal tract, Cowden syndrome should be part of the differential diagnosis in a patient presenting with a juvenile polyp.

CLINICAL PRESENTATION

Clinically, juvenile polyposis can present in two forms. The first is called juvenile polyposis of infancy. This is a generalized form occurring in infants with polyps in the stomach, small bowel and colon. The polyps vary in size from 1 to 30 mm and may be sessile or pedunculated. These infants suffer from diarrhoea, haemorrhage, malnutrition and intussusception. Death usually occurs at an early age. In addition, many of these patients have congenital abnormalities, including macrocephaly and generalized hypotonia[23]. Some investigators suggest that this rare form of juvenile polyposis is caused by continuous deletion of *BMPR1A* and *PTEN* genes located on chromosome 10q23.2 and 10q23.3 respectively, although others disagree[24, 25].

In addition, generalized juvenile polyposis and juvenile polyposis coli (juvenile polyps restricted to the colorectum) have been defined[26]. However, these forms appear to be variable expressions of the same disease, because patients of both forms have been reported to segregate according to a dominant mode in the same family[24,27]. These forms may be sporadic, i.e., 'de novo', or inherited, and usually present later in childhood or in adult life. They are characterized by the presence of gastrointestinal juvenile polyposis and an increased risk of gastrointestinal cancer[28]. A variety of extra-intestinal manifestations have been reported in these patients[23]. In approximately 50% of juvenile polyposis coli or generalized JPS cases, a heterozygous germline mutation in the *SMAD4* or *BMPR1A* gene is identified[24]. Several differences in phenotypic expression between carriers of a *SMAD4* and *BMPR1A* mutations have been noted. *SMAD4* mutations are associated with a more aggressive gastrointestinal phenotype, involving higher incidence of colonic adenomas and carcinomas and more frequent upper gastrointestinal polyps and gastric cancer than patients with a *BMPR1A* mutation[6,29,30]. Also, the combined syndrome of JPS and hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome) is associated with germline mutations in *SMAD4*[31].

POLYP DISTRIBUTION

Polyps in JPS predominantly occur in the colorectum, varying in number from five to several hundred. In addition, polyps can be found in the stomach, duodenum, jejunum, and ileum, although the incidence of upper gastrointestinal tract polyps in JPS is less well studied. Rarely, profuse gastric juvenile polyposis is found in the absence colonic polyps[32]. As noted above, upper gastrointestinal polyposis and gastric cancer has been associated with *SMAD4* germline mutation[6,29,30].

Few studies systematically examined upper gastrointestinal tract involvement in juvenile polyposis[12,14,33]. One investigation found gastric polyps in 10 out of 12 (83%) patients, mostly located in the antrum, but throughout the stomach in six individuals. Rarely, profuse gastric juvenile polyposis is found in the absence of colonic polyps. Duodenal polyps were found in four out of 12 (33%) JPS patients, with two having multiple polyps ranging in size from 0.5 to 1.5 cm. and two others with minute polyps[14]. Using capsule endoscopy, small-bowel polyps beyond the range of standard gastroscopy were found

in 2 of 10 (20%) patients and duodenal polyps in 4 others (40%)[33]. Another study reported small bowel polyps in 8 of 56 JPS patients (14%)[12]. Moreover, a number of case reports of duodenal, jejunal, and ileal polyps in JPS patients exist[13,15-17,34], In addition, juvenile polyps are frequently found in the ileal pouch of juvenile polyposis patients who have undergone proctocolectomy[35,36].

CANCER RISK

Juvenile polyposis is associated with an increased risk of gastrointestinal cancer. A recent cancer risk analysis calculated a cumulative life-time risk for colorectal cancer in JPS of 39% and a relative risk of colorectal cancer of 34[37]. However, this may be a conservative estimate, because some patients in this study had already undergone prophylactic colectomy. Jass reported a 68% cumulative risk of colorectal cancer in patients from the St Mark's Registry, but details were not provided[38]. In addition, several cases of stomach, duodenal, and pancreatic cancer in JPS have been described in the literature, but no formal risk analysis for these malignancies exists[28]. One study found small bowel carcinoma in six out of 56 (11%) JPS patients, but four of these cancers occurred in one family[12]. Evaluation of literature reports suggests that gastric and small bowel carcinoma, together, occur at about one-fifth the frequency of colorectal cancers in this patient group[37].

CANCER PATHOGENESIS

Cancer pathogenesis in juvenile polyposis has not yet been unravelled and may develop through the so-called "landscaper mechanism". The landscaper model was proposed after the observation that the genetic alterations at chromosome 10q22 (*BMPR1A* locus) occurred predominantly in the stroma of juvenile polyps. This paradigm postulates that cancer develops as a result of an abnormal stromal environment, which leads to neoplastic transformation of the adjacent epithelium[39]. Support for a "landscaper" defect triggering juvenile polyposis came from a study in which disrupted BMP signalling, through expression of a natural pathway inhibitor, resulted in development a juvenile polyposis-like phenotype in mice[40]. BMP-4 expression is normally limited to the mesenchymal compartment of the murine intestine, suggesting that disruption of this mesenchymal signal is involved in mediating juvenile polyposis.

Notwithstanding the concept that faulty transmission or receipt of mesenchymal signals by the epithelium may trigger juvenile changes, others have found that homozygous *SMAD4* deletions are limited to the epithelium of juvenile polyps from JPS patients with germline *SMAD4* mutations and in *Smad4* knockout mice[11,41]. Although further studies are needed, this suggests that *SMAD4* may act as a "gatekeeper", instead of a "landscaper" in JPS pathogenesis, consistent with the role of *SMAD4* in other cancer types[42].

MANAGEMENT

Management of JPS is mainly based on expert opinion[23,43-45]. Patients at risk or with a high suspicion of JPS should have endoscopic screening of the colon and upper gastrointestinal tract at age 15 or at the time of first symptoms[44]. At diagnosis of JPS, the entire gastrointestinal tract should be examined for the presence of polyps[23]. Genetic testing can be useful for at-risk members from families, where germline mutations have been identified. If no germline mutation is found in at-risk persons, then they do not have JPS and can be followed according to the guidelines for screening programs for the general population[44].

Endoscopic examination of the colon and upper gastrointestinal tract is recommended every two to three years in patients with JPS. In patients with polyps, endoscopic screening should be performed yearly, until the patient is deemed polyp-free. Patients with mild polyposis can be managed by frequent endoscopic examinations and polypectomy[23,36,44]. Intraoperative enteroscopy to evaluate small intestinal polyps can be considered at the time of colorectal surgery[16]. Endoscopic treatment of gastric polyps can be difficult, and patients with symptomatic gastric polyposis (e.g., severe anaemia) may need subtotal or total gastrectomy.

Prophylactic surgery is considered in patients with colorectal polyposis unmanageable by endoscopy (> 50-100 polyps), those with severe gastrointestinal bleeding or diarrhoea, juvenile polyps with dysplasia, and patients with a strong family history of colorectal cancer[35-37]. Surgical options include subtotal colectomy with ileorectal anastomosis, or total proctocolectomy with pouch[35,36]. Analogous to familial adenomatous polyposis, surgical type may depend on the extent of rectal polyposis. Recurrence of rectal polyps in patients with subtotal colectomy is frequent, and about half of these individuals require subsequent proctectomy[35,36]. Therefore, total proctocolectomy has been advocated as the initial surgery for patients with massive juvenile polyposis, who are unable to be managed endoscopically[36]. Although the surgery of choice in JPS remains debatable, patients need frequent post-operative endoscopic surveillance because of the high recurrence rates of polyps in the remnant rectum and the pouch[35].

In JPS patients with a germline *SMAD4* mutation, screening should be considered for signs of hereditary hemorrhagic teleangiectasia, including chest radiography for arteriovenous malformations, magnetic resonance imaging of the brain, and liver sonography[31]. Digital clubbing and pulmonary osteoarthropathy are frequently described in combination with arteriovenous malformations[17].

COX-2 expression is higher in JPS polyps than in sporadic juvenile polyps and correlates with polyp size and dysplasia[46]. This observation suggests that chemoprevention using selective or non-selective *COX-2* inhibitors could be beneficial in JPS. Currently, nonsteroidal anti-in-

flammatory drugs (NSAID) chemoprevention in JPS has not been systematically studied; however, two JPS patients who had undergone proctocolectomy with pouch reconstruction and subsequent polypectomy from the pouch had no further polyp development in the pouch while on sulindac[35]. However, the value of NSAID chemoprevention in JPS requires further investigation.

CONCLUSION

JPS is a rare hamartomatous polyposis syndrome characterized by the presence of multiple distinct juvenile polyps in the gastrointestinal tract. The primary defect in JPS may be stromal rather than epithelial. This so-called 'landscaper' defect may ultimately lead to neoplastic transformation in the overlying epithelium, although the polyps are not neoplastic per se. On the contrary, juvenile polyps may be considered true hamartomas, i.e., anomalies in the developmental patterning of the gut. Juvenile polyposis syndrome is, therefore, a unique model for studying carcinogenesis in the gastrointestinal tract.

Although rare, recognition of this condition is important in view of the consequences for patients and their families. Each clinician confronted with the diagnosis of a juvenile polyp should consider the possibility of juvenile polyposis syndrome. The number of juvenile polyps should be documented, along with the family history of gastrointestinal polyps and cancer. If a patient fulfils the clinical criteria of JPS, further diagnostic evaluation is indicated.

Future studies on the molecular and clinical aspects of JPS will result in a better understanding of gastrointestinal carcinogenesis and improved management of patients afflicted by this disorder.

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EDITORIAL

Proteome-based biomarkers in pancreatic cancer

Chen Sun, Ann H Rosendahl, Daniel Ansari, Roland Andersson

Chen Sun, Ann H Rosendahl, Daniel Ansari, Roland Andersson, Department of Surgery, Clinical Sciences Lund, Skåne University Hospital and Lund University, SE-221 85 Lund, Sweden
Chen Sun, Harbin Medical University, Harbin 150081, Heilongjiang Province, China
Author contributions: Sun C collected the references and drafted the article; Rosendahl AH and Ansari D revised the manuscript; Andersson R conceived the idea and finalized the revision; all authors read and approved the final manuscript.
Correspondence to: Roland Andersson, MD, PhD, Department of Surgery, Clinical Sciences Lund, Skåne University Hospital Lund and Lund University, SE-221 85 Lund, Sweden. roland.andersson@med.lu.se
Telephone: +46-46-172359 Fax: +46-46-147298
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Abstract

Pancreatic cancer, as a highly malignant cancer and the fourth cause of cancer-related death in world, is characterized by dismal prognosis, due to rapid disease progression, highly invasive tumour phenotype, and resistance to chemotherapy. Despite significant advances in treatment of the disease during the past decade, the survival rate is little improved. A contributory factor to the poor outcome is the lack of appropriate sensitive and specific biomarkers for early diagnosis. Furthermore, biomarkers for targeting, directing and assessing therapeutic intervention, as well as for detection of residual or recurrent cancer are also needed. Thus, the identification of adequate biomarkers in pancreatic cancer is of extreme importance. Recently, accompanying the development of proteomic technology and devices, more and more potential biomarkers have appeared and are being reported. In this review, we provide an overview of the role of proteome-based biomarkers in pancreatic cancer, including tissue, serum, juice, urine and cell lines. We also discuss the possible mechanism and prospects in the future. That information hopefully might be helpful for further research in the field.

Key words: Biomarkers; Mass spectrometry; Pancreatic cancer; Proteome; Screening

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INTRODUCTION

Pancreatic cancer is a highly lethal disease and despite continuous research efforts, results have only marginally improved patient outcome with minor overall changes in death rate over the last four decades. Pancreatic cancer is the fourth cause of cancer-related death and 36 800 pancreatic cancer-related deaths were reported in the United States in 2010, corresponding to 6.5% of all deaths from cancer[1]. Similar overall observations are reported from the other parts of the Western world[2-5].

Surgical resectable pancreatic cancer is associated with an improved outcome, especially if the diagnosis is obtained in an early phase. Regrettably, most symptoms, including e.g., profound weight loss, abdominal pain, new onset type 2 diabetes mellitus, jaundice and nausea, are usually vague and occur late during the course of disease. Only 20% of patients with pancreatic cancer are candidates for a potentially curative resection[6]. Efficient tumor markers for population screening are absent. Current markers used for pancreatic cancer, especially carcinoembryonic antigen and cancer antigen 19-9 (CA19-9), lack appropriate sensitivity and specificity. Biomarkers for therapeutic assessment, detection of residual or recurrent cancer and even for targeted therapy in pancreatic cancer in a more customized fashion are needed. The identification of biomarkers in pancreatic cancer is thus essential for improving outcome.

The development of proteomic techniques has increased the interest for clinical applications of biomarkers in pancreatic cancer. However, the identification of suitable biomarkers with good sensitivity and specificity for clinical use in pancreatic cancer has been sparse. In this review, we focus on potential proteome-based biomarkers to be used in pancreatic cancer (Table 1), hopefully indicative for further research within the field.

PROTEOMIC-BASED BIOMARKERS IN PANCREATIC CANCER TISSUE

Pancreatic cancer tissue is the most direct source of proteomic biomarkers for cancer detection, as it is likely to have the highest concentrations of cancer-specific markers. However, there are two major reasons that make it less available for cancer screening. Invasive biopsy material for screening is usually not readily available, and percutaneous biopsies might even result in seeding of cancer cells. Pathological evaluation renders the final diagnosis and is the best choice when pancreatic cancer tissue is available. Ongoing biomarker research obtained from pancreatic cancer tissue is not only done for diagnostic purposes, but also for the development of potential future targeted therapies.

When comparing pancreatic cancer tissue with normal pancreatic tissue by MALDI-TOF-MS, the levels of galectin-3 and calgizzarin (S100A11) protein were found to be 3-fold higher in cancer patients[7]. Galectin-3 is a member of a family of β-galactoside-binding animal lectins, and has been found helpful in diagnosing e.g., thyroid cancer[7], but is also up-regulated in liver, stomach, and tongue cancers. In the same family, galectin-1 has also been identified as a potential biomarker[8,9]. S100A11, a calcium-binding protein and a member of the S100 protein family is expressed in the nucleus and cytoplasm. S100 proteins regulate a number of cellular processes like cell growth, cell cycle, differentiation, transcription and secretion. S100 proteins have been reported overexpressed in different cancers, like breast and thyroid cancer[10]. Other studies have shown up-regulation of S100A6, S100A8, S100A9 and S100A10 in pancreatic cancer[11-13]. Using laser capture microdissection and 2-DE to analyze protein expression in stromal components of pancreatic cancer, it was demonstrated that high levels of S100A8 and S100A9 were present in the tumor-associated stroma but not in benign or malignant epithelium[12]. Immunohistochemistry confirmed high levels of both S100A8 and S100A9 in specific stromal cells, which were later identified as monocytes or immature macrophages ($CD14^+$/CD68). In a subset of these cells, S100A8 and S100A9 were co-expressed, and this relationship appeared to be influenced by the Smad4 status of the corresponding tumor cells. This study provides further evidence of the complex tumor-stroma interaction and demonstrates that stromal tissue can become a novel and highly promising source of biomarkers.

The differential diagnosis between pancreatic cancer and mass-forming chronic pancreatitis is clinically challenging. A large-scale immunoblotting analysis with more than 900 primary antibodies was performed on cancer tissue, chronic pancreatitis and normal pancreas[14]. A total of 30 proteins were found to be differentially expressed between chronic pancreatitis and normal pancreas, while 102 proteins were different between pancreatic cancer and normal pancreas. Several proteins, such as UHRF1, ATP7A and AOX1, differed in their expression between chronic pancreatitis and pancreatic cancer, suggesting their importance in pancreatic carcinogenesis. The combination of these proteins can become a useful diagnostic tool for endoscopic ultrasonography-guided fine needle aspiration specimens obtained before surgery or treatment.

Pancreatic cancer (PDAC) develops through several phases of pancreatic intraepithelial neoplasia (PanINs) lesions from benign to fully malignant. Pancreatic cancer pathology may be helpful for diagnosis and treatment by providing knowledge on which phases the patient is in. Despite research showing different genetic alternations during different phases, such as *K-ras* in the early PanIN-1A/B, *p16* in the intermediate PanIN-2 and *p53* in the late PanIN-3 phases[15], biological mechanisms still remain largely unclear. One reason is the difficulty in studying early molecular changes in pancreatic cancer, due to lack of suitable tissue specimens, as patients in the early phase often are without existing symptoms, and thus tissue samples are not available. Plectin-1 has been shown to be up-regulated in PanINs and in PDAC in genetic defect mouse models and early-stage pancreatic cancer cell lines[16]. Plectin-1 is a large 500 kDa protein associated with filamentous-actin, microtubules and intermediate filaments. Plectin-1 was found to be exclusively associated with mitochondria and may thus provide an important link of this organelle with the intermediate filament system[17]. Plectin-1 can also bind specific peptides, which may be helpful in detecting precursor lesions and PDAC, when conjugated to magnetofluorescent nanoparticles.

Proteome changes of pancreatic cancer tissue during different stages have been identified by 2-DE. Five candidate protein biomarkers were selected from a total of 31 identified nonredundant proteins, including 14-3-3 sigma, major vault protein (MVP), anterior gradient 2 (AGR2) and Annexin A4[13]. AGR2 is increased early on during tumor progression, and is also present in pancreatic juice[18]. MVP expression, associated with the PI3K pathway, and 14-3-3 sigma were found to be increased in PanIN-2 and -3[19,20]. On the other hand, annexin A4 was down-regulated. Annexin A4 is a Ca^{2+}- and phospholipid-binding protein like annexin A2, which previously has been reported in PDAC[21].

To decrease the complexity and large dynamic range of proteins found in pancreatic tissue samples, subcellular fractionation with mass spectrometric techniques has been used to identify potential biomarkers associated with pancreatic cancer. McKinney *et al*[22] and colleagues

Table 1 A selection of potential biomarkers for pancreatic cancer

Potential biomarkers for pancreatic cancer	Expression	Proteomic tools	Ref.
Tissue			
14-3-3 sigma	+	2-D SDS PAGE, MS	[13]
Annexin A4	+	2-D SDS PAGE, MS	[13]
Anterior gradient 2	+	2-D SDS PAGE, MS	[13]
AOX1	-	PowerBlot	[14]
ATP7A	+	PowerBlot	[14]
Biglycan	+	SDS-PAGE, LC-MS/MS	[22]
Galectin-1	+	2-D SDS PAGE, MS	[9]
Galectin-3	+	MALDI-TOF-MS	[7]
Gelsolin	-	Proteomic chip	[23]
Major vault protein	+	2-D SDS PAGE, MS	[13]
Pigment epithelium-derived factor	+	SDS-PAGE, LC-MS/MS	[22]
Plectin-1	+	Western blotting	[16]
S100A6	+	2-D SDS PAGE, MS	[11]
S100A8 (stroma)	+	2DE, LC-MS/MS	[12]
S100A9 (stroma)	+	2DE, MALDI-TOF-MS	[12]
S100A10	+	2-D SDS PAGE, MS	[13]
S100A11	+	MALDI-TOF-MS	[7]
Thrombospondin-2	+	SDS-PAGE, LC-MS/MS	[22]
βIGH3	+	SDS-PAGE, LC-MS/MS	[22]
UHRF1	+	PowerBlot	[14]
Serum/plasma			
α-1B-glycoprotein precursor	+	DIGE, MS/MS	[38]
Anterior gradient 2	+	iTRAQ, MS/MS	[18]
Apolipoprotein A-Ⅱ	-	SELDI-TOF, MS	[24]
Apolipoprotein C-Ⅰ	+	SELDI-TOF, MS	[24]
Caldecrin	-	ICAT, MS	[43]
CXCL 7	-	LC-MS	[29]
DJ-1	+	DIGE, MS/MS	[38]
Fibrinogen β chain	+	ICAT, MS	[43]
HSP27	+	Protein-chip technology	[33]
Pancreatic juice			
Lithostathine I α	-	2DE, MALDI-TOF-MS	[40]
Matrix metalloproteinase-9	+	DIGE, MS/MS	[38]
Neural cell adhesion molecule L1	+	ICAT, MS	[43]
p-Akt	+	Bio-Plex suspension array	[32]
p-ERK1/2	+	Bio-Plex suspension array	[32]
Phosphor-cAMP response element binding protein	+	Bio-Plex suspension array	[32]
Phosphor-p90 ribosomal S6 kinase	+	Bio-Plex suspension array	[32]
p-IκB-α	+	Bio-Plex suspension array	[32]
Plasminogen	+	ICAT, MS	[43]
Platelet factor 4	-	MALDI-TOF	[31]
p-MEK1	+	Bio-Plex suspension array	[32]
Transthyretin	+	2DE, MALDI-TOF-MS	[45]
Urine			
Annexin A2	-	2-D SDS PAGE	[46]
CD59	-	2-D SDS PAGE	[46]
Gelsolin	-	2-D SDS PAGE	[46]
Cell lines			
Apoprotein E	+	SILAC	[48]
Cadherin	Not in metastatic tumor cell	LC-MS/MS	[52]
Catenin	Not in metastatic tumor cell	LC-MS/MS	[52]
CD9	+	SILAC	[48]
Fibronectin receptor	+	SILAC	[48]
Galectin	Not in primary tumor cell	LC-MS/MS	[52]
Glucagon	+	Protein array	[53]
Integrin	Not in metastatic tumor cell	LC-MS/MS	[52]
Perlecan	+	LC-MS/MS	[48]
Prolactin	-	Protein array	[53]

SILAC: Stable isotope labelling with amino acids in cell culture; DIGE: Difference gel electrophoresis; MS: Mass spectrometry; iTRAQ: Isobaric tags for relative and absolute quantification; ICAT: Isotope-coded affinity tag; βIGH3: Ig-h3 precursor; A1BG: α-1B-glycoprotein precursor; +: Up-regulated; –: Down-regulated expression in pancreatic cancer as compared with controls.

identified 2393 unique proteins in normal and pancreatic tissue with cancer, and determined 104 proteins that were significantly changed in pancreatic cancer. Four secreted and up-regulated proteins have been validated as potential

biomarkers for diagnosing pancreatic cancer, biglycan, pigment epithelium-derived factor, thrombospondin-2 and transforming growth factor β induced protein Ig-h3 precursor, though data for sensitivity and specificity for these markers are not yet available.

Information on lymph node metastasis is very important for the surgical strategy-making and also for deciding other additional treatments (e.g., chemotherapy). Proteome comparison of pancreatic cancer tissue with corresponding non-cancerous normal tissue obtained from the same patients on antibody capture-based proteomic chips[23] identified gelsolin as a candidate biomarker for detection of lymph node metastasis in pancreatic cancer. Gelsolin is an important actin-binding protein that plays a major role in maintaining an organized actin cytoskeleton. The expression of gelsolin in pancreatic ductal adenocarcinomas with lymph node involvement (71.4%) was reported markedly increased as compared to lymph node negative pancreatic cancers (20%)[23].

BIOMARKERS IN BODY FLUIDS

Serum and plasma

Blood is the most frequently used source for biomarkers, being minimally invasive, easily accessible, generally inexpensive and reproducible to obtain and analyse. However, some highly abundant proteins, such as albumin or globulin, can affect the detection of less abundant, but for the diagnosis, valuable proteins.

One study aimed to identify biomarkers in a total of 319 serum samples from pancreatic cancer patients and controls. Using SELDI-TOF MS technology, 21 peaks were identified to be differentially expressed between pancreatic cancer and disease controls (DC), and 18 peaks between pancreatic cancer and healthy volunteers (HV)[24]. Apolipoprotein C-Ⅰ (ApoC-Ⅰ) and apolipoprotein A-Ⅱ (ApoA-Ⅱ) were significantly increased and decreased, respectively. ApoC-Ⅰ plays an important role in controlling plasma lipid metabolism, and is expressed in gastric, breast and pancreatic cancer[25]. ApoA-Ⅱ is present on the surface of lipid particles and may play a diagnostic role in prostate cancer[26]. The receiver operating characteristic area under the curve (AUC) of ApoA-Ⅱ, ApoC-Ⅰ and CA19-9 was greater than that of CA19-9 alone for pancreatic cancer *vs* DC (0.90 *vs* 0.84) and for pancreatic cancer *vs* HV (0.96 *vs* 0.90), results supported by others[27].

CXCL-7 is a chemokine member of the angiogenic ELRb CXC chemokine family, expressed within the megakaryocyte lineage[28]. Using a novel combination of hollow fiber membrane-based low-molecular-weight protein enrichment and LC-MS-based quantitative shotgun proteomics identified a peptide derived from CXCL-7 to be significantly reduced in pancreatic cancer patients[29]. These authors compared the plasma proteome in a small cohort (24 patients with pancreatic cancer and 21 healthy controls) to get 53 009 MS peaks. They then further validated their CXCL-7 finding in an independent blinded cohort (n = 237) using a high-density reverse-phase protein microarray. Combination with CXCL-7 significantly improved the AUC value of CA19-9 to 0.961. However, in this study, the precise molecular mechanisms explaining the CXCL-7 reduction in patients with pancreatic cancer remained unclear. Platelet factor 4 (PF4) is another member of the CXC chemokine family (CXCL-4), and is present in α-granules of all mammalian platelets, as well as in the granules of mast cells[30]. PF4 had been identified as a potential marker for pancreatic cancer by MALDI-TOF-MS-based clinical serum profiling in 80 samples[31]. Validation by ELISA techniques in 40 serum samples showed the AUCs of PF4 concentrations used for the discrimination between healthy controls and pancreatic cancer was 0.833. The discrimination between patients with pancreatic cancer and acute pancreatitis was 0.829.

Protein phosphorylation is one of the most common ways of modifying biological systems, including the carcinogenic progress. Several phosphoprotein levels were significantly increased in serum from pancreatic cancer patients as compared to controls. Six candidate phosphoproteins have been found in serum of pancreatic cancer patients by using a Bio-Plex suspension array; p-ERK1/2, p-MEK1, phospho-p90 ribosomal S6 kinase (p-p90RSK), phospho-cAMP response element binding protein (p-CREB), p-Akt and p-IκB-α[32]. These phosphoproteins are associated with the Ras/Raf/MEK/ERK signalling pathway, which is a dominating growth promoting pathway in pancreatic carcinomas. Further data from the same study showed a simultaneous increase in phospho- and total-ERK1/2 with a positive correlation to pancreatic cancer patients. In detecting pancreatic cancer, a combination of p-ERK1/2 and CA19-9 can potentially avoid false-negatives (87.2%) and improve the discriminatory power.

Heat shock protein 27 (HSP27) is a powerful molecular chaperone that can prevent the aggregation of nascent and stress-induced misfolded proteins[33]. HSP27 has been identified in serum of pancreatic cancer patients by ProteinChip technology and 2-DE. HSP27 was found to be up-regulated in pancreatic cancer as compared with normal tissue, with a sensitivity of 100% and a specificity of 84% in the detection of pancreatic cancer, and has further been suggested to play an important role in gemcitabine resistance[34,35].

Pancreatic juice

Pancreatic juice is rich in proteins directly secreted from pancreatic ductal cancer cells and should therefore constitute a perfect source for specific protein biomarkers for pancreatic cancer detection. However, pancreatic juice is not readily accessible and in addition, the endoscopic retrograde cholangiopancreatography procedure per se, in order to obtain pancreatic juice, may induce acute pancreatitis in 4%-7% of patients[36]. To date approximately 170 proteins have been identified in human pancreatic juice, one third of which are enzymes[37].

When comparing pancreatic juice from 9 PDAC patients and 9 healthy volunteers by using difference gel electrophoresis and tandem mass spectrometry (MS/MS), three potential biomarkers were identified: matrix metalloproteinase-9 (MMP-9), oncogene DJ1 (DJ-1) and α-1B-glycoprotein precursor (A1BG)[38]. DJ-1 is a mitogen-dependent protein involved in the Ras signalling pathway, reported to be increased in serum from pancreatic cancer patients[33,39]. A1BG, a secreted plasma protein from the immunoglobulin superfamily, was also increased in the cytoplasm of malignant epithelia in 86.3% of pancreatic cancer tissue specimens.

Obstruction of the main pancreatic ducts may alter the protein composition of pancreatic juice. Comparing the 2-DE profiles of pancreatic juice from a patient with pancreatic body cancer and a patient with benign pancreatic disease, it was found that blockade of juice secretion strongly affected protein composition[40]. A subsequent analysis of patients with comparable obstruction of the pancreatic ducts was performed. The isomeric form of lithostathine I α was identified as one of five protein spots that were consistently reduced in pancreatic cancer.

Quantitative proteomic analysis using stable isotope labelling (iTRAQ) and MS/MS were applied to identify proteins abnormalities, elevated in the pancreatic juice from PanIN-3 patients[18]. Anterior gradient-2 (AGR2) was significantly increased in PanIN-3 juice samples among 20 differently expressed proteins. AGR2 is a secreted protein and over-expressed in many cancers, including pancreatic cancer and influences pancreatic cancer cell proliferation and invasion. Further analyses showed that AGR2 had 67% sensitivity and 90% specificity in predicting PanIN-3 in pancreatic juice samples[41,42]. Proteomics can also be used to differentiate pancreatic cancer from pancreatitis. In a comparative study between pancreatic cancer and pancreatitis by Isotope-Coded Affinity Tag and MS, 72 variable proteins were identified in pancreatic juice[43]. Some of the identified proteins, including fibrinogen β-chain, plasminogen, neural cell adhesion molecule L1 and caldecrin, demonstrated at least a 2-fold change in abundance in pancreatic juice. In addition, 9 proteins (hemoglobin, fibrinogen, trypsin Ⅰ, trypsin Ⅱ, chymotrypsinogen b, Ig-α1 chain c region, Ig-μ chain c region, ribonuclease, and human serum albumin) were up-regulated both in the pancreatic juice of pancreatitis and pancreatic cancer patients.

Transthyretin (TTR) was identified as a potential protein biomarker in pancreatic juice for the detection of pancreatic cancer. Using 2-DE and MALDI-TOF-MS, it was shown that TTR in the pancreatic juice increased more than 2-fold in pancreatic cancer as compared with chronic pancreatitis and choledocholithiasis[44]. However, TTR was only present in islet cells and not expressed in pancreatic cancer cells, in line with what has been reported by others[45].

Urine

Urine is a potential source of biomarkers, as it is easily and noninvasively available. However, a limitation of urine is the dilution of the proteins of potential interest. Secondly, the urine is derived from the kidneys, only being "in contact" with the pancreas through blood, and most of the proteomic information exists in circulating blood.

Using proteomic techniques multiple deregulated proteins were detected in urine samples from patients with pancreatic cancer, implicating urine to potentially be a valuable source of biomarkers for pancreatic cancer[46]. Five potential protein biomarkers (including annexin A2, gelsolin, CD59 and S100A9) from a total of 127 statistically valid and differentially expressed protein spots were identified, most of which have been reported associated with pancreatic cancer in other studies.

BIOMARKERS IN CELL LINES

Cell lines are the most easily obtained proteomic source. This allows analysis of secreted proteins. The most relevant limitation when using data obtained from cell lines is that it may not be representative for primary tissue samples in the clinical setting. Thus, few studies have used cell lines for identifying biomarkers of relevance in pancreatic cancer[47].

By analysis of secreted proteins between Panc-1 pancreatic cancer cells and immortalized non-neoplastic HPDE cells, 145 differentially secreted proteins were identified. Several proteins were validated by immunohistochemistry, such as CD9, perlecan, apoprotein E (ApoE) and fibronectin receptor[48]. CD9 is a membrane protein expressed on the surface of human platelets. CD9 plays a role in many cellular functions, like adhesion, migration, signal transduction and differentiation[49]. Perlecan is involved in angiogenesis and growth, as a receptor for basic fibroblast growth factor[50]. ApoE is a protein component of lipoproteins that has anti-tumor activity in pancreatic cancer[51]. Fibronectin receptor is another member of the integrin family.

Development of metastases, as part of the progress of pancreatic cancer, evidently involves a number of important proteins. Proteomic research comparing primary and metastatic PDAC cell lines can reveal functional proteins, which are helpful for the prediction of metastasis and potential therapy against this process. One metastatic PDAC cell line, AsPC-1 and one primary PDAC cell line, BxPC-3, were studied for this purpose[52]. Using SDS-PAGE and LC-MS/MS, 221 and 208 proteins were identified from AsPC-1 and BxPC-3 cells, respectively, with 109 proteins present in both cell lines. Analysis of other proteins showed different levels in the two cell lines, including catenin, cadherins, integrins, galectins, annexins and collagens. Cadherins are a class of type-1 transmembrane proteins that depend on calcium ions and combined complexes with catenin to mediate cell adhesion. They were all found in primary tumor cells (BxPC-3), but not in metastatic tumor cells (AsPC-1), suggesting a defect in cellular adhesion in metastatic AsPC-1 cells. Integrins are glycoprotein members that form heterodimeric receptors.

Integrin α2 and α5, which represent major adhesion molecules, were only identified in BxPC-3 cells. Conversely, galectins, as carbohydrate-binding proteins on the cell surface and extracellular glycoproteins, were found only in AsPC-1. Most of these proteins play a role in tumor cell adhesion and motility.

Springbio Antibody Microarrays were used to detect different proteins between the pancreatic cancer cell lines SW1990 and SW1990HM, highly liver metastatic-related cell lines[53]. Increased glucagon and decreased prolactin were selected as potential biomarkers for cancer detection from 40 reproducible, altered proteins. Glucagon induces glucose production and regulates carbohydrate and protein metabolism. Prolactin is a hormone released by the pituitary gland with effects on female breast development and milk production. Both are localized at the plasma membrane, and can influence tumor cell adhesion.

FUTURE ASPECTS

Compared with other types of cancers, pancreatic cancer is probably one of the solid tumors with the highest levels of genetic alterations resulting in aberrant expression of a large number of proteins. A panel of proteomic biomarkers with the appropriate combination of high sensitivity and specificity will likely be better than a single biomarker. Many researchers have focused on proteomic profiling for pancreatic cancer detection using a combined biomarker approach and results so far have gained interest[54-56]. In addition, some studies have investigated differentially expressed proteins of pancreatic cancer stem cells, where increasing evidence indicates an important role in tumorigenesis, growth and formation of metastasis[57]. Targeting and eliminating pancreatic cancer stem cells may significantly improve the prognosis and avoid recurrence in pancreatic cancer patients after pancreatic resection[58].

CONCLUSION

Due to the characteristics of pancreatic cancer, with often vague symptoms, but associated tumor aggressiveness, resistance to standard therapy and a poor prognosis, identification of sensitive and specific biomarkers is essential. Such biomarkers would be of extreme value for disease detection during an earlier phase. Up to now, despite the development of novel techniques and potential markers reported, only a limited number may be of potential use in the clinical situation. Research on pancreatic cancer biomarkers is, however, intensive and the use of proteomic technology may provide a completely novel tool and possibility of potential improvement, achieving early diagnosis, targeted therapy, and discovery of recurrence in patients with pancreatic cancer.

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REVIEW

Hepatitis B virus infection and the risk of hepatocellular carcinoma

Ya-Jun Tan

Ya-Jun Tan, Department of Laboratory Medicine, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
Author contributions: Tan YJ wrote the paper.
Correspondence to: Ya-Jun Tan, MS, Department of Laboratory Medicine, The First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. tyj7713@163.com
Telephone: +86-571-87236383 Fax: +86-571-87236383
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Abstract

Epidemiological studies have provided overwhelming evidence for a causal role of chronic hepatitis B virus (HBV) infection in the development of hepatocellular carcinoma (HCC). However, the pathogenesis of HBV infection and carcinogenesis of HBV-associated HCC are still elusive. This review will summarize the current knowledge on the mechanisms involved in HBV-related liver carcinogenesis. The role of HBV in tumor formation appears to be complex, and may involve both direct and indirect mechanisms. Integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion, and it has been shown to enhance the host chromosomal instability, leading to large inverted duplications, deletions and chromosomal translocations. It has been shown that the rate of chromosomal alterations is increased significantly in HBV-related tumors. Prolonged expression of the viral regulatory HBV x protein may contribute to regulating cellular transcription, protein degradation, proliferation, and apoptotic signaling pathways, and it plays a critical role in the development of hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; Hepatitis B virus infection; Hepatitis B virus genotypic variations; Hepatitis B virus x protein

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the third most common cause of cancer mortality[1,2]. This tumor, which arises from hepatocytes, is often associated with liver cirrhosis resulting from chronic liver diseases. Among the environmental risk factors, the prevalence of chronic hepatitis B and C virus infections is linked directly to the incidence of HCC. There is now evidence for persistence within the tumor cells of a low level HBV multiplication potential. Hepatitis B virus (HBV) DNA replicative molecules and covalently closed circular DNA (cccDNA) are detectable by polymerase chain reaction (PCR). Moreover, the association between HCC and HBV recurrence after liver transplantation, and the detection of cccDNA in HCC cells point toward the possibility of HBV replication in tumor cells. The latter could act as potential reservoirs for HBV recurrence, especially in patients who present with a recurrence of HCC[3]. So far, chronic and persistent infection with hepatitis B virus is a major risk factor for the development of HCC.

Globally, it is estimated that 350 million people are chronically infected with the HBV[4]. Approximately 25% of

chronically HBV-infected individuals will develop HCC[5]. Chronic carriers of HBV have up to a 30-fold increased risk of HCC[6]. In areas of high HBV endemicity, persons with cirrhosis have an approximately 16-fold higher risk of HCC than the inactive carriers, and a 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis[7]. Although the mechanisms of oncogenesis of HBV remain obscure, several factors have been identified to be associated with a high risk of developing HCC among chronic hepatitis B (CHB) patients. HBV exerts its oncogenic potential through a multi-factorial process, which includes both indirect and direct mechanisms that likely act synergistically[8].

HEPATITIS B VIRUS INFECTION AND LIVER CARCINOGENESIS

Hepatitis B virus DNA genome is able to integrate into the cellular chromosomal DNA, causing both viral and host genome rearrangements

HBV DNA is a small, circular DNA with a highly compact genetic organization and overlapping open reading frames. It shares with retroviruses the use of reverse transcription during its replication. It is well known that the HBV DNA genome is able to integrate into the cellular chromosomal DNA, causing both viral and host genome rearrangements. HBV DNA integration enhances the instability of the host chromosome, leading to large inverted duplications, deletions and chromosomal translocations. As a result of spontaneous errors in viral reverse transcription, variations in the HBV genome occur naturally. These mutations arise during the course of chronic infection with HBV. In fact, several HBV mutant strains, including those with mutations in the Pre-C/C, core promoter and deletion in the *Pre-S/S* genes, are involved in the pathogenesis of progressive liver disease and HCC development[9]. Several studies have shown that HBV DNA insertion into cellular genes was frequent, and could occur in genes encoding for proteins that were crucial for the control of cell signaling, proliferation and apoptosis[10,11].

HBV-related HCC can also arise in the absence of significant liver damage. Many of these chromosomal segments contain key players in liver carcinogenesis such as P53, PB Wnt/β-catenin, cyclins A and D1, transforming growth factor β (TGF-β) and Ras signaling[12]. In another study HBV DNA was integrated at random sites of human DNA; the *MLL4* gene was one of the targets for integration during hepatocarcinogenesis[13]. Furthermore, viral DNA integration into the cellular DNA is not necessary for viral replication, but allows for the persistence of the viral genome in the cell. Viral DNA insertion as well as cellular DNA replication occurs during liver cell proliferation, secondary to the necrosis/apoptosis of adjacent hepatocytes.

Viral genotype and the risk of hepatocellular carcinoma

The viral genotype is another factor that affects cancer risk. Genotype C has a higher risk of causing HCC than genotype B[14,15], and genotype D has a higher cancer risk than genotype A[16]. Compared to the Asian genotypes (B and C), the European genotypes (A and D) are less well established.

Hepatitis B virus genotypic variations and the risk of hepatocellular carcinoma

Specific genotypic variations in HBV have been associated with cirrhosis and HCC. These variations include, in particular, mutations in the pre-core region (Pre-C, A1896G inside the ε structure of the genome), in the basal Core promoter (A1762T/G1764A), and in ORFs encoding PreS1/PreS2/S and Pre-C/C. There is an overlap between Pre-C or basic core promoter (BCP) mutations and genotype, since these mutations appear to be more common in genotype C as compared to other genotypes[14]. The $1762^{T}/1764^{A}$ double mutations (1762 A-to-T and 1764 G-to-A) in the BCP region were commonly found to be borne by HCC patients in some high-risk populations, and were thus suggested as potential biomarkers for hepatocarcinogenesis[17,18]. Comparison of HBV isolates from different studies indicates that the mutation rate of A1762T/G1764A is 64% for genotype C, 40% for genotype B and 35% for other genotypes[19]. Kusakabe *et al*[20] investigated a population-based cohort consisting of 19 393 subjects (middle aged or older) with a follow-up of over 13 years in Japan. They found that HBV mono-infected subjects with the A1762T/G1764A double mutation could be at high risk for HCC development during the natural course of HBV infection[20]. In addition, the 1753^{V} mutations (1753-to-C/A/G) were also associated with the progression of liver disease[21]. Li *et al*[22] evaluated the roles of genetic variations of HBV in the development of HCC in Southern Guangxi China. Their study supported the hypothesis that both the 1762T/1764A double mutations and the 1753V/1752V mutations were associated with increased risk for HCC. Fan *et al*[23] found that patients with higher viral load and genotype C had a higher incidence of 1762/1764 double mutations, and that Enhancer Ⅱ and DR1 were significantly more in the HCC group than in the CHB group, which may play an important role in HCC development via nucleotide substitution. The BCP mutations could affect the core promoter that regulates the expression of both HBeAg and the core protein, and this may be related to the higher rate of replication of genotype C. Substitutions in the BCP may increase genotype virulence by deregulating the transcription of pcARN/pgARN, increasing the risk of HCC in patients infected with genotype C[24]. Thus, the BCP overlaps with the X region of the HBV genome, and mutations in the amino acid sequence at positions 130 and 131 in this region have been proposed as prognostic markers for the development of liver cancer[9].

Yang *et al*[14] found that the Pre-C mutation (A1896G) could prevent the production of HBeAg, by introducing a premature stop codon into the ORF Pre-C/C that abolished the production of HBeAg. However, HBV DNA synthesis persisted under these conditions; this may cause liver damage with progression to cirrhosis and cancer.

Mutations in Pre-S have been reported in HCC cases compared to chronic or asymptomatic cases. These mutations, including deletions in Pre-S in the integrated HBV DNA, may impair the secretion of HBsAg, leading to increased endoplasmic reticulum and oxidative stress in hepatocytes[25]. Truncated forms of Pre-S2 have also been shown to interact with cyclin A, a critical regulator of the cell division cycle, an observation that supports a role for Pre-S2 in hepatocyte hyperplasia and a likely role in the process of HBV-related tumorigenesis[26]. Thus, deletions of Pre-S may contribute to hepatocarcinogenesis by several mechanisms.

Altogether, this combination of genomic mutations, and/or deletions, together with transcriptional and post-transcriptional regulations, will therefore allow the establishment of viral persistence, and the ongoing synthesis of HBV antigens.

DNA METHYLATION AND THE RISK OF HEPATOCELLULAR CARCINOMA

DNA methylation occurs in the early stage of cancer development, including HCC. Genomic hypomethylation increases chromosome instability while localized hypermethylation decreases tumor suppressor gene expression, thus increasing the risk of HCC development[27]. Aberrant methylation of *RASSF1A* (Ras association domain family member 1) is thought to be an early event in the development of HCC[28]. The process is catalyzed by DNA methyltransferases (DNMT). DNMT inhibitors directly repress tumor angiogenesis, indicating that epigenetic modifications mediated by DNMT are involved in the regulation of gene expression during tumor angiogenesis[29]. Another significant link has been suggested between HCC development and the silencing by DNA hypermethylation of several tumor suppressor genes (*TSGs*). A number of TSGs, including $p16^{INK4A}$, *SOCS-1*, *APC*, *GSTP1* and *E-cadherin*, are silenced by DNA methylation in a large proportion of liver tumors, and this process often starts at the preneoplastic stage[30]. In some reports, a higher rate of promoter methylation for specific genes, such as $p16^{INK4A}$ and *E-cadherin*, has been observed in HBV-related tumors compared to nonviral tumors[31].

HEPATITIS B VIRUS X PROTEIN AND THE RISK OF HEPATOCELLULAR CARCINOMA

The hepatitis B virus x protein (HBx) protein is a 154 amino acid polypeptide with a molecular mass of about 17 kDa. HBx appears to play a critical role in the development of HCC. HBx is important for HBV replication and can regulate cellular transcription, protein degradation, proliferation, and apoptotic signaling pathways (reviewed by Bouchard and Schneider[32]). HBx protein does not bind directly to DNA, but rather acts on cellular promoters by protein-protein interactions and by modulating cytoplasmic signaling pathways. The cell cycle inhibition effect of HBx was validated through a liver regeneration experiment reported by Sidorkiewicz *et al*[33]. Kuo *et al*[34] reported that HBx can downregulate Wnt/β-catenin expression and suppress cell growth by not only repressing cell proliferation, but also triggering cell apoptosis. Furthermore, Hsien *et al*[35] have found that HBx protein interacts with the tumor suppressor adenomatous polyposis coli to activate Wnt/β-catenin signaling. Wnt/β-catenin has been shown to up-regulate the epithelial cell adhesion molecule in HCC cells to promote tumor initiation and stemness[36]. Thus HBx activation of Wnt/β-catenin may promote directly the transformation of liver cells into cancer initiating cells[37]. A number of ways in which HBx protein may induce antiapoptotic effects have been described. The most important of these is the ability of HBx to inhibit p53-mediated apoptosis. Recent experiments have suggested that HBx protein may increase the expression of telomerase reverse transcriptase and telomerase activity, prolonging the lifespan of hepatocytes and contributing to malignant transformation. The protein also interferes with nucleotide excision repair through both p53-dependent and p53-independent mechanisms. Carboxyl-terminal truncated HBx protein loses its inhibitory effects on cell proliferation and pro-apoptotic properties, and it may enhance the protein's ability to transform oncogenes. Dysregulation of IGF-Ⅱ enhances the proliferation and anti-apoptotic effects of oncogenes, resulting in uncontrolled cell growth. Another possible explanation for the anti-apoptotic effect of HBx protein involves the accumulation of the anti-apoptotic protein, survivin[37]. Guo *et al*[37] found that Hep3B cells expressing HBx protein increased the levels of hepatoma upregulated protein (HURP) RNA and protein, and showed resistance to cisplatin-induced apoptosis. Knockdown of HURP in these cells reversed this effect. The anti-apoptotic effect of HBx protein was shown to require activation of the p38/mitogen activated protein kinase (MAPK) pathway. In addition, the expression of survivin was upregulated by HBx protein in an HURP-dependent manner. High levels of HURP favored the expression of the anti-apoptotic survivin in HBx-expressing cells. These results indicate that HBx protein activates the expression of HURP *via* the p38/MAPK pathway, culminating in the accumulation of survivin. In recent years, evidence has accumulated that HBx protein modulates the transcription of methyl transferases, causing regional hypermethylation of DNA that results in silencing of tumor suppressor genes, or global hypomethylation. This, in turn results in chromosomal instability, thereby playing a role in hepatocarcinogenesis.

The $p16^{INK4A}$ gene is known as an abnormal tumor suppressor gene and critical cancer-related gene in human hepatocarcinogenesis. Several studies have shown that hypermethylation of the $p16^{INK4A}$ promoter is an important early event in carcinogenesis[38]. Zhu *et al*[39] found that HBx upregulates *DNMT1* and *DNMT3A* expression at both the mRNA and protein levels, and that HBx represses $p16^{INK4A}$ expression by inducing hypermethylation of

the $p16^{INK4A}$ promoter. Moreover, HBx induces the hypermethylation of the $p16^{INK4A}$ promoter through *DNMT1* and *DNMT3A*. Regulation of *DNMT1* and *DNMT3A* by HBx promotes the hypermethylation of the $p16^{INK4A}$ promoter region[39].

Among the activities of HBx, its trans-activation function may play a crucial role in hepatocarcinogenesis, because it is involved in the activation of a large number of signaling pathways and cellular genes that are involved in oncogenesis, proliferation and inflammation. For example, HBx transactivates a number of cellular promoters and enhancers containing binding sites for nuclear factor-kappa-B, activator protein 1 (AP-1), AP-2, cellular promoters of genes associated with cell proliferation such as IL-8, TNF, TGF-β, and epidermal growth factor receptor, and cytosolic signal transduction pathways including Src kinases, Cjun N-terminal kinase, JaK1/STAT and protein kinase, which have overlapping effects on cell proliferation and viability[40,41].

CONCLUSION

The studies we have reviewed here illustrate that HBV constitutes a major environmental etiological factor for primary liver cancer in humans. It will therefore be important to analyze gene expression and proteomic changes in a large series of samples from CHB at different stages, to identify suitable prognostic markers and therapeutic targets. Furthermore, detection of the viral genomes using sensitive, PCR-based, assays is mandatory to enable an accurate appraisal of their prevalence. Genomic alterations and epigenetic factors like methylation-associated gene silencing may play an important role in the deregulation of cellular functions, leading to malignant transformation. A better understanding of the complex role of HBV in liver tumorigenesis will undoubtedly contribute to the improvement of the management of liver diseases induced by CHB.

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ORIGINAL ARTICLE

Erlotinib inhibits progression to dysplasia in a colitis-associated colon cancer model

Beatriz Pagán, Angel A Isidro, Myrella L Cruz, Yuan Ren, Domenico Coppola, Jie Wu, Caroline B Appleyard

Beatriz Pagán, Angel A Isidro, Myrella L Cruz, Caroline B Appleyard, Department of Physiology and Pharmacology, Ponce School of Medicine, Ponce, PR 00732, United States
Yuan Ren, Jie Wu, Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States
Domenico Coppola, Department of Anatomic Pathology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States
Author contributions: Appleyard CB, Wu J and Pagán B designed the research; Pagán B, Cruz ML and Ren Y performed the research; Pagán B, Isidro AA, Cruz ML, Coppola D, Appleyard CB and Wu J analyzed the data; Pagán B, Appleyard CB and Wu J wrote the paper.
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Correspondence to: Caroline B Appleyard, PhD, Professor, Department of Physiology and Pharmacology, Ponce School of Medicine, PO Box 7004, Ponce, PR 00732, United States.cappleyard@psm.edu
Telephone: +1-787-8402575 Fax: +1-787-8411040
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Abstract

AIM: To investigate the role of epidermal growth factor receptor (EGFR) in colitis-associated dysplasia using the EGFR tyrosine kinase inhibitor erlotinib.

METHODS: Sprague-Dawley rats received trinitrobenzene sulfonic acid (TNBS; 30 mg in 50% ethanol, ic), followed 6 wk later by reactivation with TNBS (5 mg/kg, iv) for 3 d. To induce colitis-associated dysplasia, rats then received TNBS (iv) twice a week for 10 wk. One group received erlotinib (10 mg/kg, ip) for 1 wk before the start of the reactivation of the colitis and 2 wk after (21 d); the rest received the vehicle. After rats were euthanized, the colons were removed and analyzed for damage and expression of the EGFR downstream effectors Erk1/2 and c-Myc.

RESULTS: Ninety percent of the vehicle-treated animals had dysplasia in any region of the colon. Erlotinib-treated animals had a significant decrease in the incidence of dysplasia compared to vehicle-treated animals in all regions of the colon (50.00% ± 11.47% *vs* 90.00% ± 10.00% in proximal, $P < 0.05$; 15.00% ± 8.19% *vs* 50.00% ± 16.67% in mid, $P < 0.05$; and 20.00% ± 9.17% *vs* 70.00% ± 15.28% in distal, $P < 0.01$). Erlotinib-treated animals also had reduced cell proliferation, reduced active Erk1/2, and reduced c-Myc in colon epithelium compared with the vehicle-treated animals. *In vitro*, erlotinib treatment was shown to markedly decrease c-Myc and pErk1/2 levels in rat epithelial cells. Proliferation of rat epithelial cells was stimulated by epidermal growth factor and inhibited by erlotinib ($P < 0.05$).

CONCLUSION: Erlotinib can decrease the development of colitis-associated dysplasia, suggesting a potential therapeutic use for erlotinib in patients with long-standing colitis.

Key words: Animal model; Epidermal growth factor receptor; Colitis; Dysplasia; Erlotinib

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INTRODUCTION

Patients with inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are at increased risk of developing colorectal cancer (CRC)[1,2]. The known association of IBD with CRC presents an identifiable population for preventative intervention. This preventive effort, however, relies on understanding the molecular events critical for progression from IBD to dysplasia and CRC and on the identification of suitable molecular targets to block the disease development.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase. Aberrant EGFR activity has been linked to different types of carcinoma, including CRC[3]. EGFR activates several signaling pathways that include Ras/Raf/Mek/Erk1/2 and phosphoinositide 3-kinase/PDK1/Akt to control epithelial cell proliferation and survival[4]. Aberrant EGFR activity, resulting in proliferative effects and anti-apoptosis, can be targeted with small molecule tyrosine kinase inhibitors or with specific antibodies. A number of EGFR tyrosine kinase inhibitors, such as erlotinib, gefitinib and lapatinib, have been developed to treat cancer patients or are in clinical development to treat various types of human cancer[3]. Moreover, two monoclonal antibodies to EGFR, cetuximab and panitumumab, have been approved by the US Food and Drug Administration to treat CRC patients[3,5].

During inflammatory processes like IBD, EGFR and its ligands play a repair role in colonic mucosa[6]. EGFR expression is increased in inflamed tissues of the bowel in animal models and in patients with IBD and colon cancer[7-9]. For example, in a study by Malecka-Panas, it was found that EGFR is increased in colonic mucosa by 35.2% in patients with adenomatous polyps, by 40.6% in patients with ulcerative colitis, and by 123% in patients with colon cancer[10]. One of the complications of long-standing IBD is the development of cancer[11]. The risk of cancer in patients with colitis increases with longer duration of the disease[1]. While it is not understood completely how IBD leads to neoplastic transformation and progression to CRC, higher levels of EGFR and its ligands could cause hyper-activation of growth promoting signaling pathways and may contribute to development of dysplasia. In the rat model of colon carcinogenesis induced by the carcinogen azoxymethane, EGFR is involved in the development of dysplastic lesions and colon cancer[12].

Erlotinib (Tarceva®) is the first EGFR tyrosine kinase inhibitor approved by the US Food and Drug Administration. It is currently used in clinics to treat lung and pancreatic cancer. In this study, we tested the effects of erlotinib on the occurrence of colitis-associated dysplasia in a rat model developed recently by us[13]. We hypothesized that, by inhibiting EGFR, the progression from chronic inflammation to dysplasia will be halted, as a result of the blockade of EGFR activity. Our data show that erlotinib significantly inhibits the colitis-induced dysplasia in this animal model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200-220 g at the start of the initial treatment were maintained in restricted-access rooms with controlled temperature (23 ℃) and 12-h light-dark cycle. Standard laboratory chow (8640 Teklad Rodent Diet, Harlan Laboratories; Tampa, FL) and drinking water were provided ad libitum. One week before beginning the protocol animals were acclimatized to avoid additional stress. Animal protocols were approved by the Institutional Animal Care and Use Committee at Ponce School of Medicine.

Induction and reactivation of the colitis and development of dysplasia

Chronic colitis was induced by intracolonic administration of trinitrobenzene sulfonic acid (TNBS; 0.5 mL of 60 mg/mL; Sigma Aldrich; St. Louis, MO) in 50% ethanol followed by reactivation with systemically administered TNBS 6 wk later[14]. The induction was performed by using a rubber catheter, and TNBS was introduced rectally into the colon, approximately 8 cm proximal to the anus. The reactivation was performed 6 wk after the induction. Briefly, the rats were lightly anesthetized with ether, and TNBS (5 mg/kg in 0.9% saline) was administered intravenously *via* a tail vein every 24 h for three consecutive days[14]. Dysplasia was developed by continuing to administer the TNBS (5 mg/kg) twice a week intravenously for 10 wk[13]. The rats were weighed weekly until they were sacrificed at 10 wk with an overdose of pentobarbital (about 1.5 mL of 65 mg/kg for rats of > 500 g). The experiments reported herein were performed in accordance with the principles described in the "Guide for the Care and Use of Laboratory Animals," publication No. DHHS (NIH) 86-23.

Drug treatment

One group of animals was treated with the EGFR inhibitor erlotinib (a kind gift of OSI Pharmaceuticals, Farmingdale, NY). Erlotinib was administered at a dose of 10 mg/kg per day (i.p. dissolved in 0.5% methyl cellulose) from 1 wk before the start of the reactivation of the colitis until 2 wk after (21 d, Figure 1)[15]. Methyl cellulose alone was administered to a control group for the same amount of time.

Measurement of macroscopic damage

After animals were euthanized, a macroscopic analysis of the colon was performed based on the criteria of Appleyard and Wallace[14]. Four variables were examined: the presence of diarrhea (0 or 1 for absence or presence), adhesions between the colon and other organs (0, 1 or 2 for none, minor or major, respectively), the thickness of each

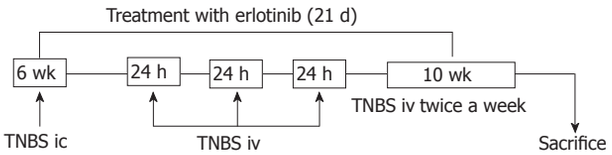

Figure 1 Experimental design for development of colitis-associated dysplasia in a rat model and timeline for administration of erlotinib (10 mg/kg, ip). TNBS: Trinitrobenzene sulfonic acid.

colon segment (in millimeters), and the degree of ulceration (0 for no damage; with increasing scores up to 10, depending on the extent of ulceration). These variables were added to give a total macroscopic damage score.

Sample collection

The colon length was measured in centimeters and cut in equal thirds representing the proximal, mid and distal parts of the colon. These segments were cut longitudinally; one half was weighed and stored at -80 ℃ for molecular analysis, and the other half was fixed in 10% buffered formalin for histological procedures. The Swiss-roll technique was used to evaluate each colon segment microscopically, allowing us to see the entire length of the intestine at once. Briefly, the tissue was rolled into a small piece with the help of forceps and then fixed and placed in a cassette for the remainder of the histological procedures[13].

Microscopic assessment

The tissues were scored microscopically for damage by a blinded observer, as previously described[13]. Criteria included loss of mucosal architecture (0-3: absent, mild, to severe), cellular infiltration (0, none; 1, in muscularis mucosae; 2, in lamina propria/villi; 3, in serosa), muscle thickening (0, muscle < 1/2 of mucosal thickness; 1, muscle = 1/2 to 3/4 of mucosal thickness; 2, muscle = mucosal thickness; 3 = all muscle), goblet cell depletion (0, absent; 1, present), and crypt abscess formation (0, absent; 1, present). The score of each variable was added to give a total microscopic damage score (maximum of 11).

Pathologic evaluation

Colonic sections (2-4 μm) stained with hematoxylin and eosin were analyzed by our pathologists in a blinded manner for dysplasia. Histologic analysis for dysplasia was scored based on previously published criteria[16,17]. Briefly, tissue sections were classified as either negative for dysplasia or positive for dysplasia or carcinoma. The tissues classified as negative for dysplasia adhered to one of the following: normal (small basally located nuclei and normal architecture), non-specific inflammation (cryptitis and glandular invasion by neutrophils), or active colitis (cryptitis, glandular invasion by neutrophils, crypt abscesses, microabscesses). A classification of positive dysplasia was characterized by low-grade dysplasia, which included hyperchromasia, loss of mucin, increased nuclear/cytoplasmic ratio, nuclear elongation and stratification, irregular nuclear outline, and increased number of normal mitoses. The criteria for high-grade dysplasia included the characteristics of low-grade dysplasia plus mucosal architectural distortion including fusion of glands (cribiform pattern) and presence of vesicular polygonal nuclei. For a diagnosis of carcinoma, the characteristics of high-grade dysplasia were included in addition to presence of atypical mitosis and/or of single tumor cells within the lamina propria[16].

Immunohistochemistry

Formalin-fixed 4 μm tissue sections were deparaffinized with xylene, 2 changes, 15 min each, and then hydrated through descending grades of ethanol to deionized water. Antigen retrieval was performed on a hot plate using a beaker with distilled water with the appropriate buffer (0.01 mol/L citrate-ethylene-diamine-tetra-acetic acid (EDTA) buffer, pH 6.0 - high to boiling or EDTA - high to boiling). Slides were cooled at room temperature for 20 min, rinsed with deionized water, and placed in phosphate-buffered saline (PBS) for 5 min. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide. After slides were washed with PBS for 5 min, they were blocked with normal serum for 20 min, followed by incubation with the primary antibody. Antibodies were used as follows: phosphorylated epidermal growth factor receptor-pY1068 (Cell Signaling; Danvers, MA), 1:400, overnight; antigen retrieval-EDTA buffer; and 5-bromo-2' deoxyuridine (BrdU), mouse monoclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA), 1:100, overnight, antigen retrieval-citrate-EDTA buffer. The secondary antibody (Bio-Genex Kit; San Ramon, CA) was added to the sections for 20 min and washed again with PBS for 4 minutes. Using the Bio-Genex Kit, we incubated sections with streptavidin-LSab-Peroxidase for 20 min and washed them with PBS for 4 min. The development of the sections was performed using 3,3'-diaminobenzidine tetrahydrochloride (Bio Genex, San Ramon, CA). All samples were lightly counterstained with Mayer's hematoxylin for 15 s, dehydrated through graded alcohol, cleared with xylene, and mounted with resinous mounting medium.

Immunohistochemistry of c-Myc was performed using a Ventana Discovery XT automated slide staining instrument. The antigen retrieval method was Ventana Cell Conditioning-1. Immunohistochemical conditions for c-Myc (ab32072, Abcam) were as follows: 1:25 dilution (60 min), Ventana UltraMAP anti-rabbit (20 min).

The stains were semiquantitatively examined by two independent pathologists using the Allred 8-unit system with a combination of a proportion score from 0 to 5 and an intensity score on a scale from 0 to 3 (none, weak, moderate, strong). A total score of 2-3 was considered low, a score of 4-5 was considered intermediate, and a score of 6-8 was considered high[18].

Western and immunoblot analysis

Proteins were extracted using lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 25 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, 1% Triton X-100, 1 mmol/L Na_3VO_4, 20 mmol/L

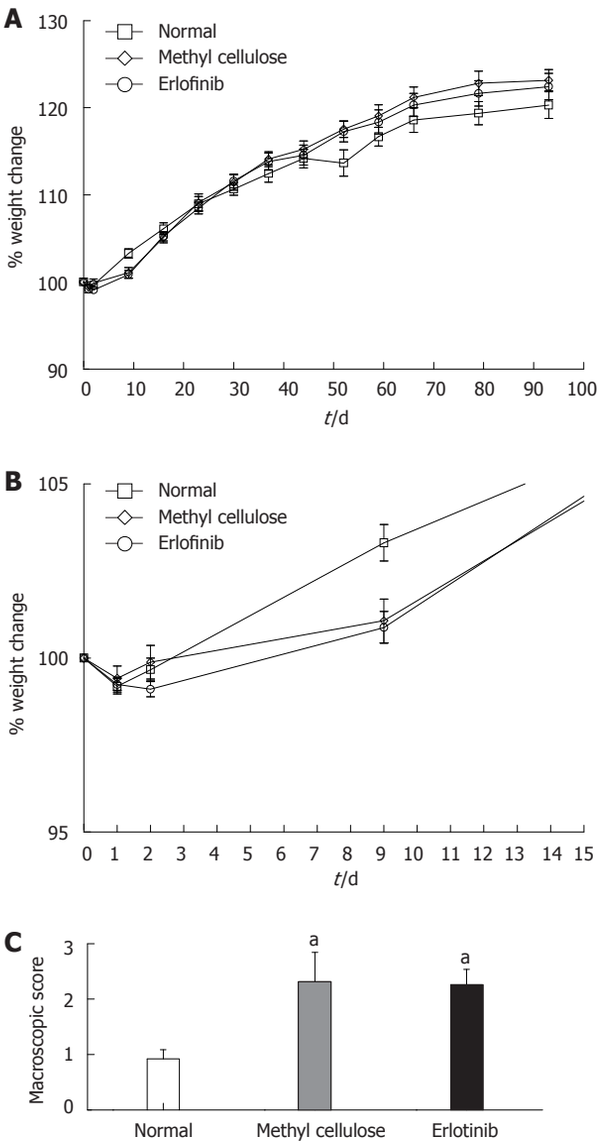

Figure 2 Erlotinib treatment did not worsen inflammation. A: Effect of erlotinib treatment on weight change in an animal model of colitis-associated dysplasia (n = 10-20 per group ± SE); B: Weight change following initial reactivation of colitis; C: Effect of erlotinib treatment on macroscopic damage score in an animal model of colitis-associated dysplasia. The average macroscopic score (including the presence of adhesions, thickness of the tissue, presence or absence of diarrhea, and grade of ulceration) was significantly higher in all trinitrobenzene sulfonic acid-treated animals whether treated with vehicle or drug ($^aP < 0.05$ *vs* normal animals, n = 12-20 ± SE).

p-nitrophenyl phosphate, 2 mg/mL leupeptin, 2 mg/mL aprotinin, and 1 mmol/L phenylmethyl-sulfonyl fluoride). Equal amounts (30 μg) of protein were separated on 12% sodium dodecyl sulfate gel and transferred to a polyvinylidene fluoride membrane (Bio Rad; Hercules, CA). Membranes were blocked with 5% non-fat dry milk in tris-buffered saline-tween (TBST) and incubated with one of the following primary antibodies: Erk1/2, pErk1/2, Akt, pAkt, Src, or Src-pY416 (Cell Signaling Technology; Danvers, MA). Membranes were washed with tris-buffered saline-tween and incubated with horseradish peroxidase-labeled secondary antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA). The bands were detected using ECL-Plus reagent kit (GE Amersham; Piscataway, NJ). Western blotting bands were quantified by densitometry using ImageQuant 5.2 Software (Typhoon 9410; GE Amersham; Piscataway, NJ). pErk1/2, pAkt, and Src-pY416 bands were normalized for the corresponding total kinase.

Cell cultures

Rat intestinal epithelial-1 (RIE-1) cell line (American type culture collection CRL-1592) was cultured in RPMI 1640 containing 5% fetal bovine serum. For analysis of c-Myc, cells were treated with erlotinib (LC Laboratories) as indicated in the figure legends and epidermal growth factor (EGF, 10 ng/mL; Rocky Hill, NJ) for 24 h. For analysis of pErk1/2, erlotinib- or mock-treated RIE-1 cells were stimulated with EGF (10 ng/mL) for 5 min. Cell lysates (20 μg/each) were analyzed by immunoblotting.

Cell proliferation was assayed by plating cells in quadruplet in 96-well plates (1000 cells/well). Twenty-four hours after plating, EGF (10 ng/mL) or erlotinib (10 μmol/L) was added. Four days later, viable cells were measured using CellTiterGlo reagent (Promega) as reported previously[19].

Statistical analysis

Values are presented as means ± SEM where "*n*" represents one tissue from one animal used for a single replicate of an experiment. Statistical analyses were performed using GraphPad Instat V3.0 and Graph Pad Prism V4.0 (Graph Pad Software, San Diego, CA). Groups were analyzed using one-way analysis of variance with Turkey's post-test, and $P < 0.05$ was considered to represent a significant difference.

RESULTS

Erlotinib treatment did not worsen inflammation

During the study (10 wk), all of the rats increased their weight in comparison with their original starting weight, apart from the first 3 d of treatment, where all groups (normal, vehicle-treated, and erlotinib-treated) lost weight (Figure 2A and B). We have observed this phenomenon in prior studies and attribute it to a combination of the intravenous administration of TNBS and the stress initially associated with the procedure[13,16]. No differences in weight change were observed between the normal, vehicle-treated, and erlotinib-treated animals during the study, suggesting no major toxicity of the drug or vehicle.

After animals were euthanized, the colons were removed to score for ulceration, adhesions, diarrhea, and thickness and to measure colon length. As expected, animals treated with TNBS and receiving the vehicle methyl cellulose had significantly higher macroscopic damage scores than the normal animals (Figure 2C). Erlotinib treatment had no effect on the macroscopic score when compared with the vehicle-treated group, with damage scores still significantly higher than normal ($P < 0.05$; Figure 2C). The average length of the colon in TNBS/vehicle-treated animals was shorter that normal (10.55 ± 0.42 cm *vs* 12.17 ± 0.38 cm). This shortening was not at-

Table 1 Effect of erlotinib treatment on microscopic damage score (means ± SE, *n* = 10-20)

	Loss of mucosal architecture	Cell infiltration	Muscle thickness	Goblet cell depletion	Crypt abscess formation	Total microscopic score
Proximal						
Normal	0.67 ± 0.19	1.58 ± 0.23	0.83 ± 0.11	0.83 ± 0.11	0.33 ± 0.14	4.25 ± 0.55
Vehicle	1.40 ± 0.16[b]	2.50 ± 0.12[b]	1.60 ± 0.22[a]	1.00 ± 0.00	0.80 ± 0.13	7.50 ± 0.27[b]
Erlotinib	1.40 ± 0.11[b]	2.35 ± 0.11[b]	1.55 ± 0.17[a]	1.00 ± 0.00	0.60 ± 0.11	6.90 ± 0.27[b]
Mid						
Normal	0.75 ± 0.18	1.50 ± 0.67	0.92 ± 0.29	0.83 ± 0.39	0.45 ± 0.51	4.42 ± 0.51
Vehicle	1.50 ± 0.22[a]	2.70 ± 0.15[b]	2.00 ± 0.26[b]	1.00 ± 0.00	0.70 ± 0.15	7.90 ± 0.53[b]
Erlotinib	1.50 ± 0.14[b]	2.35 ± 0.49[b]	1.85 ± 0.59[b]	1.00 ± 0.00	0.45 ± 0.51	7.15 ± 0.31[b]
Distal						
Normal	1.08 ± 0.26	1.92 ± 0.23	1.50 ± 0.19	0.92 ± 0.08	0.25 ± 0.13	5.67 ± 0.66
Vehicle	1.80 ± 0.20	2.70 ± 0.15[a]	2.20 ± 0.20[b]	1.00 ± 0.00	0.50 ± 0.11	8.20 ± 0.49[b]
Erlotinib	2.05 ± 0.15[b]	2.50 ± 0.14[a]	2.45 ± 0.14[b]	1.00 ± 0.00	0.75 ± 0.10[a]	8.75 ± 0.32[b]

Vehicle is methyl cellulose. One-way analysis of variance between groups. [a]$P < 0.05$, [b]$P < 0.01$ *vs* normal animals within the same colonic region.

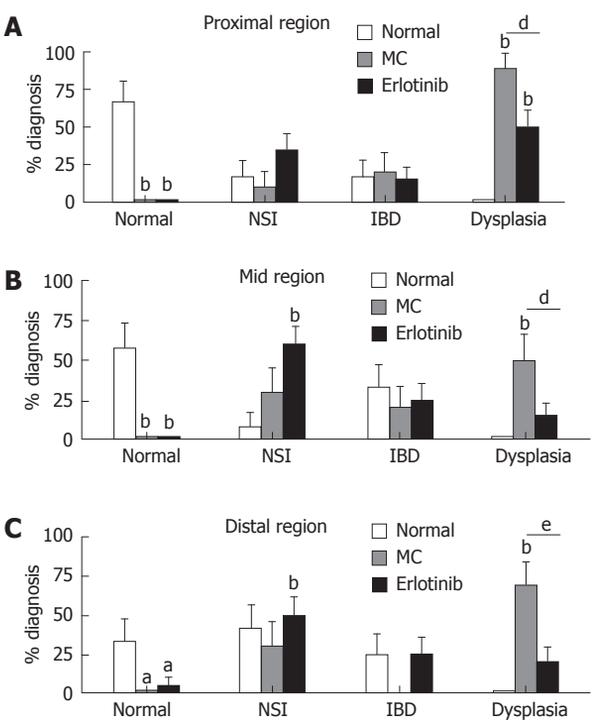

Figure 3 Effect of erlotinib treatment on pathological analysis in an animal model of colitis-associated dysplasia. The percentage of animals with the most severe diagnosis found in each region of the colon is shown ([a]$P < 0.05$, [b]$P < 0.01$ *vs* normal animals; [d]$P < 0.05$, [e]$P < 0.01$ *vs* vehicle-treated animals; *n* = 10-20 ± SE). MC: Methyl cellulose; NSI: Non-specific inflammation; IBD: Inflammatory bowel disease.

tenuated in erlotinib-treated animals (10.85 ± 0.45 cm).

Microscopic analysis of the colon revealed that total microscopic damage score was higher in all regions of the colon in animals receiving TNBS. The damage found was significantly higher in all regions of the colon from these animals than that shown in normal animals ($P < 0.01$, Table 1). Erlotinib had no effect on damage found.

Erlotinib treatment significantly decreased the occurrence of dysplasia

Pathological analysis identified areas of the colon as normal, showing inflammation (IBD and non-specific inflammation), and showing dysplasia. Ninety percent of the vehicle-treated animals had dysplasia in any region of the colon. This was decreased in the erlotinib-treated group such that only 55% of the animals had dysplasia in any area of the colon. When specific regions were analyzed, a decrease in dysplasia incidence was found in the proximal, mid, and distal regions with erlotinib treatment (50%, 15% and 20% in erlotinib *vs* 90%, 50% and 70% in vehicle). Moreover, in the erlotinib-treated group, a close to normal mucosal architecture was found, while in vehicle-treated animals a normal pathology was never observed (Figure 3). Erlotinib treatment increased non-specific inflammation in the mid-region when compared with both normal and vehicle-treated animals. No differences were observed in the identification of IBD in each region of the colon, suggesting that erlotinib may maintain animals in a milder stage of pathology, preventing progression to a more severe diagnosis (Figure 3). It was noted that some "normal" animals were found to have a finding of IBD in some areas; this may be explained by the fact that the normal animals were age-matched and, with increased age, inflammation in response to normal microflora begins to appear[20]. None of the normal animals developed dysplasia.

Erlotinib may decrease cell proliferation through Erk pathway

Proliferation of colon cells was examined by analyzing incorporation of BrdU. A significant increase in positively stained cells was found in animals treated with the vehicle compared with that shown in normal animals, suggesting that those animals have more cell proliferation (Figure 4). Erlotinib-treated animals had decreased cell proliferation compared to vehicle-treated animals, and this was not significantly different from that shown in the normal animals (Figure 4).

Attempts to examine EGFR Y1068 phosphorylation in rat tissue samples were not successful. The activation state of EGFR downstream signaling components Erk, Akt, and Src were then measured by Western blotting using phosphor-specific antibodies. Animals treated with erlotinib showed a tendency toward a decrease in the ratio of

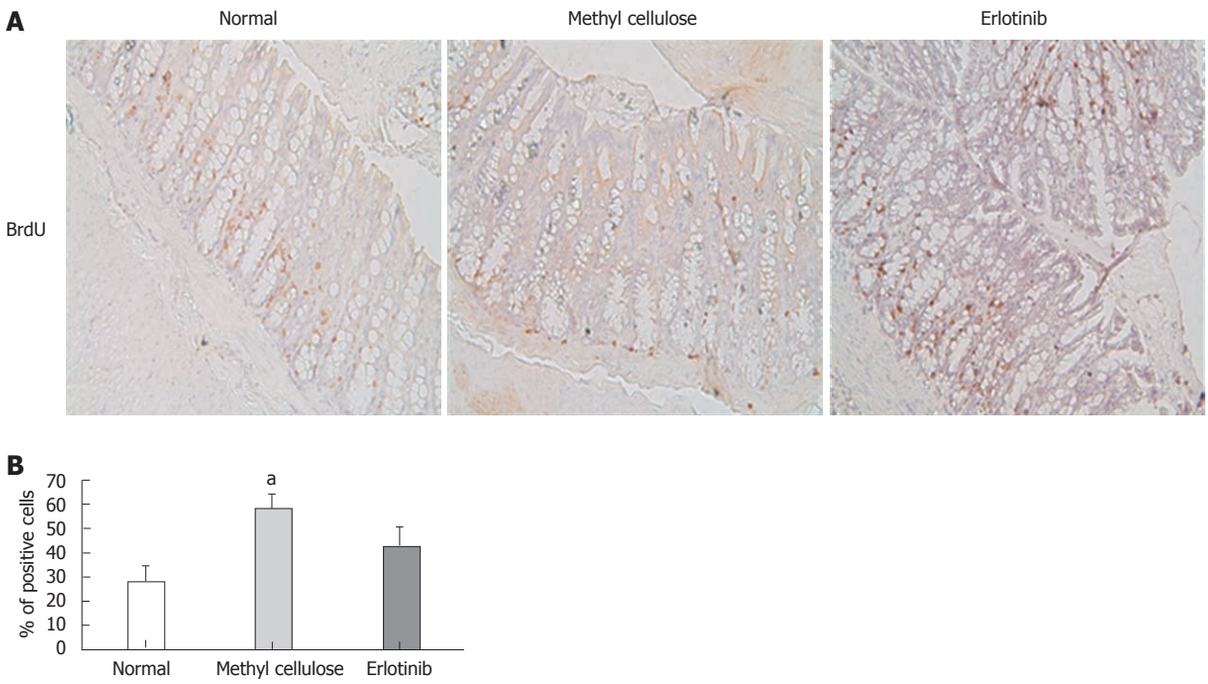

Figure 4 Erlotinib treatment inhibited cell proliferation. A: Representative immunohistochemistry for BrdU incorporation in normal, vehicle-treated and erlotinib-treated animals (200 x). B: Cell proliferation in erlotinib-treated animals was less than that shown in vehicle-treated animals and not significantly different from normal animals ($^aP < 0.05$; $n = 10 \pm SE$).

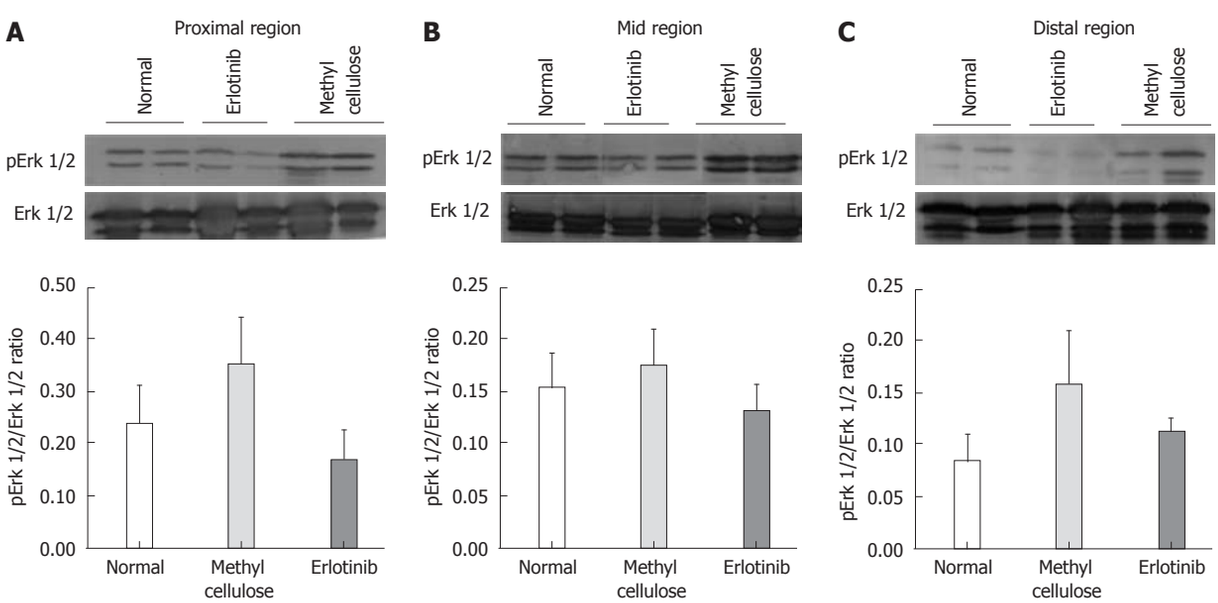

Figure 5 Effect of erlotinib in an animal model of colitis-associated dysplasia on mitogen-activated protein kinase components. Samples from (A) proximal, (B) mid, and (C) distal regions of normal rats and trinitrobenzene sulfonic acid (TNBS) + vehicle and TNBS + erlotinib treated animals were ground, and equal amounts of protein (30 μg) were separated by sodium dodecyl sulfate 12%-polyacrylamide gel electrophoresis before analysis by Western blotting. Antibodies against pErk1/2 and Erk1/2 were used. Lines are representative samples of 3 independent rats per group. Densitometry was performed by ImageQuant 5.2 Software (Typhoon 9410) (n = 10-20 ± SEM).

pErk1/2 when compared with the vehicle-treated group in the proximal, mid and distal regions, with an inhibition of the pErk1/2-to-Erk1/2 ratio of 63%, 24% and 31% in proximal, mid and distal regions, respectively, in animals treated with erlotinib compared to vehicle-treated animals (Figure 5). The ratios of pAkt/Akt and pSrc-pY416 /Src were unchanged in vehicle- and erlotinib-treated animals compared with normal animals (data not shown).

Erlotinib inhibits upregulation of c-Myc

c-Myc is frequently up-regulated in colon cancer and plays an important role in the tumorigenesis of colon cancer. We examined the presence of strong c-Myc staining in the nuclei of rat colon epithelial cells. Compared with the normal group, elevated c-Myc staining was observed in methyl cellulose-treated group, although the difference did not reach statistical significance. Importantly,

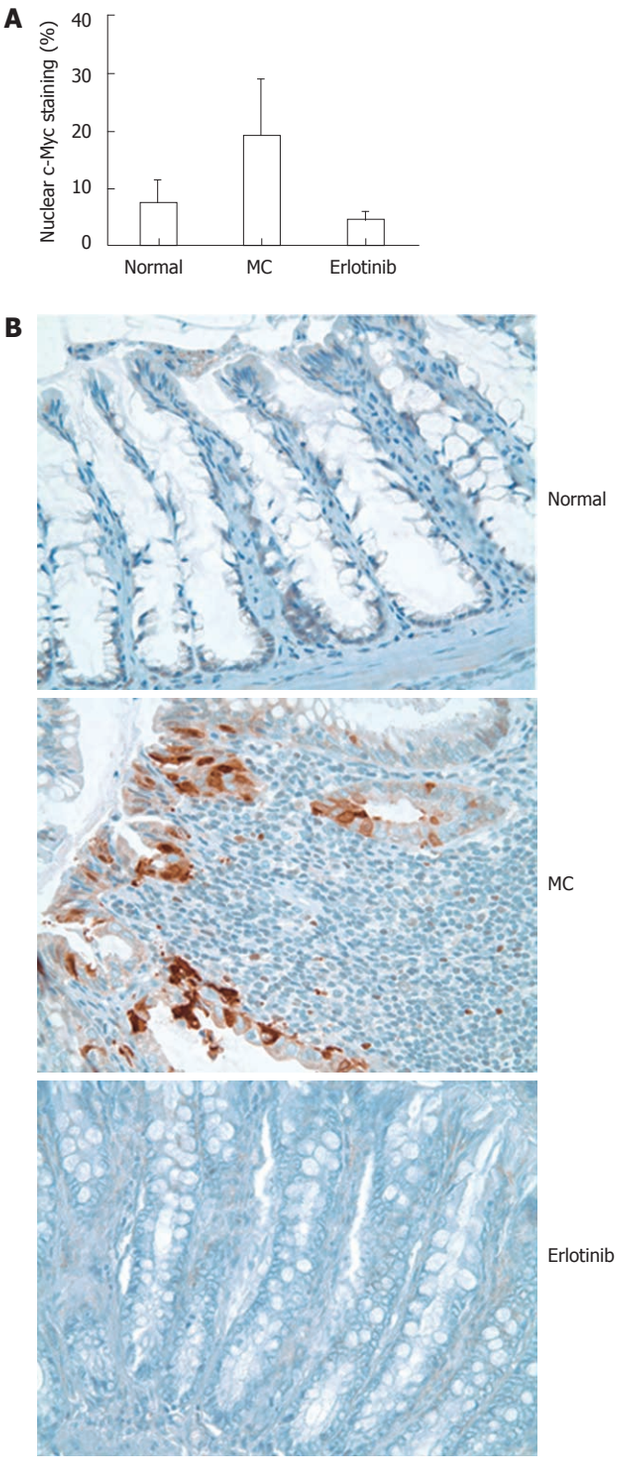

Figure 6 Immunohistochemistry analysis of c-Myc in rat colon tissue samples. A: Slides of formalin-fixed, paraffin-embedded tissues were stained with an anti-c-Myc antibody, and the percentage of strong nuclear c-Myc stain was enumerated. Samples (10, 8 and 11 samples, respectively) from normal, methyl cellulose-treated, and erlotinib-treated rat colon were analyzed; B: Representative immunohistochemistry staining of samples.

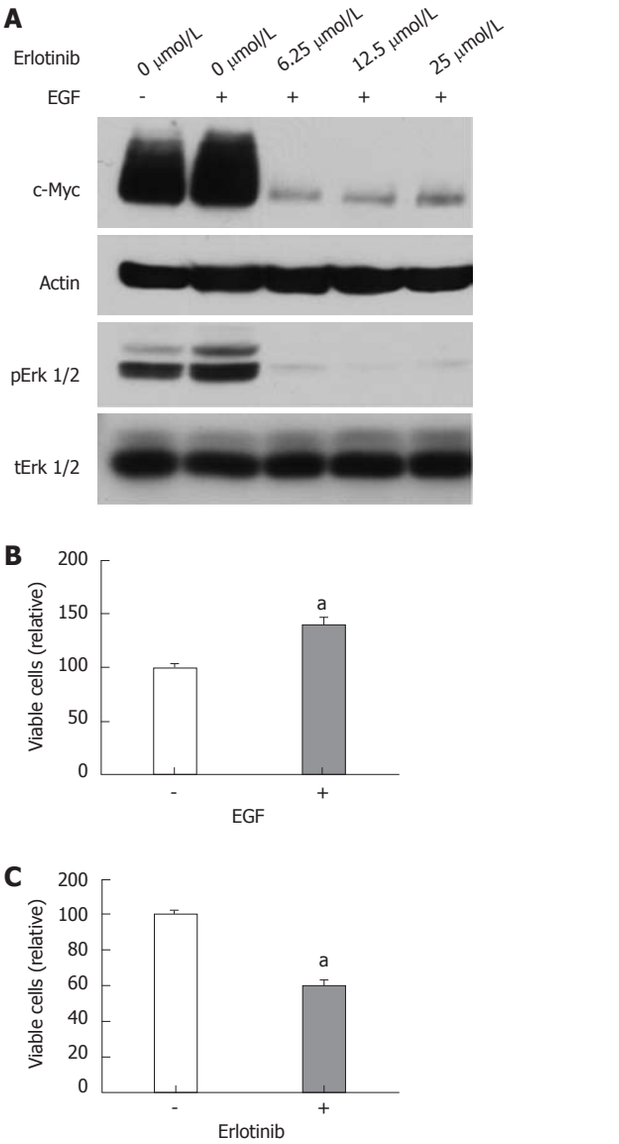

Figure 7 Effects of erlotinib on RIE-1 cells. A: Cells were incubated in RPMI 1640-5% FBS with or without EGF (10 ng/mL) and the indicated concentrations of erlotinib for 24 h. Cells were also pretreated with indicated concentration of erlotinib for 24 h and then stimulated with EGF (10 ng/mL) for 5 min. Cell lysates were analyzed by immunoblotting with indicated antibodies; B and C: Cell proliferation was assayed in the presence or absence of EGF (10 ng/mL) or erlotinib (10 μmol/L) as described in the Materials and Methods. EGF: Epidermal growth factor.

the c-Myc was reduced to a level similar to that found in the normal group in erlotinib-treated rats (Figure 6).

To further assess the effects of erlotinib on c-Myc expression and proliferation in rat epithelial cells, we examined the effects of erlotinib on c-Myc expression in RIE-1 cells. RIE-1 cells appeared very sensitive to serum starvation. In the presence of 5% fetal bovine serum, EGF slightly increased c-Myc and pErk1/2 levels in RIE-1 cells. Erlotinib treatment markedly reduced c-Myc and pErk1/2 levels (Figure 7A). RIE-1 cell proliferation was stimulated by EGF and inhibited by erlotinib (Figure 7B and C).

DISCUSSION

Although the underlying molecular mechanisms involved in colitis-associated cancer need to be further studied, EGFR has been implicated in the development of colonic dysplasia and CRC. The unraveling of molecules that play crucial roles in the transition from chronic inflammation to dysplasia and cancer is essential for iden-

tification of novel drug targets to develop for early intervention measures. Tumorigenesis and tumor promotion depend on cellular signaling pathways that control cell proliferation and survival, and many of these pathways are regulated by EGFR[4]. During chronic inflammation and tissue repair, EGFR activity is elevated. An overactive EGFR may promote the aberrant colonic epithelial cell proliferation and contribute to the development of dysplasia and CRC.

The investigation of the underlying events occurring in colitis-associated dysplasia is complicated by the fact that there are a limited number of animal models available to study the transition of inflammation to dysplasia. Our laboratory recently modified a well-established rat model of chronic colitis to develop an animal model that can be used to investigate ulcerative colitis-associated dysplasia[13,16]. The model uses a prolonged reactivation of inflammation with a proinflammatory drug (TNBS) to create an environment similar to that shown with long-standing colitis in humans, which progresses to cancer. This model shows a degree of dysplasia in 60%-70% of the rats[13,16], similar to what occurs in humans where not all patients develop cancer after a long period with colitis.

We show here that, in our TNBS-induced colitis-associated dysplasia model, animals that received an EGFR inhibitor (erlotinib) had significantly less dysplasia than vehicle-treated animals. This suggests that the EGFR inhibitor is effective in preventing the progression to dysplasia in this animal model. Importantly, we did not observe toxicity of erlotinib in the colon of animals treated with this EGFR inhibitor. It was reported previously that EGFR may have a protective role during acute and chronic inflammation in both the TNBS and dextran sulfate sodium animal models[6,21,22]. The signaling pathway proposed by those researchers involves substance P-NK-1R-EGFR, suggesting that the protective role of this pathway may be due to its effects on fibroblasts[6]. This cell type helps in the remodeling of damaged and/or dead cells or tissues; thus use of an EGFR inhibitor might have been expected to interfere with this process. However, our animals treated with erlotinib showed a milder expression of the disease than that shown in vehicle-treated animals (fewer animals treated with erlotinib progressed to IBD or dysplasia). Thus, erlotinib does not appear to worsen inflammation in our animal model of colitis-associated dysplasia. These data may also help to substantiate the idea of administering erlotinib in conjunction with an anti-inflammatory agent to treat the inflammation and prevent the risk of developing cancer.

The BrdU incorporation assay was used to measure proliferation activity of the colonocytes. Normal animals incorporated BrdU by 26%; this was more than doubled in our model of colitis-associated dysplasia, where vehicle-treated animals showed a 61% incorporation of BrdU, suggesting that these animals possess a higher proliferation rate. In contrast, erlotinib-treated animals showed less BrdU incorporation (37%). Consistently, higher levels of active Erk1/2 and c-Myc were observed in the colon mucosa of vehicle-treated animals but were reduced in erlotinib-treated animals. The Ras-Erk1/2 MAP kinase pathway is known to be activated by EGFR to control cell proliferation. c-Myc overexpression is commonly observed in colon cancer. In the adenomatous polyposis coli-mutant associated CRC, c-Myc is induced by β-catenin to promote colon tumorigenesis. Our data suggest that c-Myc is also up-regulated in colitis-induced dysplasia and erlotinib can inhibit such an increase. In support of this notion, we found that erlotinib is very effective in suppressing c-Myc expression in RIE-1 cells.

In summary, we found that erlotinib is effective in preventing colitis-associated dysplasia without causing unwanted side effects in our novel rat model. Several investigations are already underway to use erlotinib for the treatment of colorectal metastasis[23-25]. In contrast, there have been no investigations into its use as a possible treatment for colitis-associated cancer. Our results suggest that EGFR plays an important role in the progression from inflammation to dysplasia and that inhibition of EGFR, possibly in combination with an anti-inflammatory agent, is a potential approach for use in IBD patients to prevent the development of CRC.

ACKNOWLEDGMENTS

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COMMENTS

Background

Patients with ulcerative colitis are at increased risk of developing colorectal cancer. Epidermal growth factor receptor (EGFR) up-regulation is related to the development of some cancers including colorectal cancer. Erlotinib, a potent inhibitor of the EGFR tyrosine kinase, has been shown to inhibit the EGFR signaling pathway inside the cell and block tumor cell growth in pancreatic and non-small cell lung cancer; however, its role in the transition to dysplasia is unknown.

Research frontiers

The underlying mechanisms in ulcerative colitis-associated dysplasia are poorly understood. Understanding the mechanisms responsible for the transition from chronic inflammation to dysplasia and cancer might be helpful to diminish the risk that many patients have of developing colon cancer.

Innovations and breakthroughs

This is the first study to report that erlotinib is effective in preventing colitis-associated dysplasia without causing unwanted side effects in a rat model. Although erlotinib is currently under study for the treatment of colorectal metastasis, there have been no investigations into its use as a possible treatment for colitis-associated cancer. The results of this study demonstrated that erlotinib significantly inhibits the colitis-induced dysplasia in this animal model suggesting that EGFR plays an important role in the progression from inflammation to dysplasia.

Applications

Erlotinib may be an effective treatment for patients with long-standing colitis and with higher risk of developing cancer. In addition, it may be possible to combine an anti-inflammatory agent with erlotinib to reduce inflammation, and therefore the occurrence of dysplasia.

Terminology

EGFR: EGFR is a cell surface receptor involved in several downstream signaling pathways, and is also associated with many types of cancer including colorectal cancer. EGFR expression is increased in inflamed tissues of the

bowel in animal models and in patients with inflammatory bowel disease and colon cancer; Erlotinib: Erlotinib is a small molecule inhibitor which is already approved to treat non-small cell lung carcinoma and pancreatic cancer. Erlotinib exerts its biological action by reversible inhibition of tyrosine kinases on the intracellular domain.

Peer review

In the present paper, the authors used ertolinib, an EGFR tyrosine kinase inhibitor, and determined its effect on the occurrence of colitis-associated dysplasia in rat. They showed that this compound significantly inhibits colitis-induced dysplasia. This is an interesting and elegant study. The model of dysplasia, validated previously by the authors, is very original. These data have potential therapeutic implications in the domain of inflammatory bowel diseases.

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ORIGINAL ARTICLE

Vascular endothelial growth factor 165b expression in stromal cells and colorectal cancer

Makoto Tayama, Tomohisa Furuhata, Yoshiko Inafuku, Kenji Okita, Toshihiko Nishidate, Toru Mizuguchi, Yasutoshi Kimura, Koichi Hirata

Makoto Tayama, Tomohisa Furuhata, Yoshiko Inafuku, Kenji Okita, Toshihiko Nishidate, Toru Mizuguchi, Yasutoshi Kimura, Koichi Hirata, the First Department of Surgery, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan

Author contributions: Tayama M and Furuhata T contributed equally to this work; Tayama M, Furuhata T, Inafuku Y, Okita K, Nishidate T, Mizuguchi T, Kimura Y and Hirata K designed the research; Tayama M, Furuhata T and Inafuku Y performed the research; Tayama M and Furuhata T provided new reagents/analytic tools; Tayama M, Furuhata T and Okita K analyzed data; and Tayama M, Furuhata T and Hirata K wrote the paper.

Correspondence to: Makoto Tayama, MD, the First Department of Surgery, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan. tayama@sapmed.ac.jp
Telephone: +81-11-6112111-3281 Fax: +81-11-6131678

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Abstract

AIM: To characterize the implications of vascular endothelial growth factor (VEGF)-A in stromal cells and colorectal cancer and the expression of VEGF-A splice variants.

METHODS: VEGF-A expression in tumor and stromal cells from 165 consecutive patients with colorectal cancer was examined by immunohistochemistry. The association between VEGF-A expression status and clinicopathological factors was investigated. Twenty fresh-frozen samples were obtained for laser capture microdissection to analyze the splice variants of VEGF-A.

RESULTS: VEGF-A was expressed in 53.9% and 42.4% of tumor and stromal cells, respectively. VEGF-A expression in tumor cells (t-VEGF-A) was associated with advanced clinical stage (stage 0, 1/9; stage 1, 2/16; stage 2, 32/55; stage 3, 38/66; stage 4, 16/19, $P < 0.0001$). VEGF-A expression in stromal cells (s-VEGF-A) increased in the earlier clinical stage (stage 0, 7/9; stage 1, 6/16; stage 2, 33/55; stage 3, 22/66; stage 4, 5/19; $P = 0.004$). Multivariate analyses for risk factors of recurrence showed that only s-VEGF-A expression was an independent risk factor for recurrence (relative risk 0.309, 95% confidence interval 0.141-0.676, $P = 0.0033$). The five-year disease-free survival (DFS) rates of t-VEGF-A-positive and -negative cases were 51.4% and 62.9%, respectively. There was no significant difference in t-VEGF-A expression status. The five-year DFS rates of s-VEGF-A-positive and -negative cases were 73.8% and 39.9%, respectively. s-VEGF-A-positive cases had significantly better survival than s-VEGF-A-negative cases ($P = 0.0005$). Splice variant analysis revealed that t-VEGF-A was mainly composed of VEGF165 and that s-VEGF-A included both VEGF165 and VEGF165b. In cases with no venous invasion (v0), the level of VEGF165b mRNA was significantly higher (v0 204.5 ± 122.7, v1 32.5 ± 36.7, v2 2.1 ± 1.7, $P = 0.03$). The microvessel density tended to be lower in cases with higher VEGF165b mRNA levels.

CONCLUSION: s-VEGF-A appears be a good prognostic factor for colorectal cancer and includes VEGF165 and VEGF165b.

Key words: Colorectal cancer; Vascular endothelial growth factor-A; Vascular endothelial growth factor165; Microvascular density; Stromal cell

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INTRODUCTION

The growth and metastasis of cancer depend on angiogenesis, and vascular endothelial growth factor (VEGF)-A. VEGF-A is known to be one of the most important angiogenic factors. VEGF-A protein was discovered by Ferrara in 1989 as a specific growth factor and a blood vascular permeability factor for endothelial cells[1-2]. As a result of alternative splicing, 6 VEGF isoforms of 121, 145, 165, 183, 189 and 206 amino acids are produced from a single gene[3-7]. Most studies suggest that VEGF165 is the most abundant and biologically active isoform[3,8]. The biological effects of VEGF165 are mediated by tyrosine kinase receptors, i.e., VEGF receptor (VEGFR) 1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4)[9-11]. In colorectal cancer, VEGF-A is highly expressed in the case of hematogenous metastasis; therefore, VEGF-A is assumed to have value as a prognostic factor. VEGF-A and its receptor system are deeply involved in tumor angiogenesis. Thus, they are important molecular targets in the therapeutic strategy against colorectal cancer. It has been reported that the combined chemotherapy and an anti-VEGF antibody improves the response ratio of the tumor and extends the length of survival[12-15]. Tumor cells are the predominant source of VEGF; however, stromal cells surrounding the tumor have also been shown to produce VEGF[16]. Researches on the invasive and metastatic mechanisms mainly focused on the characteristics of the cancer cell itself, and there are few reports concerning the stromal cells[17-19]. Over the past decade, the role of stromal cells has gradually become a matter of interest to many researchers. The median survival in stromal VEGF-A-positive patients was 9.7 years *vs* 4.3 years in stromal VEGF-A-negative patients with stage Ⅱ and Ⅲ colorectal cancers[20]. However, the reason why VEGF-A expression in stromal cells resulted in a better prognosis has not been clarified.

VEGF165b was recently isolated from kidney epithelial cells as an angiogenesis inhibitor[21]. This variant is identical to VEGF165 except for the last six amino acids encoded by alternative splicing. VEGF165b also binds to both the VEGF receptor 1 (VEGF-R1) and the VEGF receptor 2 (VEGF-R2) with a similar affinity to that of VEGF165. VEGF165b was shown to bind to VEGF-R2, but not to stimulate phosphorylation, and to inhibit VEGF165-mediated phosphorylation in human umbilical vein endothelial cells[22-25].

We examined the association between VEGF-A expression status and clinicopathological characteristics in order to determine how VEGF-A in stromal cells affects tumor progression. We also analyzed the expression of VEGF-165 and VEGF165b using fresh-frozen specimens.

MATERIALS AND METHODS

Patients

Tumor specimens were obtained from 165 consecutive patients with colorectal cancer who underwent resection at the First Department of Surgery, Sapporo Medical University from 1997 through 2001. Of these 165 patients, 146 at stages 0-Ⅲ received curative resection. None of the patients received radiation or chemotherapy before surgery. The pathological stages, depth, histology, venous invasion, and lymphatic invasion of the primary tumor are shown in Table 1. Venous invasion and lymphatic invasion were both classified into four grades according to the Japanese Classification of Colorectal Carcinoma. v0 and ly0 represent no invasion, v1 and ly1, slight invasion, v2 and ly2, moderate invasion, and v3 and ly3, high invasion. immunohistochemical (IHC) analysis was performed in these 165 cases. We also obtained 20 fresh-frozen samples from patients with colorectal cancer in 2006-2007 to analyze the expression of VEGF165 and VEGF165b mRNAs.

Table 1 Characteristics of patients

	n	%
Gender		
Female	75	45.5
Male	90	54.5
Primary tumor location		
Ascending colon	29	17.6
Transverse colon	18	10.9
Descending colon	6	3.6
Sigmoid colon	30	18.2
Rectum	82	49.7
TNM stage		
0	9	5.5
Ⅰ	16	9.7
Ⅱ	55	33.3
Ⅲ	66	40.0
Ⅳ	19	11.5
T factor		
Tis	9	5.5
T1	7	4.2
T2	25	15.2
T3	111	67.3
T4	13	7.9
Histological differentiation		
Well	47	28.5
Moderate	95	57.6
Poor	8	4.8
Mucinous	10	6.1
Other	5	3.0
Venous invasion		
v0	46	27.9
v1	73	44.2
v2	32	19.4
v3	14	8.5
Lymphatic invasion		
ly0	53	32.1
ly1	79	47.9
ly2	28	17.0
ly3	5	3.0
Recurrence except stage IV cases		
No	95	65.1
Yes	51	34.9

Immunohistochemistry

For IHC staining, paraffin-embedded tissues were cut at 4 μm. Slides were deparaffinized in xylene for 3 min three times, 3 min in absolute alcohol, 3 min in 90% ethanol,

3 min in 70% ethanol, and finally, 3 min in phosphate-buffered saline (PBS) for three times. After being deparaffinized, sections were incubated in 3% H_2O_2-methanol for 20 min to inactivate endogenous peroxidase. Deparaffinized and rehydrated sections were heated in DAKO Target Retrieval Solution (DAKO Japan, Tokyo, Japan) for 15 min in an autoclave at 105 ℃. Nonspecific binding was blocked with 10% goat serum for 15 min at room temperature followed by incubation with the primary antibody in a moist chamber at 4 ℃ overnight. After rinsing in PBS for 3 min three times, the sections were incubated with a biotinylated secondary antibody, ENVISION + Mouse/HRP (Dako Japan, Tokyo, Japan), for 30 min. Sections were stained using aminoethylcarbazole (Dako Japan, Tokyo, Japan). Slides were mounted prior to observation under conventional light microscope.

Monoclonal antibodies

The primary antibodies were mouse monoclonal antibodies against VEGF-A, anti-human VEGF (N5) (IBL, Takasaki, Japan), CD34, anti-human CD34 (QBEnd10) and mouse monoclonal antibody Dako N1632 (Dako, Japan, Tokyo, Japan).

Evaluation of immunohistochemistry

VEGF-A expression was examined under light microscope, and both the tumor and the stromal cells were separately classified into stained cells and unstained cells. Three sections of tumor cells and stromal cells were counted respectively at × 400 magnification for marginal cancer tissue to determine whether the cells were positive for VEGF-A, and the percentage of stained cells was averaged. Specimens were regarded as VEGF negative if less than 5% of the cells were stained and as VEGF positive if more than 5% were stained. These criteria were used in many previous reports[26-27]. Microvessel density (MVD) was assessed using light microscopy in invasive tumors containing the highest number of capillaries and small venules per unit area. Any single endothelial cell or cell cluster stained with CD34 was counted as a single vessel at × 400 magnification for marginal cancer tissues[28]. Three sections were counted in one case, and the number of vessels was averaged.

Laser capture microdissection

Laser capture microdissection (LCM) is a method for obtaining pure populations of cells from heterogeneous samples. Using this technique, colorectal tumor tissues were separated into tumor and stromal tissues. The frozen tissues were sectioned at a thickness of 8 μm using a cryostat and mounted on nonadhesive glass slides. Tissue sections were rehydrated using 70% ethanol for 3 min and rinsed twice in distilled water (Invitrogen Corp., Carlsbad, CA). They were then stained using hematoxylin for 30 s and rinsed in distilled water, followed by dehydration with 95% and 100% ethanol for 10 s in each case. Counterstaining was performed three times with eosin. Dehydration with xylene was conducted twice for 1 min each time, followed by air drying for 20 min. The PixCell LM200 system (Arcturus Engineering, Mountain View, CA) was used to microdissect the tumor cells and the stromal cells from the colorectal tissue sections. Ten sections were used to obtain sufficient RNA for reverse transcription polymerase chain reaction (RT-PCR), and each section needed at least 10 000 pulses. Processing of the total RNA began immediately following LCM. Extraction and isolation were performed using a QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA).

Real-time polymerase chain reaction

We constructed the following primers to amplify fragments of human VEGF165 and VEGF165b specifically. The forward primer was located in exon 7a (TGTTTG TACAAGATCCGCAGACGTG). One reverse primer complementary to exon 8 (TCACCGCCTCGG CTTGT-CACATCTGCAAGTACGTT) detected VEGF165 but not VEGF165b, and the other reverse primer complementary to exon 9 (GTTCTGTATCAGTCTTTCCTG-GTGAGAGATCTGCA) detected VEGF165b but not VEGF165. Denaturing was conducted at 96 ℃ for 30 s, with annealing at 55 ℃ for 30 s and extension at 72 ℃ for 60 s in reactions cycled 30 times. PCR products were run on 3% agarose gels containing 0.5 μg/mL ethidium bromide and visualized under a UV transilluminator. This reaction consistently resulted in amplicons of 121 bp consistent with VEGF165b and 119 bp consistent with VEGF165. To confirm the amplification of VEGF165 and VEGF165b, we performed sequence analysis of these PCR products.

Real time PCR was performed on a LightCycler (Roche, Basel, Switzerland) for the semi-quantitation of VEGF165 and VEGF165b mRNA levels. The primer sequences were the same as those of the primers used for RT-PCR. The calculated amounts of VEGF165 and VEGF165b mRNAs were normalized to the endogenous reference control gene, human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH). All data were presented as the ratio of the target gene/GAPDH expression.

Statistical analysis

The χ^2 test and Mann-Whitney *U* test were used to examine the association between the expression status of VEGF and clinicopathological characteristics. To analyze the risk factors for recurrence, logistic regression analysis was conducted. Survival curves were computed according to the Kaplan-Meier method. The log-rank test was used to compare the survival curves. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of VEGF-A in tumor and stromal cells

VEGF-A expression in tumor cells was positive in 53.9% (89/165) of the cases (Figure 1A). VEGF-A immunoreactivity was observed mainly in the cytoplasm of tumor cells. VEGF-A expression in stromal cells was observed in 42.4% (73/165) of the cases (Figure 1B).

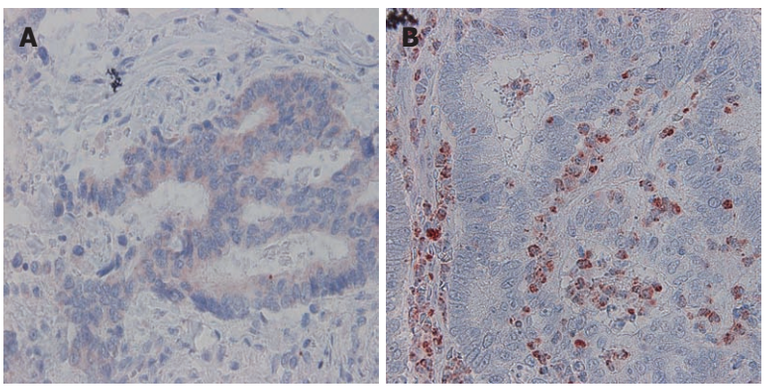

Figure 1 Immunohistochemical of colorectal cancer tissues used the anti-vascular endothelial growth factor-A antibody. A: Vascular endothelial growth factor (VEGF)-A was expressed in tumor cells but not in stromal cells; B: VEGF-A was expressed in stromal cells but not in tumor cells.

Table 2 Association between vascular endothelial growth factor-A expression and clinicopathological characteristics *n* (%)

	n	Tumor VEGF positive cases	Stromal VEGF positive cases
TNM stage			
0	9	1 (11.1)	7 (77.8)
I	16	2 (12.5)	6 (37.5)
II	55	32 (58.2)	33 (60.0)
III	66	38 (57.6)	22 (33.3)
IV	19	16 (84.2)	5 (26.3)
Total	165	89 (53.9)	73 (44.2)
		$P < 0.0001$	$P = 0.004$
T factor			
Tis	9	1 (11.1)	7 (77.8)
T1	7	0 (0.0)	6 (85.7)
T2	25	9 (36.0)	11 (44.0)
T3	111	70 (63.1)	45 (40.5)
T4	13	9 (69.2)	4 (30.8)
Total	165	89 (53.9)	73 (44.2)
		$P = 0.0002$	$P = 0.01$
Histological differentiation			
Well	47	15 (31.9)	23 (48.9)
Moderate	95	63 (66.3)	41 (43.2)
Poor	8	3 (37.5)	4 (50.0)
Mucinous	10	5 (50.0)	2 (20.0)
Other	5	1 (20.0)	3 (60.0)
		NS	NS
Venous invasion			
v0	46	14 (30.4)	27 (58.7)
v1	73	43 (58.9)	29 (39.7)
v2	32	23 (71.9)	15 (46.9)
v3	14	9 (64.3)	2 (14.3)
		$P = 0.001$	$P = 0.015$
Lymphatic invasion			
ly0	53	16 (30.1)	26 (49.1)
ly1	79	48 (60.8)	39 (49.4)
ly2	28	20 (71.4)	7 (25.0)
ly3	5	5 (100.0)	1 (20.0)
		$P < 0.0001$	$P = 0.04$

NS: Not significant; VEGF: Vascular endothelial growth factor.

Association between VEGF-A expression status and clinicopathological characteristics

A summary of the correlation between VEGF-A expression and clinicopathological characteristics is shown in Table 2. Tumor VEGF-A (t-VEGF-A) expression rates in tumors were 11.1% (1/9) in stage 0, 12.5% (2/16) in stage Ⅰ, 58.2% (32/55) in stage Ⅱ, 57.6% (38/66) in stage Ⅲ, and 84.2% (16/19) in stage IV. t-VEGF-A expression was associated with the clinical stage ($P < 0.0001$). VEGF-A (s-VEGF-A) expression rates in stromal cells were 77.8% (7/9) in stage 0, 37.5% (6/16) in stage Ⅰ, 60.0% (33/55) in stage Ⅱ, 33.3% (22/66) in stage Ⅲ, and 26.3% (5/19) in stage Ⅳ. The s-VEGF-A expression rate increased in the earlier clinical stage ($P = 0.004$). The t-VEGF-A expression rate increased with the depth of invasion ($P = 0.0002$). Conversely, the s-VEGF-A expression rate decreased with the depth of invasion ($P = 0.01$). There was no significant association between VEGF-A expression and the histological type. t-VEGF-A expression became significantly higher with the grade of venous and lymphatic invasion, while s-VEGF-A expression became significantly lower with the grade of venous and lymphatic invasion.

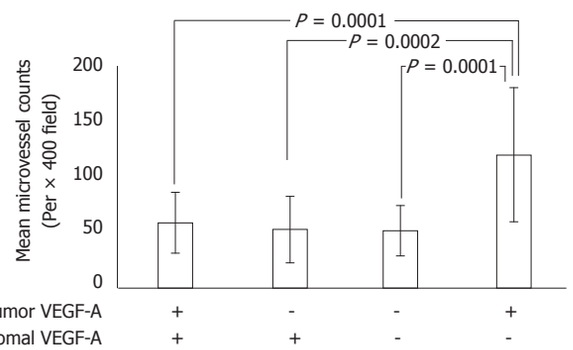

Figure 2 Microvessel density of vascular endothelial growth factor-A expression status. In s-vascular endothelial growth factor (VAEG)-A positive cases, microvessel density (MVD) was maintained at a low score regardless tumor VEGF-A (t-VEGF-A) expression. In s-VEGF-A negative cases, MVD was influenced by t-VEGF-A expression.

Microvessel density

MVD was calculated by counting CD34-positive vascular endothelial cells. The association between VEGF-A expression status and MVD is shown in Figure 2. The MVDs of t-VEGF-A and s-VEGF-A expression (+, +), (-, +), (-, -), and (+, -) were 58.5, 52.4, 51.2 and 119.0, respectively. In s-VEGF-A-positive cases, the low MVD score

Table 3 Logistic regression analysis for recurrence in colorectal carcinoma except for stage Ⅳ cases

Factor	*n* (Recurrence)	Univariate analysis			Multivariate analysis		
		Relative risk	95% CI	*P* value	Relative risk	95% CI	*P* value
Clinical stage							
0	9(1)	2.120	1.302-3.451	0.0250	1.718	0.980-3.010	0.0586
Ⅰ	16(3)						
Ⅱ	55(15)						
Ⅲ	66(32)						
Venous invasion							
v0	46(12)	1.500	1.050-2.143	0.0260	0.812	0.504-1.307	0.3907
v1	63(27)						
v2	27(6)						
v3	10(6)						
Lympathic invasion							
ly0	52(13)	2.094	1.354-3.238	0.0010	1.27	0.714-2.261	0.4155
ly1	68(24)						
ly2	23(12)						
ly3	3(2)						
s-VEGF-A positive	68(14)	0.269	0.135-0.535	0.0002	0.309	0.141-0.676	0.0033
t-VEGF-A positive	73(31)	2.340	1.218-4.495	0.0110	1.918	0.768-3.718	0.1918
Total	146(51)						

CI: Confidence interval.

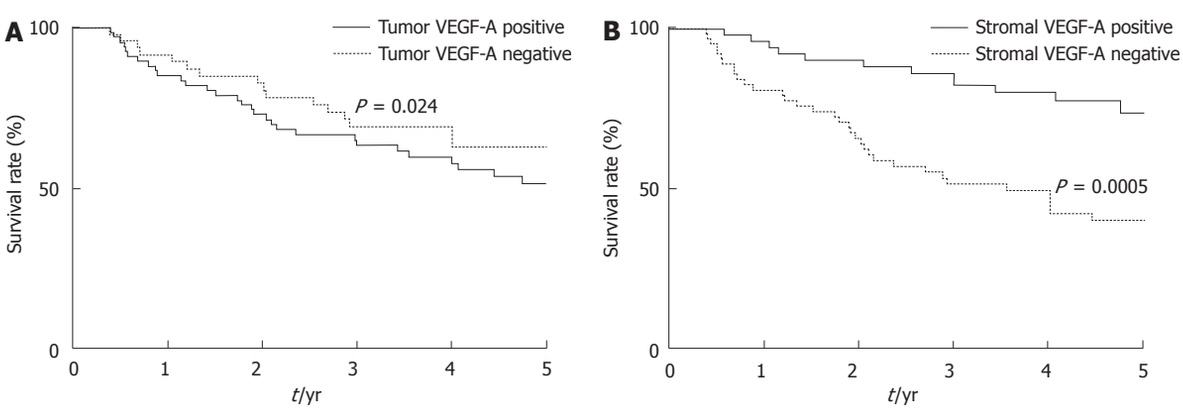

Figure 3 Disease-free survival of patients with stages Ⅱ and Ⅲ colorectal cancer. A: t-vascular endothelial growth factor (VAGE)-A positive *vs* negative. The log-rank test indicates *P*= 0.24 (not significant); B: s-VEGF-A positive *vs* negative. The log-rank test statistical analysis indicates a significant difference (*P* = 0.0005). VEGF: Vascular endothelial growth factor.

was almost the same regardless of t-VEGF-A expression. t-VEGF-A-positive and s-VEGF-A-negative cases had significantly higher MVD scores.

Recurrence

Risk factors for recurrence in the 146 cases excluding stage Ⅳ cases were examined using logistic regression analysis. In univariate analysis, clinical stage, venous invasion, lymphatic invasion, t-VEGF-A positivity and s-VEGF-A negativity were risk factors for recurrence (Table 3). Multivariate analyses of these risk factors were performed, which showed that only s-VEGF-A expression was an independent risk factor for recurrence ($P = 0.0033$) (Table 3).

Survival analysis

Survival analysis was performed for stage Ⅱ and Ⅲ patients ($n = 121$). The five-year disease-free survival (DFS) rates of t-VEGF-A-positive ($n = 70$) and -negative cases ($n = 51$) were 51.4% and 62.9%, respectively. There was no significant difference in t-VEGF-A expression status (Figure 3A). The five-year DFS rates of s-VEGF-A-positive ($n = 55$) and -negative ($n = 66$) cases were 73.8% and 39.9%, respectively. s-VEGF-A-positive cases had significantly better survival than negative cases ($P = 0.0005$) (Figure 3B).

Expression analysis of VEGF165 and VEGF165b

Expression analysis of VEGF165 and VEGF165b was performed using specimens of 20 cases obtained by LCM. RT-PCR was performed using specific primer sets (exon7/exon8 and exon7/exon9) to investigate the expression of VEGF165 and VEGF165b. Sequence analysis revealed that the PCR products were VEGA165 and VEGF165b (data not shown)[26]. IHC analysis was performed in the same 20 cases. Expression of s-VEGF-A and t-VEGF-A was positive in 40% (8/20) and 70% (14/20), respectively. mRNA levels of VEGF165 and VEGF165b were semi-quantified by real time PCR for each VEGF-A expres-

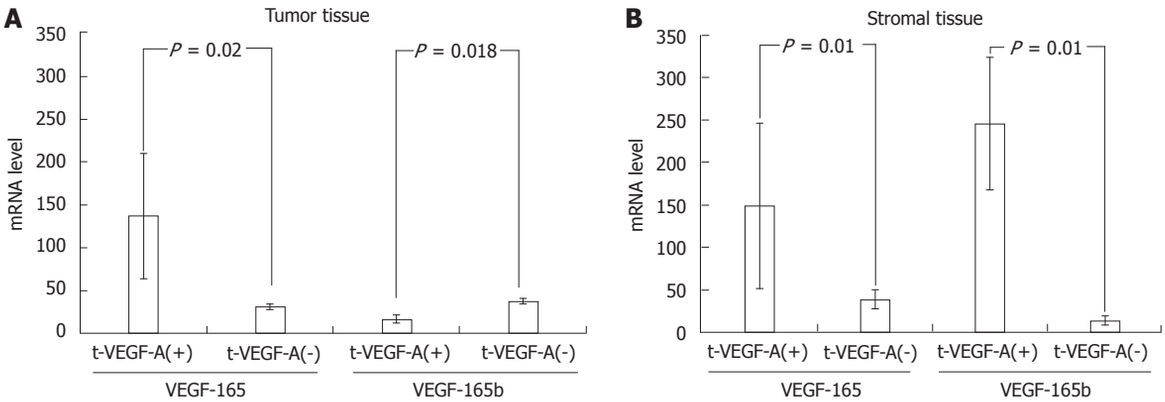

Figure 4 mRNA level of VEGF165 and VEGF165b semi-quantified by real-time polymerase chain reaction in tumor and stromal tissues. A: In tumor tissue, only vascular endothelial growth factor (VEGF) 165 expressed in t-VEGF-A positive cases; B: In stromal tissues, both VEGF165 and VEGF165b expressed in s-VEGF-A positive case.

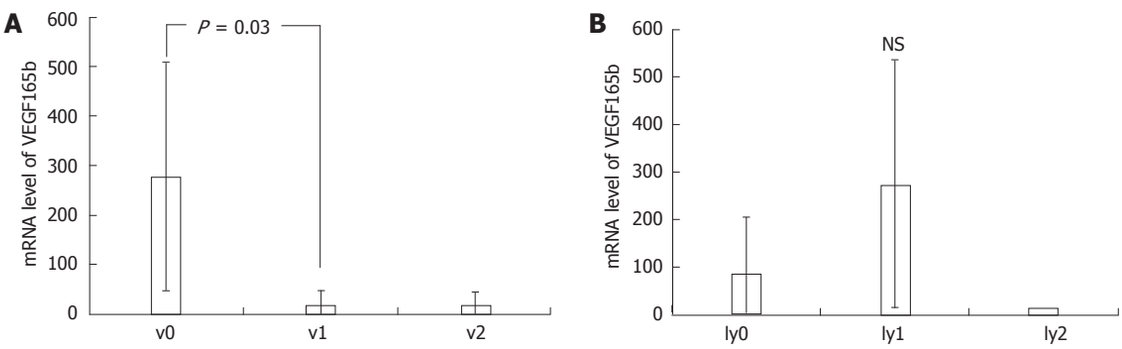

Figure 5 Correlations of vascular endothelial growth factor 165b expression in stromal tissue and tumors with venous and lymphatic invasion. A: Vascular endothelial growth factor (VEGF) 165b mRNA level in v0 cases was significantly higher than those in v1 cases; B: There were no significant differences of VEGF165bmRNA levels among degrees of the lymphatic invasion. NS: Not significant.

sion status determined by IHC. In tumor tissues, only VEGF165 was expressed in t-VEGF-A-positive cases (P = 0.02) (Figure 4A). In stromal tissues, both VEGF165 and VEGF165b were expressed in s-VEGF-A-positive cases (Figure 4B).

Correlation between VEGF165b expression in stromal tissues and venous invasion, VEGF165b expression in stromal tissues and lymphatic invasion

The VEGF165b mRNA level in v0 cases was significantly higher than in v1 cases (Figure 5A). There were no significant differences of VEGF165bmRNA levels among various degrees of lymphatic invasion (Figure 5B).

VEGF165 and VEGF165 mRNA levels and MVD in each case

In cases with lower VEGF165b mRNA levels (numbers 1-8), MVD depended on the VEGF165 mRNA level, while in cases with higher VEGF165b mRNA levels (numbers 14-20), MVD did not reach a high score regardless of the VEGF165 mRNA level (Figure 6).

DISCUSSION

Neoangiogenesis plays an important role in the progression and metastasis of colorectal cancer, and VEGF-A, among many molecules, is known to be of paramount importance because VEGF-A secreted from tumor cells chiefly binds to VEGFR-2 and induces angiogenesis. In colorectal cancer, it is well known that VEGF-A is highly expressed in cases with hematogenous metastasis[29,30]. Therefore, it is assumed that VEGF-A is one of the biomarkers for prognosis[31]. VEGF-A expression in tumor cells was examined to evaluate the degree of risk in many studies. However, there have been few reports focusing on stromal cells surrounding tumor cells. Concerning VEGF-A expression in stromal cells, stromal VEGF-A positivity generally results in a better prognosis than VEGF-A negativity[20].

In this report, IHC staining was performed in 165 consecutive patients with colorectal cancer to detect VEGF-A expression in tumor and stromal cells. Our results showed that s-VEGF-A expression might be a factor indicating a better prognosis. These results were consistent with a previous report[20] and implied that the functions of VEGF-A expressed in stromal cells might be different from those in tumor cells. Since VEGF has 6 splicing isoforms[2-6], we focused on one of them, VEGF165b, which was reported to inhibit neoangiogenesis. Our report demonstrated that s-VEGF-A, including VEGF165 and VEGF165b expressed in stromal cells, might inhibit

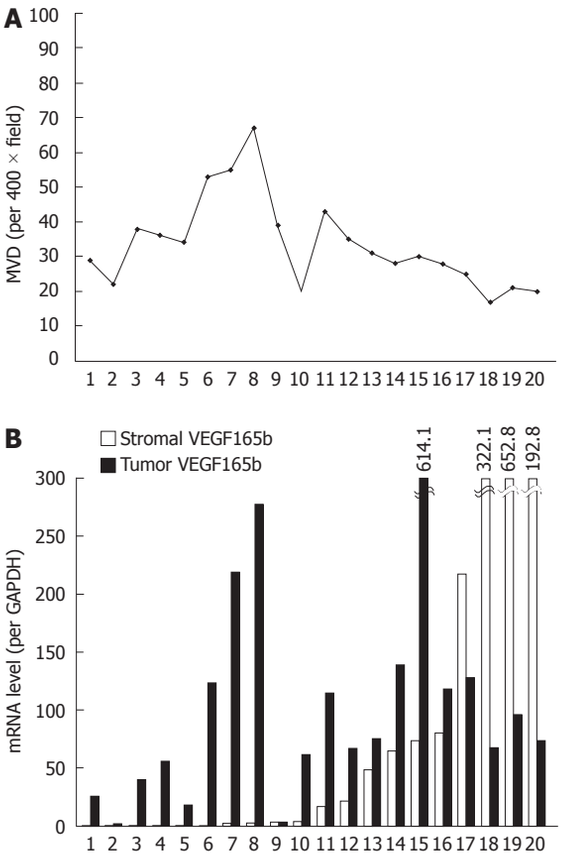

Figure 6 Relationship between the level of vascular endothelial growth factor 165 mRNA in tumor, vascular endothelial growth factor 165b mRNA in stromal tissues and microvessel density. Twenty cases are arrayed on the X axis in ascending order of the amount of vascular endothelial growth factor (VEGF)165b expression. A: The score of microvessel density (MVD); B: The mRNA level of VEGF165 and VEGF165b. MVD was maintained at a low level in the cases in which VEGF165b expressed in stromal tissues.

angiogenesis and reduce MVD. However, we could not conclude that VEGF165b expression improved the prognosis of colorectal cancer patients because the association between VEGF165b expression and the prognosis has not been investigated in a large series.

In this study, we clarified that s-VEGF-A, including VEGF165b, had a function to inhibit neoangiogenesis. However, it remains unexplained what kinds of cells secrete VEGF165b and what factors induce VEGF165b expression. A previous report showed that a subset of macrophages expressed VEGF-A resulting from CD68 (a macrophage-specific immunostain) macroIHC staining[32]. In our series, 76% of CD68-positive cases were s-VEGF-A positive and most of the s-VEGF-A(+) cells were identical to CD68(+) cells under light microscope (data not shown). CD68(+) stromal cells, and tumor-associated macrophages (TAMs) have been reported to have dual potential to improve and worsen the prognosis[33]. We speculate that CD68(+) stromal cells may secrete VEGF165b and inhibit the angiogenesis induced by VEGF165 from tumor cells to interfere with tumor progression. In the future, we will study TAMs in colorectal cancer, especially those expressing VEGF165b, which may be a key to developing a novel therapeutic strategy.

In summary, the s-VEGF-A appears be a good prognostic factor for colorectal cancer and includes VEGF165 and VEGF165b.

COMMENTS

Background

Neoangiogenesis plays an important role in the progression and metastasis of colorectal cancer and vascular endothelial growth factor (VEGF)-A, among many molecules, is known to be highly important because VEGF-A secreted from tumor cells chiefly binds to VEGFR-2 and induces angiogenesis. In colorectal cancer, it is well known that VEGF-A is highly expressed in cases with hematogenous metastasis. Therefore, VEGF-A is assumed to have value as a prognostic factor. VEGF-A and its receptor system are deeply involved in tumor angiogenesis. Thus, they are important molecular targets in the therapeutic strategy against colorectal cancer.

Research frontiers

It has been reported that combined chemotherapy and an anti-VEGF-A antibody improves the response ratio of the tumor and extends the length of survival. Tumor cells are the predominant source of VEGF-A; however, stromal cells surrounding the tumor have also been shown to produce VEGF-A. In many reports, VEGF-A expression in tumor cells was examined to evaluate the degree of risk. However, there have been few reports focusing on stromal cells surrounding tumor cells.

Innovations and breakthroughs

In this report, immunohistochemical staining was performed in 165 consecutive patients with colorectal cancer to detect VEGF-A expression in tumor and stromal cells. The results showed that s-VEGF-A expression might be a factor indicating a better prognosis. These results implied that the functions of VEGF-A expressed in stromal cells might be different from those in tumor cells. This report demonstrated that s-VEGF-A, including VEGF165 and VEGF165b, expressed in stromal cells, might inhibit angiogenesis and reduce microvessel density.

Applications

The authors clarified that s-VEGF-A, including VEGF165b, had a function to inhibit neoangiogenesis. However, it remains unexplained what kinds of cells secrete VEGF165b and what factors induce VEGF165b expression. Studies of TAMs in colorectal cancer, especially those expressing VEGF165b, may be a key to developing a novel therapeutic strategy.

Peer review

This is an excellent manuscript, with a well done methodological approach, and showing a correlation with stromal VGEF expression and colorectal cancer prognosis.

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ORIGINAL ARTICLE

Inhibition of tumor angiogenesis by TTF1 from extract of herbal medicine

Chao Liu, Xiao-Wan Li, Li-Min Cui, Liang-Chang Li, Li-Yan Chen, Xue-Wu Zhang

Chao Liu, Xiao-Wan Li, Liang-Chang Li, Li-Yan Chen, Xue-Wu Zhang, Basic Medical College, Yanbian University, Yanji 133002, Jilin Province, China
Chao Liu, Li-Min Cui, Clinical Medical College, Yanbian University, Yanji 133002, Jilin Province, China
Author contributions: Liu C constructed the chick embryo chorioallantoic membrane model; Liu C and Li XW constructed the BALB/c nude mouse model, and measured the molecular biology parameters; Cui LM performed drug extraction and separation; Li LC and Chen LY measured immunohistochemical parameters; Zhang XW designed the project, analyzed the data and wrote the manuscript.
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Correspondence to: Xue-Wu Zhang, Professor, Basic Medical College, Yanbian University, Yanji 133002, Jilin Province, China. zhangxuewu@ybu.edu.cn
Telephone: +86-433-2435102 Fax: +86-433-2435104
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Abstract

AIM: To study the inhibition of tumor angiogenesis by 5,2,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1) isolated from an extract of herbal medicine *Sorbaria sorbifolia*.

METHODS: Angiogenic activity was assayed using the chick embryo chorioallantoic membrane (CAM) method. Microvessel density (MVD) was determined by staining tissue sections immunohistochemically for CD34 using the Weidner capillary counting method. The mRNA and protein levels of vascular endothelial growth factor (VEGF), vascular endothelialgrowth factor receptor 2 (VEGFR2, Flk-1/KDR), basic fibroblast growth factor (bFGF), cyclo-oxygenase (COX)-2 and hypoxia-inducible factor (HIF)-1α were detected by quantitative real-time polymerase chain reaction and Western blotting analysis.

RESULTS: The TTF1 inhibition rates for CAM were 30.8%, 38.2% and 47.5% with treatment concentrations of 25, 50 and 100 μg/embryo × 5 d, respectively. The inhibitory rates for tumor size were 43.8%, 49.4% and 59.6% at TTF1 treatment concentrations of 5, 10, and 20 μmol/kg, respectively. The average MVD was 14.2, 11.2 and 8.5 at treatment concentrations of 5 μmol/kg, 10 μmol/kg and 20 μmol/kg TTF1, respectively. The mRNA and protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α in mice treated with TTF1 were significantly decreased.

CONCLUSION: TTF1 can inhibit tumor angiogenesis, and the mechanism may be associated with the down-regulation of VEGF, KDR, bFGF, HIF-1α and COX-2.

Key words: Chinese herbal medicine; *Sorbaria sorbifolia*; TTF1; Inhibition; Tumor angiogenesis

Peer reviewer: Dr. Jeff Butterworth, MB, FRCP, Department of Gastroenterology, Shrewsbury and Telford Hospital NHS Trust, Mytton Oak Road, Shrewsbury, Shropshire SY3 8XQ, United Kingdom

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INTRODUCTION

Angiogenesis is the process by which a new blood-vascular system grows from the existing vascular bed through the interaction of cytokines, the cellular matrix and proteolylic enzymes. Tumor angiogenesis is closely associated with tumor growth, metastasis, recurrence and overall prognosis. For this reason, tumor angiogenesis is a desir-

able target for tumor treatment[1]. Anti-angiogenesis is an important strategy for tumor therapy[2]. Many studies have demonstrated that tumor angiogenesis can be inhibited by the flavones present in Chinese herbal medicines, including apigenin, silibinin, quercetin, wogonin, genistein and luteolin[3-9]. Previously, we have reported that acetic ether extracts of the medicinal plant *Sorbaria sorbifolia* (*S. sorbifolia*) inhibits the growth of HepG-2 cells[10] and mouse S180 sarcoma, down-regulates the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-2, and reduced the cellular activity of natural killer cells[11]. In addition, extracts inhibit the placental glutathione S transferase formation of positive foci in hepatoma precancerous rats and down-regulated the expression of p53 and Bcl-2. They increase the activity of superoxide dismutase and glutathione peroxidase and decrease the nitrogen monoxide (NO) synthase activity and malondiadehyde and NO concentrations[12,13]. Six compounds have been identified in the *S. sorbifolia* acetic ether extracts, including 5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1), 5,7- dihydroxy-8-methoxyflavone, rutin, quercetin, daucosterol, benzoate and p-hydroxybenzoic acid, and TTF1 was the first active flavonoid compound identified[11]. After testing the six compounds, we found that TTF1 inhibited vascular endothelial growth factor (VEGF) expression in HepG-2 cells and VEGF165-induced human umbilical vein endothelial cells proliferation and vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR) protein expression[10]. This study focused on the effect of TTF1 specifically on the inhibition of tumor angiogenesis.

MATERIALS AND METHODS

Extraction of TTF1

TTF1 was separated using the water extraction and alcohol precipitation method from 10 kg *S. sorbifolia* (collected from Jilin Province) as previously described[11].

Cell culture

The HepG-2 cell line was purchased from KeyGEN Co., Ltd. (Nanjing, China). Cells were grown in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin. Cells were cultured at 37 ℃ in a humidified incubator containing 5% CO_2. Cells in the logarithmic growth phase were used for tests.

Chick embryo chorioallantoic membrane assay

Angiogenic activity was assayed using a chick embryo chorioallantoic membrane (CAM) as described previously[14]. HepG-2 cell resuspensions (1×10^6) were inoculated into the chick embryo CAM. Using 4-d-old chick embryos in shells, 50 μL of different concentrations of TTF1, apigenin (KeyGEN), and normal saline were added to the chick chorioallantoic membrane once per day for 5 d. Each experimental group included five eggs, and experiments were repeated five times. Chorioallantoid membranes were collected for microscopy and photographic documentation. Five visual fields were randomly chosen for analyzing the angiogenesis inducing rate and inhibitory rate using the SmartScape microscope photography analysis system.

Inducing rate (%) = (vascular branchpoint number after inoculating tumor cells minus the vascular branchpoint number in non-inoculated tumor cells the vascular branchpoint number in non-inoculated tumor cells) × 100%

Inhibitory rate (%) = (vascular branchpoint number after inoculating tumor cells minus the vascular branchpoint number with drug treament/the vascular branchpoint number after inoculating tumor cells) × 100%

Nude mouse HepG-2 tumor model

BALB/c nude mice were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Jilin, China). All studies were in compliance with guidelines of the Institutional Animal Care and Use Committee. 0.1 mL HepG-2 cell resuspensions (1×10^6) were transplanted into the armpits of test mice subcutaneously as an experimental model. Ten days after HepG-2 cell transplantation, 40 mice bearing tumors were selected and divided into five groups, and orally administered 5, 10 or 20 μmol/kg of TTF1 or 10 μmol/kg of apigenin once a day for 10 d. The control group was treated with normal saline. Mice were sacrificed and the tumors were collected and weighed. The tumor inhibition rate was calculated as follows: inhibition rate (%) = (1- the tumor weight in treatment group/the tumor weight in control group) × 100%. Samples were fixed in a 10% formaldehyde solution to prepare the slides for hematoxylin and eosin staining and microscopy.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Immunodetection of blood vessels in mouse tumor sections was performed with an anti-CD34 Ab (Boshide Biotechnology Company, Wuhan, China). Sections were incubated with a biotinylated anti-rat Ab (CD34) and then with peroxidase-conjugated streptavidin (Boshide Biotechnology Company, Wuhan, China). To quantify angiogenesis, microvessel density (MVD) was determined by staining tissue sections immunohistochemically for CD34 using the Weidner capillary counting method[15]. Entire sections were scanned under low magnification, and vascularization was subjectively graded. Three highly vascularized areas per tumor were then evaluated at low magnification (× 200). Any brown-staining CD34 distinct from adjacent microvessels, tumor cells, or other stromal cells was considered a single countable microvessel. The total number of microvessels was determined from five vessels in each area, and the average number was recorded for each tumor. To test TTF1 treatment effect on VEGF and basic fibroblast growth factor (bFGF) expression in tumor, the slides were prepared by following the protocol of S-P Kit. Using the double-blind method, the pictures from at least five representative high-power fields were observed in each slice, and no less than 100 cells in each field were counted for analysis.

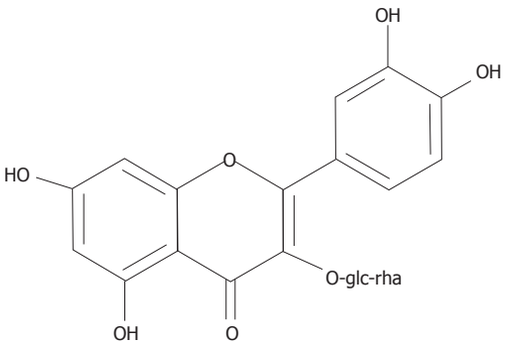

Figure 1 Chemical structure of TTF1.

Western blotting analysis

Tumors were lysed in lysis buffer (Pierce Roche, United States) and then centrifuged at 12 000 *g* for 15 min. Protein concentration was determined using the BCA kit (Pierce Rockford, United States) following the manufacturer's instructions. Seventy μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY, United States). After blocking for 1 h with 5% milk in tris-buffuered saline and tween 20, the primary antibody (anti-VEGF, KDR, bFGF, COX-2 or HIF-1α; 1:400) (Boshide Biotechnology Company) was added and incubated at 4 ℃ overnight. After incubation with secondary antibodies (1:5000), membranes were visualized by chemiluminescence. The intensity of protein bands was quantitatively determined using a ultraviolet crosslinkers (Bio-Rad, United States) and normalized with the intensity of Actin band in each gel.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from tumors using the RNeasy Plus Mini Kit (KeyGEN) following the manufacturer's instructions. cDNA was generated with the iScript Select cDNA Synthesis Kit (KeyGEN) and analyzed by quantitative real-time polymerase chain reaction (PCR) using SyberGreen qPCR primer assays (KeyGEN) and the iCycler iQ multicolor real time PCR detection system (KeyGEN). Relative expression levels were normalized against β-actin expression run concurrently as a reference control. The primers used were as follows: VEGF (forward, 5'-TACGTTGGTGCCCGCTGCTG-3'; reverse, 5'-GCCCTCCGGACCCAAAGTGC-3'; amplicon size of 400 bp), KDR (forward, 5'-AGCGTGTGGCACCCACGATC-3'; reverse, 5'-GGCAATCACCGCCGTGCCTA-3'; amplicon length of 338 bp); COX-2 (forward, 5'-TTGCCCGACTCCCTTGGGTGT-3'; reverse, 5'-CTCCTGCCCCACAGCAAACCG-3'; amplicon length of 397 bp); HIF1-α (forward, 5'-ACAGCAGCCAGACGATCATGCAG-3'; reverse, 5'-TGGCTACCACGTACTGCTGGCA-3'; amplicon length of 724 bp); β-actin (forward, 5'-GCTCGTCGTCGACAACGGCTC-3'; reverse, 5'-CAAACATGATCTGGGTCA TCTTCTC-3'; amplicon length of 353 bp).

Statistical analysis

Data in all experiments are shown as mean ± SD. Statistical difference was evaluated using a one-way ANOVA and independent *t* test of sample pairs with SPSS 13.0 software.

RESULTS

Effect of TTF1 on angiogenesis in chick embryo chorioallantoic membrane

The antiangiogenic activities of TTF1 (Figure 1) were tested using the CAM assay. HepG-2 cells induced CAM angiogenesis (Figure 2B). Capillary vessels were intensively spread in HepG-2 cell-inoculated regions, vessel branching significantly increased ($P < 0.05$) (Figure 2A, B and D), and the inhibitory rate was 53.9%. TTF1 inhibited angiogenesis: the number of capillary vessels significantly decreased ($P < 0.05$) in the TTF1 treatment group (Figure 2C and D), with inhibitory rates of 30.8%, 38.2% and 47.5% with TTF1 treatment concentrations of 25, 50 and 100 μg/embryo × 5 d, respectively (Figure 2D and E). The inhibitory effect on angiogenesis *in vivo* by TTF1 was dose-dependent. These results indicate that TTF1 inhibited angiogenesis induced by HepG-2 cells in CAM.

Changes in mouse tumor weight

To test whether TTF1 inhibited tumor growth, we measured tumor weight after TTF1 treatment. Compared to the control group, the tumor weights in the TTF1-treated group were significantly lower ($P < 0.01$), with inhibitory rates of 43.8%, 49.4% and 59.6% at treatment concentrations of 5, 10 and 20 μmoL/kg, respectively (Figure 3). These results suggest that TTF1 administration blocked the growth of HepG-2 cell-induced tumors in mice and that the inhibitory rate of TTF1 was dose-dependent.

Tumor pathology

Compared to the tumors in the control group, the tumors in the TTF1 and apigenin-treated groups were smaller in size with gray surfaces. Their texture was hard, and necrosis was present in the central area but few capillary hemorrhages were observed (data not shown). Microscopy of tumors from the TTF1 and apigenin-treated groups revealed that they had fewer tumor cells, increased cell gaps with clearly visible cell boundaries, and few capillaries in the central area (data not shown).

Changes in microvessel density in a mouse model

To quantify the HepG-2 cell-induced angiogenesis in mouse tumors, MVD was determined by staining tissue sections immunohistochemically for CD34. The positive staining of CD34 was brown and mainly located in the vascular endothelium of the cytomembrane and the cytoplast of capillary vessels, venules and arterioles (Figure 4A). The results showed that the number of capillary vessels greatly increased in tumor tissues in the control group, while they significantly decreased in the TTF1 treatment group (Figure 4B and C). The average MVD was 14.2, 11.2 and 8.5 at the treatment concentrations

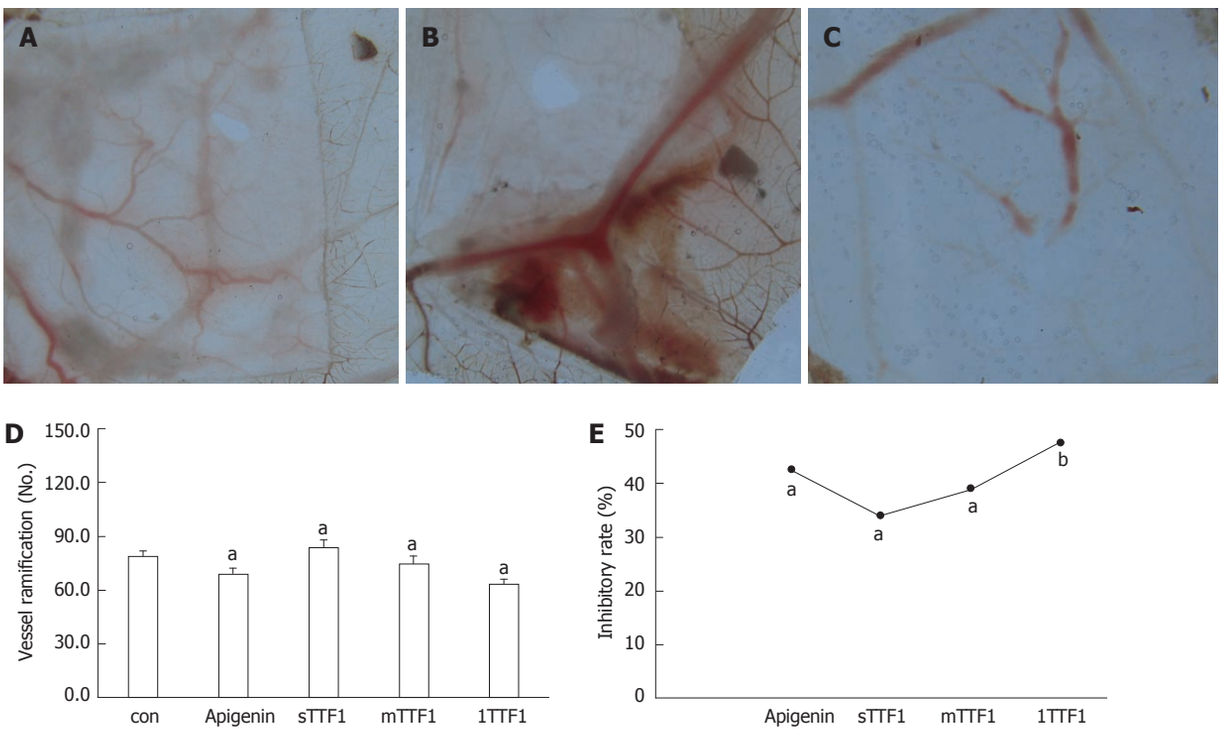

Figure 2 Inhibitory effect of TTF1 on HepG-2 cell-induced angiogenesis in chorioallantoic membrane. A: Control, inoculated with normal saline; B: Inoculated with HepG-2 cells; C: Inoculated with HepG-2 cells and treated with TTF1; D and E: Different doses of compound were used as treatments in the HepG-2 cell-induced angiogenesis in chorioallantoic membrane (CAM) as follows: sTTF1 (25 μg/embryo × 5 d); mTTF1 (50 μg/embryo × 5 d); lTTF1 (100 μg/embryo × 5 d); apigenin (100 μg/embryo × 5 d); and Con (control group treated with normal saline). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

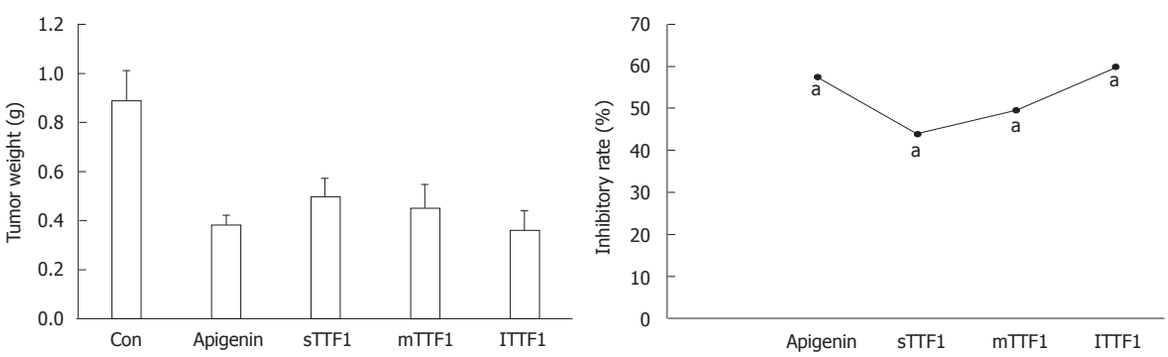

Figure 3 Effect of TTF1 on tumor weight in HepG-2-transplanted nude mice. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); lTTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline). Compared to the control group. $^{a}P < 0.05$ *vs* control group.

of 5 μmol/kg, 10 μmol/kg and 20 μmol/kg TTF1, respectively, and it decreased in a dose-dependent manner (Figure 4C). These results indicated that TTF1 inhibited HepG-2 cell-induced angiogenesis in mouse tumors.

Effect of TTF1 on angiogenesis regulation factors

To test whether TTF1 affects the expression of the angiogenesis regulation factors including VEGF, KDR, bFGF, COX-2 and HIF-1α, we analyzed the protein levels of these factors in HepG-2 cell-induced tumors in mice. Immunohistochemistry results (as shown in Figures 5 and 6) showed the effect of TTF1 on the expression of VEGF and bFGF. In the control group, expression of VEGF and bFGF was demonstrated by brown staining of the cytoplasm and membrane of cancer cells, with a focal or diffuse distribution (Figures 5E and 6E). In the TTF1 treatment group, the brown-stained VEGF and bFGF cancer cells were significantly reduced, and most of the cells were stained blue (negative), as shown in Figures 5C and 6C. Combining these results showed that treatment with TTF1 resulted in significant down-regulation of VEGF and bFGF expression in tumors (Figure 7). Western blotting indicated that the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α were lower in tumors that were treated with TTF1 than in control tumors (Figure 8A and B). We found that the decrease in protein levels occurred in a dose-dependent manner and showed significant differences at the 10 μmol/kg and 20 μmol/kg doses (as shown in Figure 8A and B) when compared to the controls. To explore whether TTF1

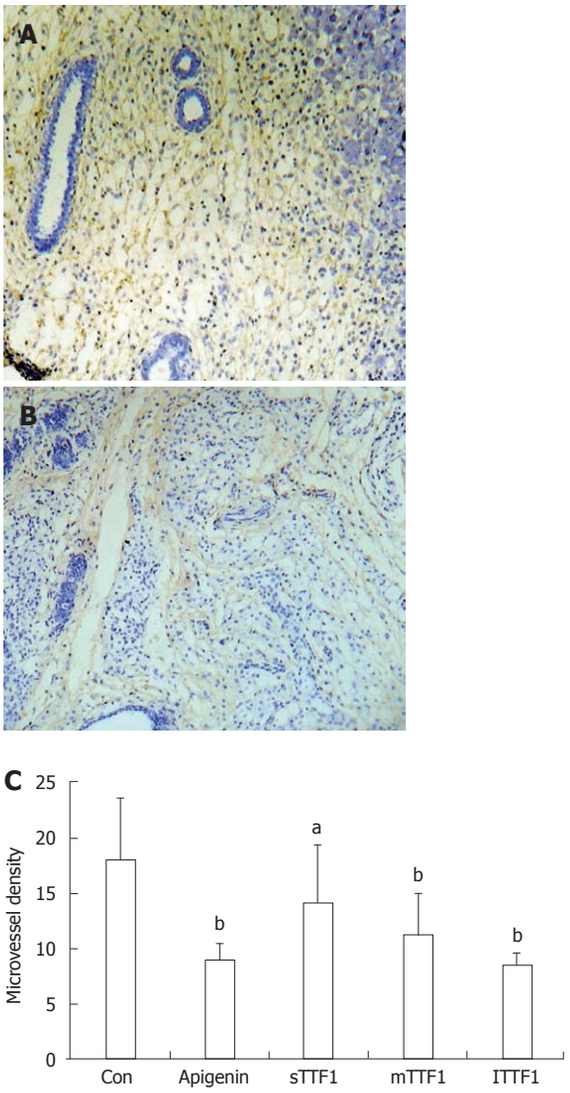

Figure 4 Microvessels in tumor tissue angiogenesis in HepG-2-transplanted nude mice. Brown staining indicates CD34 positive cells. A: Control, HepG-2-transplanted nude mouse treated with normal saline; B: TTF1, HepG-2-transplanted nude mouse treated with TTF1; C: Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); ITTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

inhibits gene transcription to decrease the expression of these angiogenesis regulation factors, quantitative real-time PCR (qRT-PCR) was performed to determine the mRNA levels of VEGF, KDR, bFGF, COX-2 and HIF-1α in mice treated with TTF1. Representative qRT-PCR graphs for these genes is shown in Figure 8C-E. The effect of TTF1 on the mRNA levels of VEGF, KDR, bFGF, COX-2 and HIF-1α was consistent with the effect TTF1 on their protein levels. Our results indicate that TTF1 inhibits tumor angiogenesis by decreasing the RNA and protein levels of angiogenesis regulation factors (VEGF, KDR, bFGF, COX-2 and HIF-1α).

DISCUSSION

S. sorbifolia is a Chinese medicinal plant that grows on

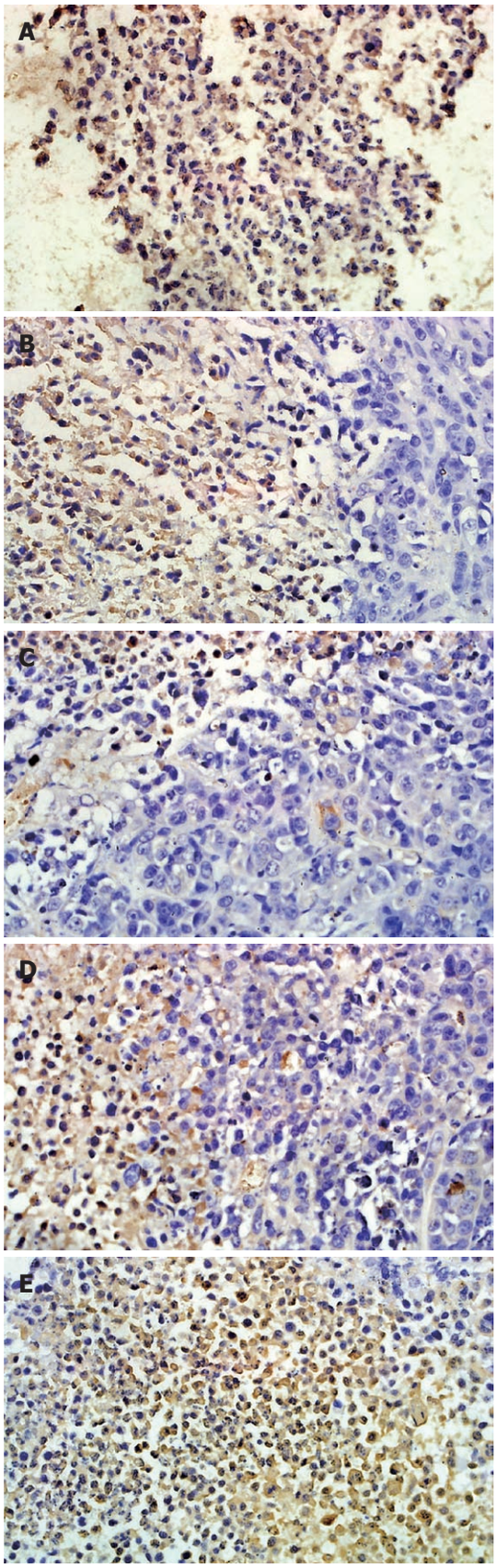

Figure 5 Down-regulation of expression of vascular endothelial growth factor by TTF1 (× 200). Brown staining indicates vascular endothelial growth factor positive. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: A: sTTF1 (5 μmol/kg); B: TTF1 (10 μmol/kg); C: ITTF1 (20 μmol/kg); D: Apigenin (40 μmol/kg); E: Con (control group treated with normal saline).

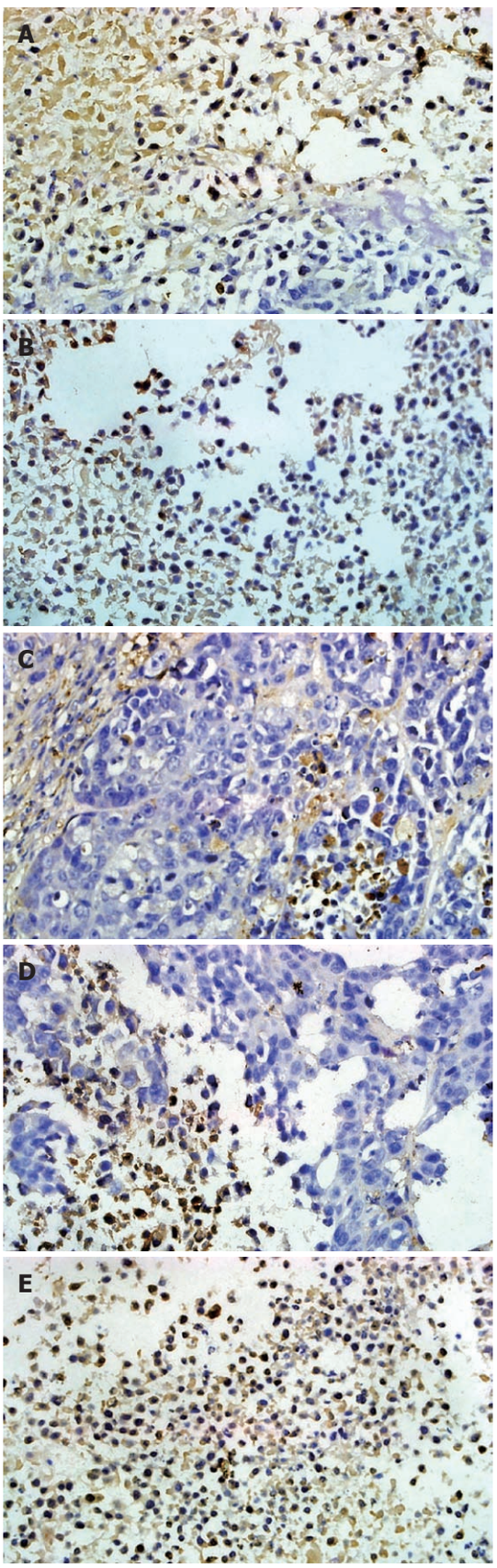

Figure 6 Down-regulation of expression of bFGF by TTF1 (× 200). Brown staining indicates bFGF-positive cells. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: A: sTTF1 (5 μmol/kg); B: TTF1 (10 μmol/kg); C: lTTF1 (20 μmol/kg); D: Apigenin (40 μmol/kg); E: Con (control group treated with normal saline).

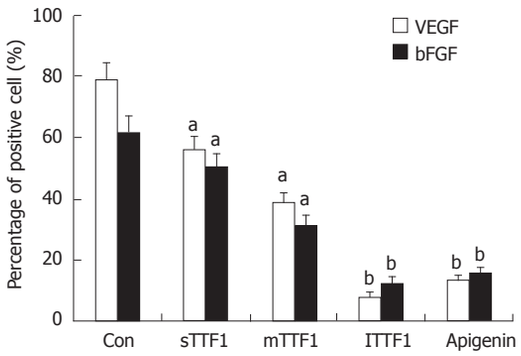

Figure 7 Quantify the effects of TTF1 on the expressions of vascular endothelial growth factor and basic fibroblast growth factor. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg), TTF1 (10 μmol/kg), lTTF1 (20 μmol/kg), apigenin (40 μmol/kg), and Con (control group treated with normal saline). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group. VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor.

Changbai Mountain. Our group began systematic research on its medicinal properties in 2002. An earlier study showed that an acetic ether extract of *S. sorbifolia* has anti-tumor, liver protective and anti-inflammatory effects. Six chemicals were identified in the acetic ether extract, and the novel monomeric compound TTF1 was separated for the first time.

Angiogenesis mainly occurs during embryo development as well as in some pathological conditions, such as damage repair, inflammation, and in particular, tumor growth and metastasis[16]. CAM is the ideal *in vivo* model to study angiogenesis and anti-vascular formation. Our research demonstrated that TTF1 inhibited HepG-2-induced CAM angiogenesis. We also found that TTF1 inhibited tumor growth in HepG-2-transplanted nude mice with an inhibition rate similar to that of apigenin, a flavone extracted from another Chinese medicinal plant that is currently in clinical use.

MVD is a marker to assess the level of tumor angiogenesis. An increase in MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor. After treatment with TTF1 on the transplanted tumors of nude mice, MVD decreased, suggesting that it inhibited tumor angiogenesis. VEGF is the most important inducing factor for angiogenesis, which specifically stimulates the proliferation of vascular endothelial cells and angiogenesis. VEGF proteins function in association with VEGF receptor (VEGFR) proteins. The five types of VEGFR include VEGFR-1 (Flt-1), VEGFR-2 (KDR), VEGFR-3 (Flt-4), NP-1 and NP-2. VEGF primarily functions through dimerization with KDR, and its intracellular tyrosine residues autophosphorylate after VEGF and KDR bind together. bFGF is another important inducing factor for angiogenesis. Tumor cells produce bFGF, and induce the vascular endothelial cells to produce bFGF, at the same time, increasing angiogenesis[17,18]. The expression levels of VEGF, VEGFR and bFGF were down-regulated after treatment with TTF1, suggesting that TTF1 may inhibit tumor growth through decreasing angiogenesis-inducing factors in HepG-2-transplanted nude mice.

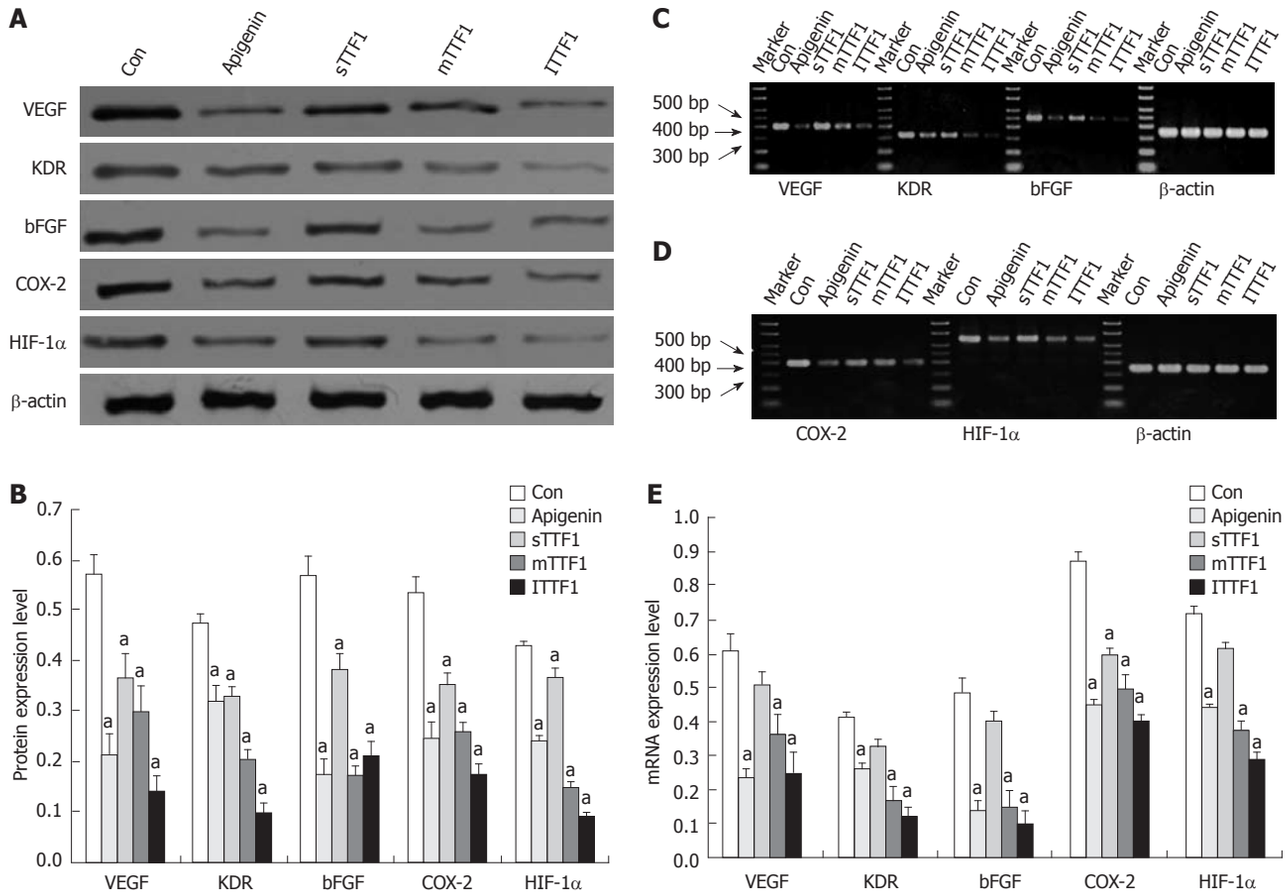

Figure 8 TTF1 decreases the gene and protein level of angiogenesis regulation factors. Western blotting analysis was used to determine the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α. A: Tumor tissues were centrifuged at 12 000 *g* for 15 min and the supernatant (70 μg/lane) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting analysis was performed to detect the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α. Different compounds and dosages were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); ITTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline); B: The intensity of the VEGF, KDR, bFGF, COX-2 and HIF-1α protein bands were determined and normalized with β-actin's intensity by using the ultraviolet crosslinkers imager and plotted ($^aP < 0.05$ *vs* control group); C-E: Quantitative real-time PCR was performed to determine the mRNA expression levels of VEGF, KDR, bFGF, COX-2 and HIF-1α ($^aP < 0.05$ *vs* control group).

An insufficient blood supply in fast-growing tumor tissues may cause hypoxia. HIF-1α is the transcription factor that regulates gene transcription during tissue hypoxia. TTF1 may inhibit expression of HIF-1α by suppressing its association with the regulatory sequences of VEGF and bFGF, and therefore resulting in decreased transcription. The expression of VEGF and bFGF may further decrease the expression of KDR through negative feedback. Recent studies have shown that COX-2 is associated with tumor formation, development, and angiogenesis[19]. COX-2 was down-regulated after TTF1 treatment in tumor tissues, in accordance with the down-regulation of the other angiogenesis-inducing factors VEGF, bFGFand VEGFR.

Identification of the compounds responsible for the anti-tumor angiogenesis effects of Chinese herbal medicines is a research hotspot. Our study used the anti tumor drug apigenin as a positive control and comparison for TTF1 treament. Its mechanism of anti tumor activity includes inhibition of tumor angiogenesis, induction of tumor cell apoptosis, disturbing cellular signal pathways, and anti oxidation. Our experiments showed that the inhibitory effect of TTF1 on tumor angiogenesis surpassed that of apigenin.

S. sorbifolia is a rosaceous plant that grows extensively in Changbai Mountain, in Yunnan, Guizhou, Sichuan, Hubei, Gansu and Ningxia Provinces. It is traditionally used in activating blood, dissolving stasis, reducing swelling, easing pain, and healing fractures and injuries from falls[20]. It is a perennial herbaceous plant that has low toxicity and is liver-protective. Our study explored the inhibitory effect of TTF1 on tumor growth and angiogenesis. Further study needs to focus on the different regulatory factors and their interaction using molecular biological techniques after TTF1 inhibition of tumor angiogenesis. The relationship of the chemical structure of TTF1 to its activity should be studied, so that further structural modification may lead to new inhibitors of tumor angiogenesis with better curative effect and easier production. Moreover, further study is also needed to determine whether there are other pathways (such as inducing apoptosis, regulation of nuclear factor-κB or mitogen-activated protein kinase pathways) through which TTF1 inhibits tumor growth.

COMMENTS

Background

Anti-angiogenesis is an important strategy for tumor therapy. Many studies have demonstrated that tumor angiogenesis can be inhibited by the flavones present in Chinese herbal medicine. Previously, the authors reported that acetic ether extracts of the medicinal plant *Sorbaria sorbifolia* (*S. sorbifolia)* inhibites the growth of HepG-2 cells and mouse S180 sarcoma, down-regulates the levels of tumor necrosis factor-α and interleukin-2, and reduces the activity of natural killer cells. 5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1) was the first active flavonoid compound identified in *S. sorbifolia*. This study focused on the effect of TTF1 specifically on the inhibition of tumor angiogenesis.

Research frontiers

Identification of the compounds responsible for the anti-tumor angiogenesis effects of Chinese herbal medicine is a research hotspot. The study used the anti-tumor drug apigenin, which is currently used clinically, as a positive control and comparison for TTF1 treatment. Its mechanism of anti-tumor activity includes inhibition of tumor angiogenesis, induction of tumor cell apoptosis, disturbing cellular signal pathways, and anti-oxidation. The experiments showed that TTF1 had an inhibitory effect on tumor angiogenesis, as did apigenin.

Innovations and breakthroughs

Six compounds were identified in acetic ether extracts of *S. sorbifolia*, including TTF1,5,7- dihydroxy-8-methoxyflavone, rutin, quercetin, daucosterol, benzoate, and p-hydroxybenzoic acid and TTF1 was the first active flavonoid compound identified in *S. sorbifolia*. After testing the six compounds, the authors found that TTF1 inhibited vascular endothelial growth factor (VEGF) expression in HepG-2 cells and VEGF165-induced human umbilical vein endothelial cell proliferation and vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR) protein expression. The study explored the inhibitory effect of TTF1 on tumor growth and tumor angiogenesis.

Applications

The study results suggest that the TTF1 extracts of the medicinal plant *S. sorbifolia* is a potential therapeutic compound that could be used for tumor inhibition.

Terminology

Sorbaria sorbifolia (*S. sorbifolia)* is a Chinese medicinal plant that grows on Changbai Mountain. An earlier study has shown that an acetic ether extract of *S. sorbifolia* has anti-tumor, liver protective and anti-inflammatory effects. Chick embryo chorioallantoic membrane, is the ideal *in vivo* model for studying angiogenesis and anti-vascular formation. Microvessel density (MVD), is a marker to assess the level of tumor angiogenesis. An increase in MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor.

Peer review

The present paper examining the effects of extracts of the Chinese herb *S. sorbifolia* (TTF1) on tumor growth is work that extends and builds upon previously published work by this research group. The paper will gather a lot of interest amongst practicing gastroenterologists and oncologists.

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BRIEF ARTICLE

Human intestinal acyl-CoA synthetase 5 is sensitive to the inhibitor triacsin C

Elke Kaemmerer, Anne Peuscher, Andrea Reinartz, Christian Liedtke, Ralf Weiskirchen, Jürgen Kopitz, Nikolaus Gassler

Elke Kaemmerer, Department of Pediatrics, RWTH Aachen University, Aachen, Germany and Institute of Pathology, RWTH Aachen University, 52074 Aachen, Germany
Anne Peuscher, Department of Plant Biotechnology, Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), 52074 Aachen, Germany
Andrea Reinartz, Nikolaus Gassler, Institute of Pathology, RWTH Aachen University, 52074 Aachen, Germany
Christian Liedtke, Department of Medicine Ⅲ, RWTH Aachen University, 52074 Aachen, Germany
Ralf Weiskirchen, Institute of Clinical Chemistry and Pathobiochemistry, RWTH Aachen University, 52074 Aachen, Germany
Jürgen Kopitz, Institute of Pathology, University of Heidelberg, 69120 Heidelberg, Germany
Author contributions: Kopitz J designed and coordinated the biochemical studies; cloning and biochemical experiments were performed by Kaemmerer E, Peuscher A, Reinartz A, Liedtke C and Weiskirchen R; Gassler N designed the study; all authors read and approved the final manuscript.
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Correspondence to: Nikolaus Gassler, MA, Professor, Institute of Pathology, RWTH Aachen University, Pauwelsstrasse 30, 52074 Aachen, Germany. ngassler@ukaachen.de
Telephone: +49-241-8088897 Fax: +49-241-8082439
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Abstract

AIM: To investigate whether human acyl-CoA synthetase 5 (ACSL5) is sensitive to the ACSL inhibitor triacsin C.

METHODS: The ACSL isoforms ACSL1 and ACSL5 from rat as well as human ACSL5 were cloned and recombinantly expressed as 6xHis-tagged enzymes. Ni^{2+}-affinity purified recombinant enzymes were assayed at pH 7.5 or pH 9.5 in the presence or absence of triacsin C. In addition, ACSL5 transfected CaCo2 cells and intestinal human mucosa were monitored. ACSL5 expression in cellular systems was verified using Western blot and immunofluorescence. The ACSL assay mix included TrisHCl (pH 7.4), ATP, CoA, EDTA, DTT, $MgCl_2$, [9,10-^{3}H] palmitic acid, and triton X-100. The 200 μL reaction was initiated with the addition of solubilized, purified recombinant proteins or cellular lysates. Reactions were terminated after 10, 30 or 60 min of incubation with Doles medium.

RESULTS: Expression of soluble recombinant ACSL proteins was found after incubation with isopropyl beta-D-1-thiogalactopyranoside and after ultracentrifugation these were further purified to near homogeneity with Ni^{2+}-affinity chromatography. Triacsin C selectively and strongly inhibited recombinant human ACSL5 protein at pH 7.5 and pH 9.5, as well as recombinant rat ACSL1 (sensitive control), but not recombinant rat ACSL5 (insensitive control). The IC50 for human ACSL5 was about 10 μmol/L. The inhibitory triacsin C effect was similar for different incubation times (10, 30 and 60 min) and was not modified by the N- or C-terminal location of the 6xHis-tag. In order to evaluate ACSL5 sensitivity to triacsin C in a cellular environment, stable human ACSL5 CaCo2 transfectants and mechanically dissected normal human intestinal mucosa with high physiological expression of ACSL5 were analyzed. In both models, ACSL5 peak activity was found at pH 7.5 and pH 9.5, corresponding to the properties of recombinant human ACSL5 protein. In the presence of triacsin C (25 μmol/L), total ACSL activity was dramatically diminished in human ACSL5 transfectants as well as in ACSL5-rich human intestinal mucosa.

CONCLUSION: The data strongly indicate that human ACSL5 is sensitive to triacsin C and does not compensate for other triacsin C-sensitive ACSL isoforms.

Key words: Acyl-CoA synthetase 5; Fatty acid metabolism; Mitochondria; Triacsin C

Peer reviewers: Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; Haruhiko Sugimura, MD, PhD, Professor, Department of Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, 431-3192, Japan

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INTRODUCTION

Acyl-CoA derivatives play a fundamental role in the lipid metabolism of eukaryotic cells including enterocytes. Several biological processes are influenced by acyl-CoA thioesters (acyl-CoAs), ranging from intermediary and mitochondrial metabolism to nuclear gene transcription[1]. The formation of long-chain acyl-CoA derivatives is catalyzed by acyl-CoA synthetases (ACSLs; E.C. 6.2.1.3.), which convert long-chain fatty acids (FAs) into acyl-CoAs[2]. In humans and rodents, five ACSL isoforms have been identified so far, differing in their substrate preferences, enzyme kinetics, cellular and organelle locations, as well as their expression[3]. Human ACSL5 is strongly expressed by enterocytes of the small and large intestine, and is suggested as a modifier of enterocytic maturation and cell death[4-6]. Impaired ACSL5 expression and synthesis has been found in colorectal carcinogenesis[7]. The diversity of ACSL proteins is of functional interest because recent studies suggest that ACSL proteins may play a role in channelling fatty acids toward diverse and complex lipid functions with high relevance for cellular behaviour[8,9].

Triacsin C [1-hydroxy-3-(E,E,E-2',4',7'-undecatrienylidine) triazene], an alkenyl-N-hydroxytriazene fungal metabolite, has been reported to be a potent competitive inhibitor of acyl-CoA synthetase activity[10]. The inhibitory capacity of triacsin C depends on the N-hydroxytriazene moiety of the molecule. In different cellular systems, consequences of ACSL inhibition by triacsin C were found, including a dramatic reduction in cholesterol as well as triglyceride synthesis with non-transition of macrophages to foam cells or enhanced eicosanoid release in leucocytes[11,12]. In endothelial cells, arachidonoyl-CoA synthesis was considerably inhibited by triacsin C[13]. Interference of triacsin C with cellular proliferation *via* inhibition of hu-ACSLs has been found[14,15]. It has been speculated that the plethora of triacsin C effects results from differences in the triacsin C susceptibility of ACSL isoforms. In accordance with this hypothesis, it has been demonstrated that triacsin C inhibits recombinant rat ACSL1 (r-ACSL1), rat ACSL3 (r-ACSL3), and rat ACSL4 (r-ACSL4), but not ACSL5 (r-ACSL5) enzyme activity and may therefore, be useful for discriminating amongst ACSL functions[16,17]. The activity of recently described rat ACSL6 subtypes (r-ACSL6_v1 and r-ACSL6_v2) was not affected by triacsin C at concentrations as high as 50 μmol/L[17]. However, ACSL6 is not essentially expressed in intestinal tissues and enterocytes are widely negative for ACSL6 species.

The aim of the present study was to characterize the effect of triacsin C on human ACSL5 protein *in vitro* and in the intestinal cellular environment. Our findings show that human ACSL5 is, unlike rat ACSL5, sensitive to triacsin C.

MATERIALS AND METHODS

Materials

The rat anti-human ACSL5 antibody KD7 was prepared as previously described[5]. Additional antibodies and substances were anti-beta-actin (Sigma, Deisenhofen, Germany), anti-histidine antibodies (Roche, Mannheim, Germany), HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany; Dianova, Hamburg, Germany), enhanced chemiluminescence (PIERCE, Rockfort, United States), rainbow protein standard (Amersham Bioscience, United Kingdom), PVDF Immobilon-P membrane (Millipore, Bedford, United States), MitoTracker RedCMXRos (Molecular probes, Eugen, United States), and DAPI (Vysis Inc., Downer's Grove, United States). The alkenyl-N-hydroxytriazene fungal metabolite triacsin C (Biomol, Hamburg, Germany) and other materials were obtained from commercial sources. Standard cloning techniques were used for cloning hu-ACSL5 sequences (GeneBank accession no. AB033899) into the pENTRY vector of the GATEWAY cloning system (Invitrogen Life Technologies, Karlsruhe, Germany). Cytomegalovirus expression constructs were synthesized by recombination into the pcDNA_DEST40 vector. CaCo2 cell lines, stably transfected with hu-ACSL5 in pcDNA_DEST40 or empty pcDNA_DEST40, were cultured under appropriate culture conditions. In one approach, T5 promoter expression constructs with N-terminal fusion of hu-ACSL5 sequences to 6xHis-tag and factor Xa protease recognition sites were synthesized by recombination into the pQE-30Xa vector (Qiagen, Hilden, Germany). In a second approach, sequences of r-ACSL1 (EST clone IMAGp998O0814978Q in pExpress-1) and r-ACSL5 (I.M.A.G.E. full length cDNA clone IRBPp993B014D in pExpress-1), both delivered from RZPD Berlin, Germany, as well as the hu-ACSL5 sequence were cloned into pET22b(+) (Merck, Darmstadt, Germany) in *Escherichia coli (E. coli)* without a pelB leader sequence for cytosolic expression of C-terminal 6xHis-tagged proteins. Specimens of human normal intestinal mucosa (n = 10) were mechanically dissected from surgical resections and used fresh or immediately frozen in liquid nitrogen and stored at -80 ℃. The use of human tissues for study purposes was approved by each patient and by the local ethics committee at the University Hospital of the RWTH Aachen (EK019/06).

Cloning and recombinant expression of acyl-CoA synthetase species

For cloning of hu-ACSL5 into appropriate vectors, sequences were generated from human intestine by long distance RT-PCR using the following set of primers: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTACCATGCTTTTTTATCTTTAACTTTTTTGTTTTCCCCACTTCC-3' (sense) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCATCCTGGATGTGCTCATACAGGCTGT-3' (antisense). Sequences of r-ACSL1 and r-ACSL5 were amplified for recombinant expression cloning. Correctness of all ACSL target sequences (accession nos.: AB033899, AM262166, NM_012820, NM_053607) were controlled by full length sequencing. Recombinant expression of different ACSL proteins in *E. coli* M15 or *E. coli* BL21 was induced with 1 mmol/L isopropyl beta-D-1-thiogalactopyranoside (IPTG) in LB medium when an OD_{600} of 0.6-0.9 was reached. In *E. coli* M15, formation of inclusion bodies was highly diminished by an incubation period of 45 min at 20 ℃. An incubation period of 24 h at 16 ℃ was used for r-ACSL1 and r-ACSL5 expression in *E. coli* BL21, whereas 6 h at 28 ℃ was optimal for expressing hu-ACSL5 in *E. coli* BL21. Expression of soluble ACSL proteins was verified by Western or Dot blotting of ultracentrifuged fractions and anti-ACSL5 or anti-His antibody probes. In control experiments, bacteria transformed with the empty vector were used.

Protein purification

Samples of IPTG-treated bacteria were sonicated in ice-cooled buffer (buffer A) containing 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1% triton X-100, and 0.1% sodium cholate, and further processed by ultracentrifugation. The resulting supernatants were applied to a Ni^{2+}-affinity column (5 mL HisTrap HP; Amersham Pharmacia GE Healthcare, Freiburg, Germany). Elution was performed at a flow rate of 1 mL/min at 20 ℃; a linear gradient from buffer A to A/B (50:50, buffer B: buffer A + 500 mmol/L imidazole); 10 min with A/B (50:50); a linear gradient from A/B (50:50) to solvent B; finally with buffer B. Purification of ACSL proteins was controlled by SDS gel electrophoresis with subsequent silver staining, Western blotting, and ACSL activity assays of the purified proteins. ACSL proteins were purified to near homogeneity, migrating as single bands. The Lowry procedure was used for measurement of total protein, and bacteria transformed with the empty vector was used as a negative control.

Acyl-CoA synthetase activity assay

ACSL activity assays were performed as previously described[6]. The standard ACSL assay mix contained 150 mmol/L TrisHCl (pH 7.4), 40 mmol/L ATP, 1.2 mmol/L CoA, 2 mmol/L EDTA, 2 mmol/L DTT, 0.1 mol/L $MgCl_2$, 0.5 μmol/L [9,10-^{3}H] palmitic acid, and 0.2% triton X-100. The 200 μL reaction was initiated by adding solubilized purified recombinant hu- or r-ACSL proteins (ca. 0.1-2.0 μg) or cellular lysates in 0.1% sodium deoxycholate and 1% triton X-100 in 20 mmol/L Tris (pH 7.4). Reactions were terminated after 10, 30 or 60 min incubation with Doles medium. After phase separation, the watery phase was washed twice with palmitic acid-enriched n-heptane. Radioactivity was measured using Ultima Gold cocktail in a Tri-Carb liquid scintillation (2900TR) counter equipped with QuantaSmart software (PerkinElmer, Rodgau, Germany). The studies were repeated three times, and virtually identical results were obtained from each experiment. Enzyme activity was always demonstrated in percent of the related peak activity. Error bars are SEM.

Western blotting analysis

Cellular proteins were separated by one-dimensional SDS-PAGE and transferred to a PVDF Immobilon-P membrane. Immunodetection was performed with primary antibodies [mAB KD7, specific for ACSL5 (undiluted); anti-beta-actin (1:1000)], probed with HRP-conjugated secondary antibodies (1:10 000) and developed with enhanced chemiluminescence as suggested by the provider. Rainbow protein standard was used for molecular weight estimation.

Immunofluorescence

Cells were incubated with anti-histidine antibodies, specific for His-tagged proteins, followed by Cy2-labeled anti-mouse antibodies. For negative controls, the primary antibody was replaced by normal serum. MitoTracker RedCMXRos was used as a mitochondrial marker following the manufacturer's recommendations. Cells were incubated with 25 nmol/L MitoTracker for 15 min, washed, fixed (methanol at -20 ℃ for 5 min, followed by acetone at 4 ℃ for 2 min), and permeabilized with 0.2% Triton X-100. After incubation of primary and secondary antibodies, DAPI was applied for nuclear staining. Images were visualized using a confocal laser microscope (Nicon, Düsseldorf, Germany).

RESULTS

Expression and purification of recombinant hu-acyl-CoA synthetase 5 proteins

Expression of soluble recombinant hu-ACSL5 protein in *E. coli* M15 was found after incubation with IPTG for 45 min at 20 ℃. Soluble activity in supernatants after ultracentrifugation (about 10% of total ACSL activity) was further purified to near homogeneity using Ni^{2+}-affinity chromatography. The specific activity of recombinant hu-ACSL5 proteins from different preparations were in the range of 1.08-2.31 nmol/min per mg and independent of the epitope and its location at the N- or C-terminus (Figure 1).

Triacsin C strongly inhibits recombinant hu-acyl-CoA synthetase 5, but not r-acyl-CoA synthetase 5 activity

Since hu-ACSL5 displayed activity peaks at pH 7.5[6,16] and pH 9.5[6], conditions favouring maximal enzyme activity were chosen when the inhibitory effects of triacsin

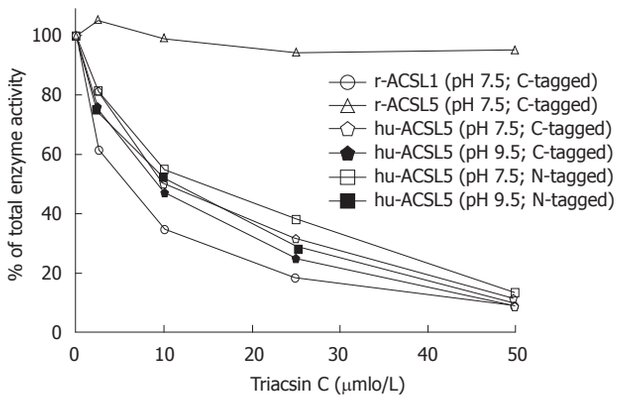

Figure 1 Sensitivity of recombinant acyl-CoA synthetase 5 proteins to triacsin C. Enzyme activity of Ni2+-affinity purified recombinant r-ACSL1, r-ACSL5, hu-ACSL5 C-terminal 6xHis-tagged enzyme, and hu-ACSL5 N-terminal 6xHis-tagged enzyme were assayed at pH 7.5 (white symbols) and pH 9.5 (black symbols) in the presence and absence of triacsin C as described in the methods section. ACSL5: Acyl-CoA synthetase 5.

C to ACSL proteins were tested. To test the effects of triacsin C on purified rat and human ACSL proteins in simultaneous reactions, the triacsin C substance dissolved in DMSO (2.5% of final assay reaction) was directly added to the reaction mixture (0-50 μmol/L) following the procedures by Kim *et al*[16]. Triacsin C selectively and strongly inhibited recombinant human ACSL5 protein (pH 7.5 and pH 9.5), as well as recombinant r-ACSL1 (sensitive control), but not recombinant r-ACSL5 (insensitive control) in a dose dependent manner (Figure 1). The IC50 for human ACSL5 proteins was about 10 μmol/L, and the inhibitory triacsin C effect was similar for different incubation times (10, 30 and 60 min) and was not modified by the N- or C-terminal location of the 6xHis-tag.

Triacsin C inhibits human acyl-CoA synthetase 5 activity in human intestine cellular systems

In order to further characterize the inhibitory potency of triacsin C on hu-ACSL5 protein in a cellular environment, stable hu-ACSL5 transfectants in CaCo2 cells (ATCC No. HTB-37) were used as previously published[6]. Transgenic and endogenous hu-ACSL5 protein expressions were controlled with Western blotting and indirect immunofluorescence (Figure 2). The shift between both ACSL5 proteins in Western blotting is due to the 6xHis-tag and a linker sequence, which result in a higher molecular weight of the transgenic ACSL5 protein (Figure 2A). N-terminal 6xHis-tagged hu-ACSL5 proteins are strongly co-localized with mitochondria. Some extramitochondrial signalling is found, including ER/ribosomes (Figure 2B). Monitoring of ACSL-activity in stable hu-ACSL5 N-terminal 6xHis-tagged transfectants and controls (wild type CaCo2 cells and CaCo2 transfectants with the empty vector) was performed between pH 5 and pH 10 (Figure 2C). In stable hu-ACSL5 CaCo2 transfectants, peak activity was found at pH 7.5 and pH 9.5, corresponding to the properties of recombinant hu-ACSL5 protein. This bimodal curve of ACSL activity was not observed in control cells, where the main ACSL activity was detectable at pH 7. The data further indicate that the bimodal distribution of ACSL activity was mainly due to hu-ACSL5 activity in the CaCo2 cellular environment.

In the presence of triacsin C (25 μmol/L), total ACSL activity was dramatically diminished in hu-ACSL5 transfectants as well as in controls. Importantly, the bimodal curve of ACSL activity with peak values at pH 7.5 and pH 9.5, due to hu-ACSL5 transgenic over-expression, was essentially smoothed in the presence of triacsin C (Figure 2C).

Next, mechanically dissected normal human intestinal mucosa with high ACSL5 expression (Figure 3A) was monitored for ACSL activity in the pH range of 5 to 10 (Figure 3B). Strong ACSL activity was found at pH 7, pH 8, and pH 9.5, partly reflecting the characteristic bimodal activity of human ACSL5 proteins. In the presence of triacsin C (25 μmol/L), ACSL activity was dramatically inhibited; the pH 9.5 peak was especially diminished (Figure 3B). The resulting smooth curve paralleled the findings with triacsin C treated purified hu-ACSL5 recombinant proteins and stable hu-ACSL5 transfected CaCo2 cells.

DISCUSSION

A considerable amount of data indicates that long chain fatty acids are essential in intestinal physiology and pathophysiology. In the modifier concept of intestinal carcinogenesis, the activity of intestinal long chain fatty acids is suggested as an important cell cycle modifier[18]. The function of ACSL mediated metabolic channelling of fatty acids in the regulation of intestinal cell behaviour includes the lipidation of proteins and translation of long chain fatty acid modifiers in several signalling cascades and receptor structures[19]. Specific inhibitors of enzyme activity are well established and powerful tools for determining enzymatic functions in cellular and non-cellular systems. Both sensitivity and specificity of inhibitors are essential prerequisites for a stringent functional analysis of target enzymes. Several molecular mechanisms in enzyme inhibition have been characterized so far, including covalent and non-covalent binding of substrate-like or non-substrate compounds. Competitive binding with the substitution of a characteristic substrate frequently underlies non-covalent enzyme inhibition. Competitive inhibition is the mechanism behind the triacsin C-mediated biochemical effects on ACSL isoforms[10,16,20].

The overwhelming number of studies concerning triacsin C mediated effects on ACSL molecules have been performed in rat models. There is convincing experimental data demonstrating the triacsin C sensitivity of rat ACSL isoforms 1, 3, 4 and 6, but insensitivity of the rat isoform 5[16,17]. However, many ACSL isoforms have splice variants, most of which have not been tested and characterized for triacsin C sensitivity. In addition, species-related differences of ACSL protein sensitivity to triacsin C have not been systematically examined up-

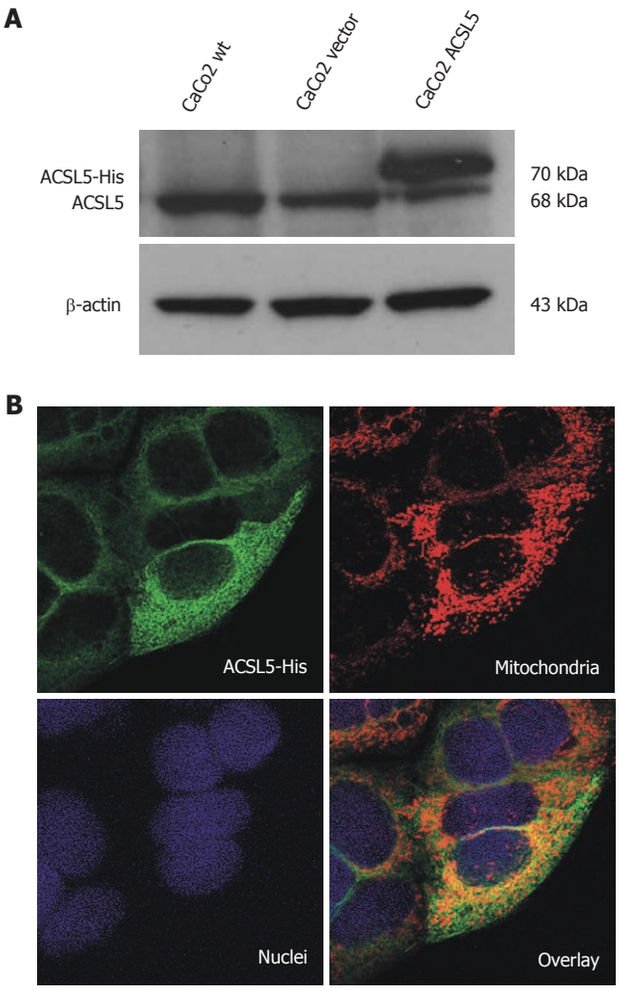

Figure 2 Human acyl-CoA synthetase 5 is triacsin C sensitive in CaCo2 cells. A: CaCo2 cells were stably transfected with the full-length ACSL5 expressing pcDNA_DEST40 plasmid or empty control vector. Expression of N-terminal 6xHis-tagged hu-ACSL5 protein in CaCo2 was analyzed by Western blot using anti-ACSL5 and anti-His antibodies; B: Indirect immunofluorescence of N-terminal 6xHis-tagged hu-ACSL5 proteins, mitochondria, and nuclei in CaCo2 cells shows the mitochondrial localization of recombinant ACSL5 proteins. Original magnification × 400; C: Analysis of ACSL activity (pH 5-10) in hu-ACSL5 CaCo2 transfectants and controls (pcDNA_DEST40 CaCo2 transfectants and CaCo2 wild type) in the presence (white symbols) or absence (black symbols) of 25 μmol/L triacsin C. ACSL5: Acyl-CoA synthetase 5.

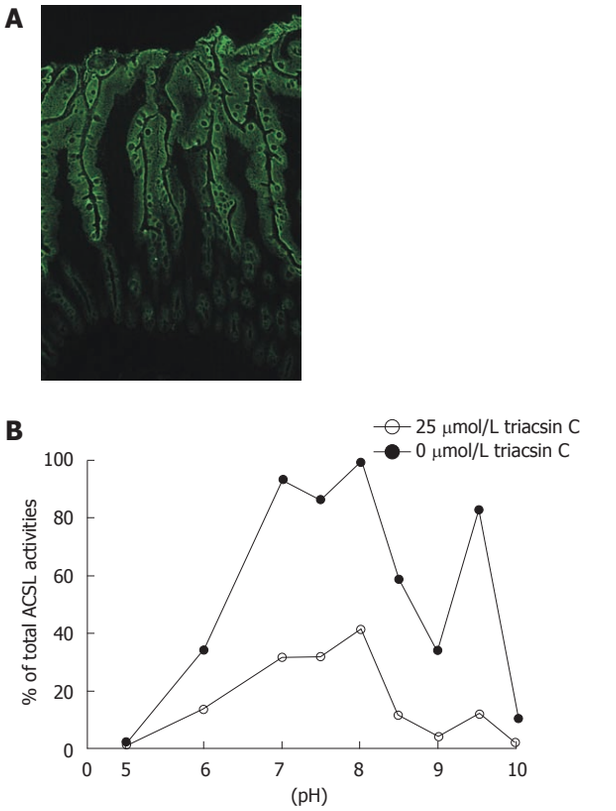

Figure 3 Acyl-CoA synthetase 5 related acyl-CoA synthetase activity is sensitive to triacsin C in human small intestinal mucosa. A: Human small intestinal mucosa after indirect immunofluorescence with anti-ACSL5 antibodies; B: Analysis of ACSL activity in human intestinal mucosa in the presence or absence of 25 μmol/L triacsin C. Triacsin C strongly inhibits ACSL activity in human intestinal mucosa. ACSL5: Acyl-CoA synthetase 5.

to-now. The present study was, therefore, designed to systematically analyze triacsin C effects on human ACSL5 proteins, which are predominantly found in enterocytes of the human small intestinal mucosa.

In order to analyze the biochemical behaviour of hu-ACSL5, we cloned the respective sequences from an intestinal or plasmid cDNA resource, expressed the 6xHis-tagged recombinant proteins, and further purified the enzymes to near homogeneity using Ni^{2+}-affinity chromatography. As demonstrated by digestion experiments and the subsequent analysis of ACSL-activity, the N- or C-terminal 6xHis-tag did not alter the enzymatic activity of ACSL5 at 30 ℃ or 37 ℃ in a broad range of pH values (pH 5-10). Monitoring ACSL-activity of recombinant hu-ACSL5 protein revealed triacsin C-sensitivity with an IC50 about 10 μmol/L. In this experimental setting, insensitivity of recombinant r-ACSL5 and sensitivity of recombinant r-ACSL1 was found, identical to previously described data by Kim *et al*[16].

Additional experiments ruled out the possibility that triacsin C-sensitivity of purified recombinant hu-ACSL5 protein could be a result of the absence of a cellular environment. Stable hu-ACSL5 transfectants and stable controls were established from CaCo2 cells, and in another set of experiments, human intestinal mucosa was investigated. The ACSL activity of cellular systems and tissues

was monitored at different pH values in the presence or absence of triacsin C (25 μmol/L), and similar results to those with recombinant proteins were seen. Especially at pH 9.5, a pH value highly characteristic for hu-ACSL5 activity[6], ACSL activity was significantly decreased in cultured cells as well as intestinal mucosa. The background ACSL activity was probably due to enzymes other than ACSL5, including triacsin C insensitive ACSL splice forms, ACSL isoforms, and fatty acid binding proteins. In conclusion, we demonstrate experimental evidence that hu-ACSL5 is triacsin C sensitive as a purified recombinant protein, in hu-ACSL5 over-expressing epithelial cell lines, and in human small intestinal mucosa, a tissue with high ACSL5 expression levels. These findings imply that human ACSL5 is not able to compensate for triacsin C-inhibited ACSL isoforms, and triacsin C cannot be used to differentiate functions of different ACSL enzymes in human cells or tissues.

The insensitivity of human ACSL5 to triacsin C has been postulated by the observation that recombinant rat ACSL5 was not inhibited by this fungal metabolite[16]. This has been addressed in several studies[15,21-24]. In all of these studies, triacsin C was preferentially used to incubate cultured cells in a concentration clearly below the $IC50^{hu\text{-}ACSL5}$ of approximately 10 μmol/L, and the substance was not directly added to the acyl-CoA activity assay mixture. In our study triacsin C was always used as a competitive inhibitor. Identical to the experimental approach of Kim *et al*[16], triacsin C was directly added to the acyl-CoA activity assay mixture.

We hypothesize that the divergent triacsin C effects on ACSL activity are species-related and determined by human and rat ACSL5 protein sequences. A sequence analysis of the proteins revealed that these were only 81% identical, and that discrepancies existed in exon 20 splicing as well as in the organization and length of functional domains, like the ATP-binding domain or the FA activation domain (NCBI data base: http//www.nlm.nih.gov/nlmhome.html). Species-related differences in ACSL5 activity are further suggested by expression experiments. Over-expression of r-ACSL5 in a rat cellular environment increases fatty acid incorporation into diacylglycerol and triacylglycerol but does not affect fatty acids used for beta-oxidation[25], whereas r-ACSL5 in a human cellular environment increases palmitate oxidation[26].

In the present study, sensitivity of human ACSL5 protein to triacsin C was demonstrated using purified recombinant protein, CaCo2 cells, and human intestinal mucosa. The divergent inhibitory effect of triacsin C with sensitivity of hu-ACSL5 and insensitivity of rat-ACSL5 is most likely species-related. Our findings indicate that human ACSL5 does not compensate for other triacsin C sensitive ACSL isoforms.

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COMMENTS

Background

Strong expression of acyl-CoA synthetase 5 (ACSL5) is found in surface lining epithelia of the large and small intestine. ACSL5 enzyme activity is probably related to enterocytic maturation and intestinal carcinogenesis. Triacsin C is an inhibitor of several ACSL isoforms and is used to differentiate amongst ACSL functions. In rat, ACSL5 is insensitive to triacsin C.

Research frontiers

Analysis of human ACSL5 *in vitro* as well as in a cellular environment revealed sensitivity to the inhibitor triacsin C, which is in contrast to rat ACSL5. The data indicate that a species-related difference in triacsin C inhibition of ACSL5 exists, and human ACSL5 does not compensate for other triacsin C-sensitive human ACSL isoforms.

Innovations and breakthroughs

In previous triacsin C related studies of ACSL activity, cultured cells were preferentially incubated with triacsin C in a concentration clearly below the $IC50^{hu\text{-}ACSL5}$ of approximately 10 μmol/L. Moreover, triacsin C was not used as a competitive inhibitor. In particular, species-related differences of ACSL protein sensitivity to triacsin C have not been systematically addressed up to now.

Applications

The recent finding that human ACSL5 is sensitive to the inhibitor triacsin C should be considered in related experiments. In human tissues, the differentiation of ACSL activities and the characterization of ACSL5 function with triacsin C are limited. The current finding suggests that human ACSL5 does not compensate for other triacsin C-sensitive ACSL isoforms.

Terminology

Acyl-CoA synthetase 5 is an enzyme that catalyzes formation of long-chain acyl-CoA derivatives and belongs to the family of acyl-CoA synthetases (ACSLs; E.C. 6.2.1.3.). Five ACSL isoforms differing in their enzyme kinetics, substrate preferences, and cellular expression have been identified so far in humans and rodents. Triacsin C [1-hydroxy-3-(E,E,E-2',4',7'-undecatrienylidine) triazene], an alkenyl-N-hydroxytriazene fungal metabolite, has been reported to be a potent competitive inhibitor of acyl-CoA synthetase activity.

Peer review

Kaemmerer *et al* showed that human intestinal ACSL5 is sensitive to triacsin C using purified recombinant protein, CaCo2 cells, and human intestinal mucosa. The experimental design is good and interpretation of results was conducted appropriately.

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BRIEF ARTICLE

Serum manganese superoxide dismutase and thioredoxin are potential prognostic markers for hepatitis C virus-related hepatocellular carcinoma

Tsutomu Tamai, Hirofumi Uto, Yoichiro Takami, Kouhei Oda, Akiko Saishoji, Masashi Hashiguchi, Kotaro Kumagai, Takeshi Kure, Seiichi Mawatari, Akihiro Moriuchi, Makoto Oketani, Akio Ido, Hirohito Tsubouchi

Tsutomu Tamai, Hirofumi Uto, Yoichiro Takami, Kouhei Oda, Akiko Saishoji, Masashi Hashiguchi, Kotaro Kumagai, Takeshi Kure, Seiichi Mawatari, Akihiro Moriuchi, Makoto Oketani, Akio Ido, Hirohito Tsubouchi, Department of Digestive and Lifestyle Related Diseases, Human and Environmental Sciences, Health Research, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
Author contributions: Tamai T and Takami Y performed the majority of experiments; Uto H and Tsubouchi H were involved in editing the manuscript; Uto H, Oda K, Saishoji A, Hashiguchi M, Kumagai K, Kure T, Mawatari S, Moriuchi A, Oketani M and Ido A coordinated the collection of and provided all the human material for this work; Tamai T and Uto H designed the study and wrote the manuscript.

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Correspondence to: Hirofumi Uto, MD, PhD, Department of Digestive and Lifestyle Related Diseases, Human and Environmental Sciences, Health Research, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. hirouto@m2.kufm.kagoshima-u.ac.jp
Telephone: +81-99-2755326 Fax: +81-99-2643504
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Abstract

AIM: To evaluate the clinical significance of oxidative stress markers in patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

METHODS: Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital were enrolled in this retrospective study. All patients had chronic liver disease (CLD) due to infection with HCV. Thirty patients with HCV-related HCC, 34 with HCV-related CLD without HCC (non-HCC), and 20 healthy volunteers (HVs) were enrolled. Possible associations between serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels and clinical parameters or patient prognosis were analyzed over a mean follow-up period of 31.7 mo.

RESULTS: The serum MnSOD levels were significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.03$) or HVs ($P < 0.001$). Similarly, serum TRX levels were also significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.04$) or HVs ($P < 0.01$). However, serum levels of MnSOD and TRX were not correlated in patients with HCC. Among patients with HCC, the overall survival rate (OSR) was lower in patients with MnSOD levels $\geqslant$ 110 ng/mL than in patients with levels < 110 ng/mL ($P = 0.01$), and the OSR tended to be lower in patients with TRX levels < 80 ng/mL ($P = 0.05$). In addition, patient prognosis with HCC was poorest with serum MnSOD levels $\geqslant$ 110 ng/mL and serum TRX levels < 80 ng/mL. Furthermore, a multivariate analysis using a Cox proportional hazard model and serum levels of five factors (MnSOD, prothrombin time, serum albumin, serum α-fetoprotein (AFP), and serum des-γ-carboxy prothrombin) revealed that MnSOD levels $\geqslant$ 110 ng/mL (risk ratio: 4.12, 95% confidential interval: 1.22-13.88, $P = 0.02$) and AFP levels $\geqslant$ 40 ng/mL (risk ratio: 6.75; 95% confidential interval: 1.70-26.85, $P < 0.01$) were independent risk factors associated with a poor patient prognosis.

CONCLUSION: Serum MnSOD and TRX levels are potential clinical biomarkers that predict patient prognosis in HCV-related HCC.

Key words: Oxidative stress; Manganese superoxide dismutase; Thioredoxin; Hepatitis C virus; Hepatocellular carcinoma

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Tamai T, Uto H, Takami Y, Oda K, Saishoji A, Hashiguchi M, Kumagai K, Kure T, Mawatari S, Moriuchi A, Oketani M, Ido A, Tsubouchi H. Serum manganese superoxide dismutase and thioredoxin are potential prognostic markers for hepatitis C virus-related hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(44): 4890-4898 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4890.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4890

INTRODUCTION

As a significant cause of global cancer morbidity and mortality, hepatocellular carcinoma (HCC) is the fifth- and seventh-most frequently diagnosed cancer worldwide in men and women, respectively, and is the second- and sixth-most frequent cause of cancer deaths in men and women, respectively[1]. HCC is most frequently caused by persistent infection with hepatitis C or B virus. Early HCC diagnosis and better treatments have helped to improve the prognosis for patients with HCC. Also, interferon (IFN)-based treatments not only eliminate hepatitis C virus (HCV) infection, but also prevent HCC in patients with chronic hepatitis C (CHC)[2]. However, IFN-based therapies do not always effectively eliminate HCV infection or prevent HCC. Thus, biomarkers that are indicative of HCC pathological condition would have many clinical benefits, including aiding in the selection of the most appropriate treatment for a patient's disease.

Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the antioxidative defenses that maintain a cellular redox state. ROS include superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide, all of which are indispensable elements in many biochemical processes[3]. ROS are mainly derived from Kupffer and inflammatory cells in the liver[4], and upon exposure to other cells are thought to induce apoptosis, necrosis, inflammation, immune responses, fibrosis and tissue regeneration[5]. In liver disease, there is an overproduction of ROS from endogenous sources such as the mitochondria, peroxisomes, and activated inflammatory cells. In particular, ROS of mitochondrial origin were recently reported to be elevated in patients with alcoholic liver disease, non-alcoholic steatohepatitis (NASH)[6,7] and HCV-related chronic liver disease (CLD)[8]. Conversely, cells are protected from oxidative stress by intracellular antioxidants such as glutathione (GSH) and thioredoxin (TRX) and by various antioxidant enzymes such as superoxide dismutase (SOD), GSH peroxidase, catalase, and heme oxygenase-1[9-11]. Collectively, the relative expression levels of these molecules may serve as biomarkers for various liver diseases, including HCV-related HCC.

Manganese SOD (MnSOD) is an antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. We have previously demonstrated that MnSOD expression was induced in primary cultured hepatocytes that were loaded with hydrogen peroxide *in vitro* and that serum MnSOD levels can be used to distinguish between NASH and simple steatosis in patients with nonalcoholic fatty liver disease[7]. However, the clinical significance of serum MnSOD levels in HCV-related CLD has not been fully investigated.

TRX was originally discovered in *Escherichia coli* as a proton donor for ribonucleotide reductase[12]. Subsequently, the human *TRX* gene was cloned as an adult T-cell leukemia-derived factor and was originally described as an interleukin-2 receptor inducer present in the cell culture supernatant of human T-lymphotropic virus type-1 -transformed cells[13]. TRX expression is induced by various oxidative stressors in patients with acquired immunodeficiency syndrome[14], Sjögren's syndrome[15], rheumatoid arthritis[16], and malignant neoplasms[17,18]. Previous studies have reported that serum TRX is an oxidative stress marker and that serum TRX levels increase in patients with HCV-related CLD during liver fibrosis progression[19]. In addition, serum TRX levels are reported to be elevated in patients with NASH compared to patients with simple steatosis[20]. However, the clinical significance of elevated TRX levels among patients infected with HCV in relation to HCC diagnosis and prognosis has not been elucidated.

In this study, we aimed to clarify the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related CLD, and in particular among patients with HCC.

MATERIALS AND METHODS

Patients

Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital between December 2006 and November 2008 were enrolled in this retrospective study. All patients had CLD due to an HCV infection and were diagnosed with HCC (30 patients; HCC group) or without HCC (34 patients; non-HCC group). Twenty healthy volunteers (HVs) were also enrolled in this study.

In this study, HCC was diagnosed based on findings from abdominal ultrasound, abdominal computed tomography, and serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP, also known as PIVKA-Ⅱ). Patients were excluded from this study if they were positive for hepatitis B surface antigen; other types of hepatitis, including autoimmune hepatitis and alcoholic liver disease; or other malignancies.

The study endpoint was patient death, the available follow-up date, or December 31, 2010. Patient follow-up

periods ranged from 5.1 to 44.6 mo, with a mean observation time of 31.7 mo. Informed consent was obtained from all study patients and healthy controls. This study was approved by the ethical committees of Kagoshima University Graduate School of Medical and Dental Sciences and Kagoshima University Medical and Dental Hospital.

Laboratory markers

The clinical laboratory parameters assessed included platelet count (Plt), prothrombin time (PT), albumin (Alb), total bilirubin (T-Bil), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), AFP and DCP. The serologically defined HCV genotype (HCV serotype) was determined using a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was evaluated using the HCV Core Genotype assay (SRL, Tokyo, Japan). HCV genotype 1b was included with serotype Ⅰ, while genotypes 2a and 2b were included with serotype Ⅱ. No other HCV genotype was detected in this study population. HCV RNA titers were quantified using either quantitative RT-PCR (Amplicor monitor version 2, Roche, Tokyo, Japan) or the Cobas TaqMan PCR assay (Roche, Tokyo, Japan). Patients were categorized as having a high viral load if their values were 100 KIU/mL or greater based on quantitative RT-PCR analysis, or 5 log IU/mL or more based on the Cobas TaqMan PCR assay.

Evaluation of clinical stage

Hepatic function was assessed in the HCC group using Child-Pugh staging based on both clinical (ascites and encephalopathy) and laboratory (Alb, T-Bil, and PT) parameters. HCC clinical stage was assessed based on a patient's Cancer of the Liver Italian Program (CLIP) score, which was calculated by adding points for the following four variables: Child-Pugh stage, tumor morphology, AFP value, and portal venous invasion[21,22]. The Japan Integrated Staging (JIS) system[23,24], developed by the Liver Cancer Study Group of Japan and based on a combination of Child-Pugh stage and HCC TNM classification, was used to clinically stage HCC.

Serum MnSOD and TRX levels

Serum was obtained from peripheral blood samples by centrifugation at 4000 *g* for 5 min at room temperature. Serum samples were frozen at -80 ℃ until further use. Serum MnSOD or TRX levels were measured using the Human Superoxide Dismutase 2 (AbFRONTIER, Seoul, Korea) and human thioredoxin (Redox Bio Science, Kyoto, Japan) ELISAs, respectively.

Statistical analysis

Results are expressed as the mean and standard deviation. *P* values less than 0.05 were regarded as statistically significant. Statistical analyses were performed using the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. The area under the curve (AUC) was calculated for the receiver operating characteristic (ROC) curve in order to measure the overall accuracy of the test. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of diagnostic test were additionally determined according to the protocol described previously[25]. Differences among the three groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests. Correlation coefficients were calculated using Spearman's rank correlation analysis. The Kaplan-Meier method was used to estimate death for each parameter that had been identified at enrollment, and the death distribution curves were compared using the log-rank test. Univariate and multivariate analyses of patient outcome risk ratios were performed using Cox's proportional hazards regression analyses. All statistical analyses were conducted using PASW Statistics v. 18 (SPSS Inc., Chicago, IL).

Table 1 Patient clinical characteristics

Characteristics	Non-HCC group (*n* = 34)	HCC group (*n* = 30)	*P* value[1]
Age (yr)	62.3 ± 11.0	72.2 ± 7.5	< 0.001
Sex (male/female)	10/24	21/9	< 0.01
Plt (× 10^4/μL)	17.0 ± 5.5	10.3 ± 5.2	< 0.001
PT (%)	99.7 ± 13.3	77.6 ± 11.8	< 0.001
Alb (g/dL)	4.3 ± 0.4	3.6 ± 0.6	< 0.001
T-Bil (mg/dL)	0.8 ± 0.3	1.5 ± 0.8	< 0.001
ALT (IU/L)	44.8 ± 30.2	52.0 ± 28.2	0.12
γ-GTP (IU/L)	31.3 ± 16.1	56.2 ± 44.3	< 0.01
AFP (ng/mL)	7.2 ± 22.8	85.9 ± 197.6	< 0.001
DCP (mAU/mL)	22.8 ± 14.7	485.5 ± 1982.6	0.001
HCV serotype group (1/2)	18/10 (*n* = 28)	21/3 (*n* = 24)	0.06
HCV RNA level (high/low)	28/5 (*n* = 33)	21/4 (*n* = 25)	0.99

Data are shown as the mean ± SD. *n*: Number of patients or the number of samples analyzed. [1]Differences between mean values were evaluated using either the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: alpha-fetoprotein; DCP: des-γ-carboxy prothrombin; HCV: Hepatitis C virus; RNA: Ribonucleic acid.

RESULTS

Patient characteristics and classification according to the presence of hepatocellular carcinoma

Table 1 summarizes the baseline clinical characteristics of the 64 patients who were classified based on the presence or absence of HCC. Age, sex, and clinical laboratory parameters, including Plt, PT, Alb, T-Bil, γ-GTP, AFP and DCP, were significantly different between these two groups.

Serum MnSOD and TRX levels in hepatocellular carcinoma patients

Serum MnSOD levels were significantly higher in patients with HCC compared to patients without HCC ($P = 0.03$) and HVs ($P < 0.001$) (Figure 1A). The serum TRX levels were also significantly higher in the HCC group compared to the non-HCC group ($P = 0.04$) and HV group ($P < 0.01$) (Figure 1B). However, there was no correlation between these two markers in the HCC group ($P = 0.28$, $r = 0.20$) (Figure 1C).

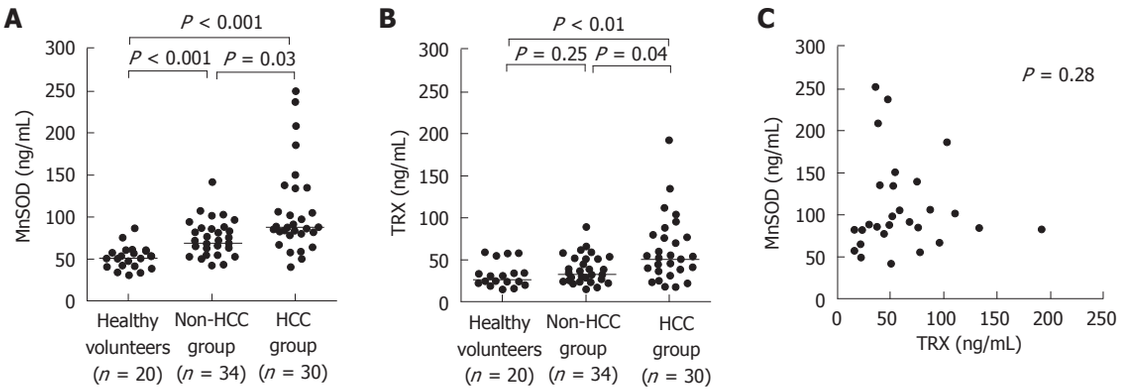

Figure 1 Serum levels of manganese superoxide dismutase and thioredoxin in the hepatocellular carcinoma, non-hepatocellular carcinoma and healthy volunteer groups. A: Serum manganese superoxide dismutase (MnSOD) levels were significantly higher in the hepatocellular carcinoma (HCC) group than in either the non-HCC group ($P = 0.03$) or the healthy volunteers (HV) group ($P < 0.001$); B: Serum thioredoxin (TRX) levels were also significantly higher in the HCC group than in either the non-HCC group ($P = 0.04$) or the HV group ($P < 0.01$); C: No significant correlation was detected between serum MnSOD and TRX levels in the HCC group.

Table 2 Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of manganese superoxide dismutase and α-fetoprotein serum levels for diagnosis of hepatocellular carcinoma in all patients (%)

Factors	Sensitivity	Specificity	PPV	NPV	Accuracy
MnSOD (≥ 110 ng/mL)	26.7	97.1	88.9	60.0	64.1
AFP (≥ 40 ng/mL)	33.3	97.1	90.9	62.3	67.2
Combination[1]	46.7	94.1	87.5	66.7	71.9

[1]MnSOD ≥ 110 ng/mL and/or AFP ≥ 40 ng/mL. PPV: Positive predictive value; NPV: Negative predictive value; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

Table 3 Correlation between serum manganese superoxide dismutase or thioredoxin levels and laboratory data in the hepatocellular carcinoma group

Factors	HCC group ($n = 30$)			
	Serum MnSOD levels		Serum TRX levels	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
Age (yr)	-0.97	0.61	0.11	0.55
Plt (× 10^4/μL)	0.03	0.89	0.66	< 0.001
PT (%)	-0.36	0.05	0.12	0.53
Alb (g/dL)	-0.63	< 0.001	0.19	0.33
T-Bil (mg/dL)	0.25	0.18	0.05	0.79
ALT (IU/L)	0.12	0.52	0.15	0.42
γ-GTP (IU/L)	0.30	0.11	0.28	0.13
AFP (ng/mL)	0.38	0.04	0.11	0.57
DCP (mAU/mL)	0.57	0.001	0.12	0.52

P values were assessed by Spearman's rank correlation analysis. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; HCC: Hepatocellular carcinoma; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin.

Diagnostic value of serum MnSOD and TRX levels for patients with hepatocellular carcinoma and hepatitis C virus infection

Serum AFP and DCP concentrations are established diagnostic markers for HCC. To evaluate the utility of MnSOD and TRX for the diagnosis of HCC, we measured AFP and DCP expression in addition to MnSOD and TRX expression. In an AUC-ROC analysis, AFP was the strongest diagnostic marker for HCC (AUC-ROC, 0.90). AUC-ROCs for MnSOD, TRX and DCP were 0.73, 0.77 and 0.77, respectively. Additional analyses showed that the accuracy of AFP (≥ 40 ng/mL) for diagnosis of HCC was higher than that of MnSOD (≥ 110 ng/mL) (Table 2), while the combination of AFP and MnSOD was a more accurate marker of HCC than either marker alone.

Association of serum MnSOD or TRX levels with laboratory data in the HCC group

Serum MnSOD levels for the 30 patients in the HCC group were positively correlated with serum AFP and DCP levels and were negatively correlated with serum Alb levels (Table 3). Serum MnSOD levels were also significantly higher in patients with two or more HCC tumors than in patients with a single HCC tumor [average ± SD (ng/mL), 125.4 ± 50.9 *vs* 87.4 ± 48.8, $P = 0.008$], although HCC tumor size was not associated with serum MnSOD levels. In addition, HCC patient serum MnSOD levels increased in parallel with the Child-Pugh stage, CLIP score and JIS score (Figure 2A-C). In contrast, serum TRX levels were only associated with platelet counts (Table 3). Serum TRX levels were not associated with HCC tumor number or size. Furthermore, there were no significant correlations between serum TRX levels for various scores (Figure 2D-F).

Overall survival rate based on serum MnSOD or TRX levels in the HCC group

In the HCC group, the overall patient survival rate was significantly lower ($P = 0.01$) in patients with MnSOD levels ≥ 110 ng/mL compared to patients with levels < 110 ng/mL (Figure 3A). In addition, the overall survival rate tended to be lower ($P = 0.05$) in patients with TRX levels < 80 ng/mL compared to those with levels ≥ 80 ng/mL (Figure 3B). Furthermore, among all HCC groups, patients who had both serum MnSOD levels ≥ 110

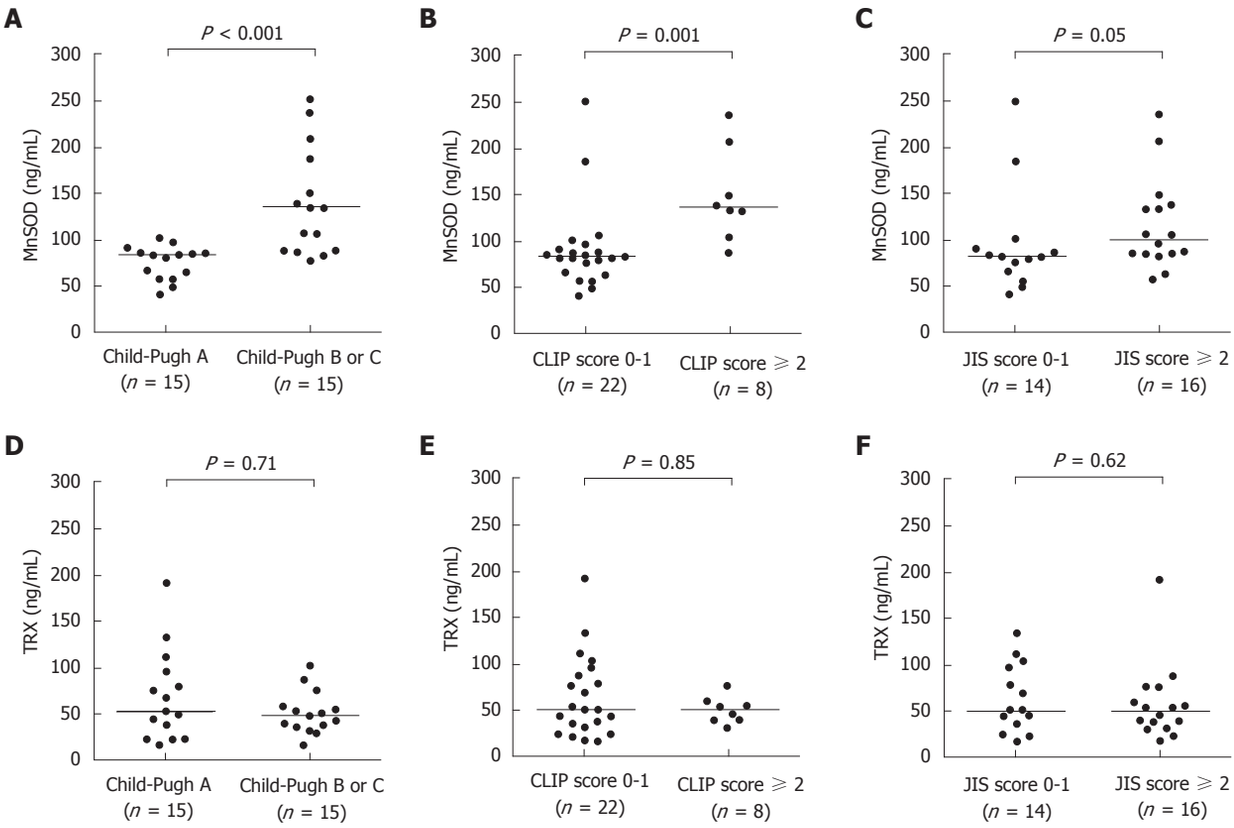

Figure 2 Clinical significance of serum manganese superoxide dismutase and thioredoxin levels in hepatocellular carcinoma. In the hepatocellular carcinoma (HCC) group, differences in serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels were evaluated based on Child-Pugh stage, cancer of the liver italian program (CLIP) score and Japan integrated staging (JIS) score. A: Serum MnSOD levels were significantly higher in patients with Child-Pugh B or C compared to those with Child-Pugh A ($P < 0.001$); B: Serum MnSOD levels in patients with a CLIP score of 2 or greater were significantly higher compared to levels in patients with a CLIP score of 0 or 1 ($P = 0.001$); C: In addition, serum MnSOD levels tended to be higher in patients with a JIS score of 2 or greater compared to patients with a JIS score of 0 or 1 ($P = 0.05$); D-F: In contrast, serum TRX levels were not significantly different based on Child-Pugh stage, CLIP score or JIS score.

ng/mL and TRX levels < 80 ng/mL had a significantly poorer prognosis. Conversely, patients with a serum TRX level ⩾ 80 ng/mL had a favorable prognosis, regardless of their serum MnSOD level (Figure 3C).

In addition to serum MnSOD and TRX levels, other possible prognostic factors were also investigated in the HCC group. A univariate analysis (log-rank test) revealed that the survival rate was significantly different between patients with high and low levels of MnSOD, PT, Alb, AFP and DCP, but not other factors such as TRX (Table 4). A multivariate analysis using a Cox proportional hazard model and five markers (MnSOD, PT, Alb, AFP and DCP) selected based on the results of the univariate analysis revealed that MnSOD levels ⩾ 110 ng/mL and AFP levels ⩾ 40 ng/mL were independent risk factors that were associated with a poor patient prognosis (Table 5). In addition, similar results were obtained from a similar multivariate analysis using the same five factors and TRX, supporting the finding that TRX is not an independent risk factor associated with HCC prognosis. Furthermore, patient Child-Pugh stage, CLIP score and JIS score, which were calculated based on several factors including clinical symptoms and laboratory data, were also prognostic factors for patients with HCC (Table 4). A multivariate analysis using the three markers of MnSOD, Child-Pugh stage and CLIP score indicated that Child-Pugh stage was also a significant prognostic factor (risk ratio: 6.19, 95% confidential interval: 1.33-28.95, $P = 0.02$).

DISCUSSION

HCV infection is the most important known contributor to the etiology of HCC. An increasing incidence of HCC has been largely attributed to a rise in HCV infections in the general population during the last 50 to 60 years[26]. During HCV infection, ROS production increases and persists throughout the infection. In addition, ROS are thought to play a major role in the pathogenesis of chronic inflammatory changes in the liver, leading to increased hepatic fibrosis and decreased hepatic function. In this study, we have shown that both serum MnSOD and TRX levels are elevated in patients with HCV-related HCC, with no correlation between these two markers. In addition, serum MnSOD and TRX levels were a useful predictor of overall patient survival. Serum MnSOD and TRX levels are reported to be biomarkers of oxidative stress in several diseases, including liver disease[7,14,17,19,27-29]. There were a small number of enrolled patients in this study and other contributors to liver diseases such as chronic hepatitis B infection should be further evaluated. However, our

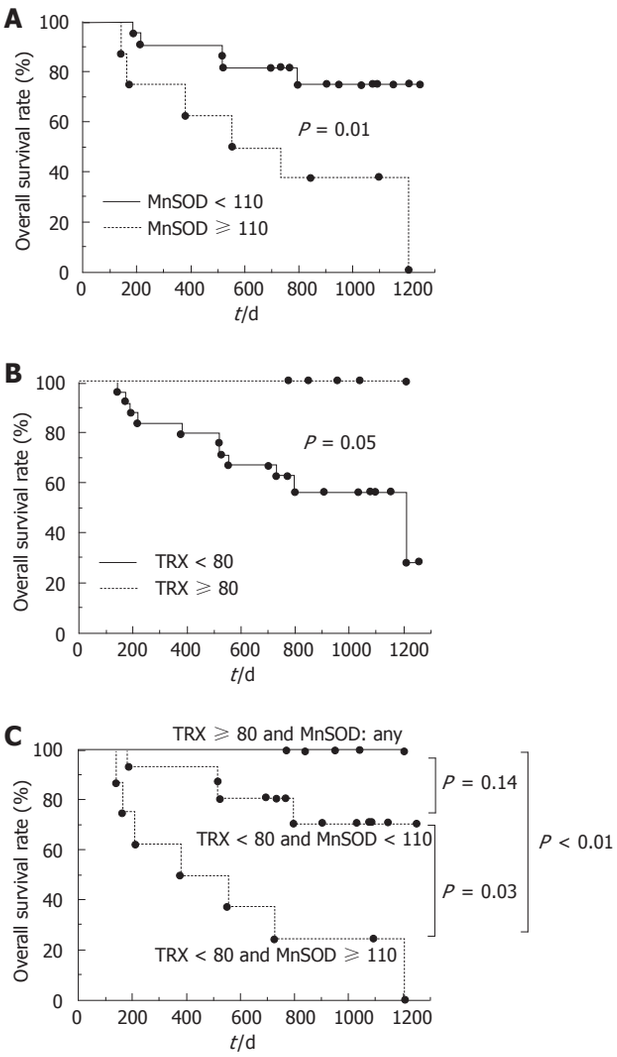

Figure 3 Overall hepatocellular carcinoma patient survival based on serum levels of manganese superoxide dismutase or thioredoxin. Overall survival was plotted using the Kaplan-Meier method after separation into two or three groups defined as follows: A: Manganese superoxide dismutase (MnSOD) < 110 ng/mL or ⩾ 110 ng/mL; B: Thioredoxin (TRX) < 80 ng/mL or ⩾ 80 ng/mL; TRX ⩾ 80 ng/mL, TRX < 80 ng/mL; C: MnSOD < 110 ng/mL, or TRX < 80 ng/mL and MnSOD ⩾ 110 ng/mL. The overall survival rate was lower in patients with MnSOD levels ⩾ 110 ng/mL (P = 0.01) (A). Also, cumulative patient survival rate tended to be lower in patients with TRX levels < 80 ng/mL (P = 0.05) (B). Among these groups, patients with serum TRX levels < 80 ng/mL and serum MnSOD levels ⩾ 110 ng/mL had the poorest prognosis (C).

Table 4 Univariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Category	Number	*P* value[1]
Single marker			
MnSOD (ng/mL)	< 110/⩾ 110	22/8	0.01
TRX (ng/mL)	< 80/⩾ 80	24/6	0.05
Age (yr)	< 70/⩾ 70	12/18	0.23
Plt (× 10^4/μL)	< 10/⩾ 10	19/11	0.38
PT (%)	< 80/⩾ 80	15/15	0.02
Alb (g/dL)	< 3.5/⩾ 3.5	15/15	0.02
T-Bil (mg/dL)	< 1.5/⩾ 1.5	18/12	0.34
ALT (IU/L)	< 40/⩾ 40	11/19	0.58
γ-GTP (IU/L)	< 50/⩾ 50	17/13	0.98
AFP (ng/mL)	< 40/⩾ 40	20/10	< 0.01
DCP (mAU/mL)	< 40/⩾ 40	16/14	0.02
Staging system			
Child-Pugh stage	A/⩾ B	16/14	< 0.01
CLIP score	0-1/⩾ 2	22/8	0.01
JIS score	0-1/⩾ 2	14/16	0.41

[1]*P* values were assessed using the log-rank test. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: Alpha-fetoprotein; DCP: Serum des-γ-carboxy prothrombin; CLIP: Cancer of the Liver Italian Program; JIS: Japan Integrated Staging.

Table 5 Multivariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Risk ratio	95% CI	*P* value
MnSOD (⩾ 110 ng/mL)	4.12	1.22-13.88	0.02
AFP(⩾ 40 ng/mL)	6.75	1.70-26.85	< 0.01

95% CI: 95% confidence interval; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

study has clearly demonstrated the clinical significance of these markers in patients with HCV-related HCC.

Serum MnSOD and TRX levels should both reflect hepatic oxidative stress. The results of the current study showed that both of these markers were increased in the HCC group relative to levels in the non-HCC group and the HV group (Figure 1A and B). However, there was no correlation between these two markers in the HCC group (Figure 1C). MnSOD is primarily localized to the mitochondrial matrix[3] and abnormal mitochondrial morphologies are frequently observed in CHC[8]. Therefore, MnSOD may be an indicator of mitochondrial disorders that are induced by oxidative stress. On the other hand, there are two TRX proteins, cytoplasmic TRX1 and mitochondrial TRX2[30]. TRX1 negatively regulates the apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase/P38 apoptotic pathway by binding to and inhibiting the kinase activity of ASK1, which plays an important role in ROS-induced cellular responses[31]. TRX2 is an essential regulator of mitochondrial ROS levels that has been associated with mitochondrial outer membrane permeability[32]. In the present study, we examined the serum levels of TRX1, but not TRX2, using a sandwich ELISA. Thus, the MnSOD and TRX proteins that were examined in this study have different origins in the mitochondria and cytoplasm, respectively, which could contribute to the lack of correlation between these two markers.

Several studies have shown that the HCV core protein directly inhibits the electron transport system and modulates apoptosis, transcription, and cell signaling[33]. Abdalla *et al*[34] reported that expression of not only the HCV core protein but also the HCV NS proteins increases ROS and further showed that the presence of these proteins can increase endogenous expression levels of antioxidant enzymes and prooxidants such as MnSOD. Several reports have shown that serum MnSOD levels in patients with HCV-related CLD[35-37] are associated with

various clinical findings, such as fibrosis and hepatic oxidative stress. However, the significance of serum MnSOD levels has not been fully examined in patients with HCC. We previously reported that serum MnSOD levels may be correlated with fibrosis in patients with NAFLD[7]. In addition, serum MnSOD levels decreased in patients with CHC after administration of an interferon-based treatment (data not shown). These results indicate that serum MnSOD levels are likely associated with hepatic fibrosis or oxidative stress in patients with CHC. In the present study, however, MnSOD levels were not associated with platelet counts, which is a simple predictor of hepatic fibrosis in this patient population[38]. Thus, advanced hepatic fibrosis or oxidative stress may be one reason why serum MnSOD levels have diagnostic and prognostic utility with HCC, but other mechanisms should also be considered.

The present study revealed that serum MnSOD levels were significantly higher in the HCC group than in the non-HCC group (Figure 1A). In the HCC group, serum MnSOD levels were negatively correlated with serum Alb and tended to negatively correlate with PT (Table 3); these results showed an association between MnSOD and Child-Pugh stage (Figure 2A). It is known that in humans, MnSOD activity is comparatively higher in the liver compared to other tissues[39]. In addition, although a previous immunohistochemical study showed that MnSOD expression was higher in both cancerous and non-cancerous liver tissues from patients with HCC, this positive immunoreactivity was strongly observed in non-cancerous liver tissues, especially in normal hepatocytes surrounding HCC, regenerative small hepatocytes in the tumor boundary, and mononuclear inflammatory cells in necroinflammatory lesions[40]. Furthermore, ROS are overproduced by Kupffer cells and inflammatory cells in liver disease[5,41]. In the present study, serum MnSOD levels were also positively correlated with the serum tumor markers AFP and DCP (Table 3) and with Child-Pugh stage and CLIP score (Figure 2). These results indicate that increased MnSOD expression reflects hepatocyte oxidative stress and correlates with decreased hepatic function, increased hepatic fibrosis and ROS production by inflammatory cells in liver cirrhosis. These features comprise the main background characteristics leading to HCC and may be associated with the indirect effects of liver cancer progression. These associations may also explain why serum MnSOD levels predicted the overall survival of patients with HCC.

It was previously reported that serum levels of TRX, which is a stress-induced protein, increase relative to the degree of hepatic fibrosis, and that high serum concentrations of TRX may indicate advanced hepatic fibrosis[19,20]. In contrast, it has also been reported that a higher degree of hepatic fibrosis is associated with lower platelet counts[38]. Therefore, the present study may present a conflict, since results indicated that serum TRX level was positively correlated with platelet count. A previous report showed that the survival rate following LPS plus GalN-induced hepatitis was much higher in transgenic mice overexpressing TRX than in wild-type mice, and that thioacetamide-induced hepatic fibrosis was suppressed in TRX transgenic mice compared to wild-type mice[42]. Although it is still unclear why TRX and platelet counts are positively correlated, we speculate that elevated serum TRX in patients with HCC and advanced hepatic fibrosis potentially improves overall survival by suppressing oxidative stress[43]. In addition, patients with HCC, low levels of TRX, and high levels of MnSOD, which may be indicative of excessive oxidative stress without TRX attenuation, have the poorest prognosis. This result supports the hypotheses presented above. In order to better assess these findings, future studies are needed that incorporate sequential observations of serum TRX and MnSOD levels over time in patients with chronic hepatitis, cirrhosis and HCC.

Serum MnSOD and TRX may be useful biomarkers for HCC diagnosis (Figure 1). AFP is also a diagnostic marker for HCC, and the present results indicate that AFP can be used to distinguish between patients with and without HCC (Table 2). However, AFP is not a sufficiently sensitive marker for identification of the majority of patients with small HCCs[44,45], and AFP testing is not currently included in the recommendations for HCC surveillance in the updated HCC guidelines published by the American Association for the Study of Liver Disease[46]. Therefore, clinicians and clinical researchers should consider using MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in a HCC surveillance program using ultrasonography or AFP. In addition, it is highly important to know whether these markers decrease in response to HCC therapy and reductions in tumor burden. These markers also may have utility in patients on a transplant waiting list who are treated with neo-adjuvant therapy for tumor downstaging.

Our study demonstrated that elevated serum AFP level is indicative of a poor prognosis for patients with HCC (Table 4), as was previously reported[47]. The CLIP score, which is calculated based on four factors such as the AFP value, was also useful to predict the prognosis of HCC patients in this study as well as in a previous report[48]. Other markers such as the protein survivin have been reported as poor prognostic factors for HCC[49]. Similarly, MnSOD was an independent predictive factor for overall survival in the HCC group (Figure 3A, Table 5). Although TRX was not an independent predictor of overall survival in patients with HCC (Table 4), we speculate that a combination assay using both MnSOD and TRX could be used to predict overall patient survival. It will be important to conduct further prospective evaluations of each individual marker as well as a combination of these markers using a large number of patients.

In conclusion, serum MnSOD and TRX levels increased as HCV-related chronic liver disease progressed, especially among patients with HCC. Although there was no correlation between serum levels of MnSOD and TRX, higher serum MnSOD levels and lower TRX levels in patients with HCC trended towards an indication of poor

patient prognosis. These results suggest that serum Mn-SOD and TRX levels are not only a potential biomarker for HCV-related progressed liver disease, but may also serve as prognostic markers in HCC.

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COMMENTS

Background

During hepatitis C virus (HCV) infection, production of reactive oxygen species (ROS) is persistently increased throughout HCV infection. ROS are thought to play an important role in the pathogenesis of chronic inflammatory changes in the liver, which may lead to the development of hepatic fibrosis, decreased hepatic function or hepatocellular carcinoma (HCC). However, there is little information currently available regarding serum oxidative stress markers in patients with HCV-related HCC.

Research frontiers

Cells are protected from oxidative stress by antioxidant enzymes such as superoxide dismutase (SOD) and by intracellular antioxidants such as thioredoxin (TRX). Serum manganese SOD (MnSOD) and TRX are thought to be biomarkers for various liver diseases, including HCV-related liver disease, but these possibilities have not been fully investigated. In this study, the authors demonstrated the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related HCC.

Innovations and breakthroughs

Although there was no correlation between serum levels of MnSOD and TRX, serum levels of both markers increased as HCV-related chronic liver disease progressed, and in particular among patients with HCC. In addition, higher serum MnSOD levels and lower TRX levels tended to indicate a poor prognosis among patients with HCC.

Applications

Serum MnSOD and TRX levels are not only potential biomarkers for progression of HCV-related liver disease, but they may also serve as prognostic markers for patients with HCC. Therefore, clinicians should consider using serum levels of MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in HCC surveillance programs. In addition, it will be important to know whether these markers change after therapy for liver disease, including HCC.

Peer review

Oxidative stress is closely associated with carcinogenesis. If oxidative stress markers could be useful in predicting clinical outcome in chronic hepatitis C and HCV-related HCC, they would provide us with a practical and informative tool. However, there are some limitations of this investigation, including a relatively small number of patients studied. Thus, the overall assessment is "good".

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BRIEF ARTICLE

Clinical presentation and management of Fasciola hepatica infection: Single-center experience

Muhsin Kaya, Remzi Beştaş, Sedat Çetin

Muhsin Kaya, Remzi Beştaş, Department of Gastroenterology, Dicle University School of Medicine, Diyarbakır 21280, Turkey
Sedat Çetin, Department of Internal Medicine, Dicle University School of Medicine, Diyarbakır 21280, Turkey
Author contributions: Kaya M designed the study, wrote the manuscript, performed all endoscopic retrograde cholangiopancreatography procedures and collected data; Beştaş R and Çetin S collected data.
Correspondence to: Muhsin Kaya, MD, Department of Gastroenterology, Dicle University, School of Medicine, Diyarbakır 21280, Turkey. muhsinkaya20@hotmail.com
Telephone: +90-532-3479458 Fax: +90-532-3479458
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Abstract

AIM: To identify the characteristic clinical, laboratory and radiological findings and response to treatment in patients with fascioliasis.

METHODS: Patients who were diagnosed with Fasciola hepatica infection were included in this prospective study. Initial clinical, laboratory and radiological findings were recorded. All patients were followed until a complete response was achieved or for 6 mo after treatment discontinuation.

RESULTS: Fasciola hepatica infection was diagnosed in 30 patients (24 females; mean age: 42.6 years) between January 2008 and February 2011. Twenty-two (73%) patients had hepatic phase fascioliasis, 5 patients had biliary phase, and 3 patients had biliary phase associated with acute pancreatitis. Of the 8 patients with biliary phase fascioliasis, 2 patients displayed features that overlapped with both hepatic and biliary phase. Abdominal pain and right upper abdominal tenderness were the most prominent signs and symptoms in all patients. Eosinophilia was the most prominent laboratory abnormality in both patients with hepatic and biliary phase (100% and 50%, respectively). Multiple nodular lesions like micro-abscesses on abdominal computerized tomography were the main radiological findings in patients with hepatic phase. Small linear filling defects in the distal choledochus were the main endoscopic retrograde cholangiopancreatography (ERCP) findings in patients with biliary phase. Patients with hepatic phase were treated with triclabendazole alone, and patients with biliary phase were treated with triclabendazole and had live Fasciola hepatica extracted from the bile ducts during ERCP.

CONCLUSION: Fasciola hepatica infection should be considered in the differential diagnosis of patients with hepatic or biliary disease and/or acute pancreatitis associated with eosinophilia.

Key words: Fasciola hepatica; Liver abscesses; Cholangitis; Pancreatitis; Triclabendazole

Peer reviewer: Kiichi Tamada, MD, Department of Gastroenterology, Jichi Medical Shool, 3311-1 Yakushiji, Minamikawachi, Kawachigun, Tochigi 329-0498, Japan

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INTRODUCTION

Fascioliasis is an infection caused by a trematode of the liver, Fasciola hepatica, that particularly affects sheep, goats and cattle. The flukes are leak-like, flat worms, measuring 2-4 cm[1]. The number of reports of humans infected with Fasciola hepatica has increased significantly since 1980, and several geographical areas have been described as endemic for the disease in humans, with preva-

lence and incidence ranging from low to very high[2,3]. In humans, the infection begins with the ingestion of watercress or contaminated water containing encysted larva. The larva excyst in the stomach, penetrate the duodenal wall, escape into the peritoneal cavity, and then pass through the liver capsule to enter the biliary tree[1]. Human fascioliasis has two phases. The hepatic phase of the disease begins one to three months after ingestion of metacercariae, with penetration and migration through the liver parenchyma toward the biliary ducts[1,4,5]. Common signs and symptoms of the hepatic phase are abdominal pain, fever, eosinophilia, and abnormal liver function tests[1,4,6-8]. The biliary phase of the disease usually presents with intermittent right upper quadrant pain with or without cholangitis or cholestasis[9-11].

In non-endemic areas, diagnosis of fascioliasis can be difficult and usually is delayed because the disease is not often encountered and the symptoms may be confused with other hepatic or biliary disorders. Diagnosis of Fasciola hepatica infection has traditionally relied on detecting the presence of eggs in fecal samples, but this method is unreliable and complex[1,4]. Among human cases in non-endemic areas, low egg outputs, e.g., 1-2 eggs per g of feces (epg) and 1-4 epg were being considered rare. These egg outputs are much lower than those found among humans in endemic areas[3]. Computerized tomographic (CT) findings in patients with hepatic phase and ultrasonographic findings in patients with biliary phase are used for the diagnosis of fascioliasis[5,6]. Confirmation of the diagnosis is necessary and should be based on serological findings and parasitic tests[12]. Triclabendazole and bithionol are effective agents for the treatment of fascioliasis[8].

The aim of this prospective study was to identify the characteristic clinical, laboratory and tomographic findings and response to treatment during follow-up in patients with fascioliasis.

MATERIALS AND METHODS

Patients who were admitted to our clinic and were diagnosed with Fasciola hepatica infection between January 2008 and February 2011 were prospectively enrolled in this study. All patients received an initial complete clinical exam, laboratory tests (including complete blood counts and routine biochemical analyses), and abdominal CT. All of the CT scans were obtained using a 4-channel multislice CT scanner (Sensation 4; Siemens Medical Solutions, Erlargen, Germany). A specific indirect hemagglutination assay (IHA) using purified adult Fasciola hepatica F1 antigen (Laboratoires Fumouze Diagnostic, Levallois Perret, France; cut-off 1/320) was used for serological diagnosis of fascioliasis. The diagnosis of Fasciola hepatica infection with hepatic phase was based on: (1) the presence of characteristic findings on the abdominal CT examination, as previously described[5-8]; (2) exclusion of all other known diseases that cause hepatic lesions on tomographic examination; and (3) a positive specific IHA for Fasciola hepatica; and/or (d) the presence of Fasciola hepatica eggs in the fecal examination. The diagnosis of Fasciola hepatica infection with biliary phase was based on the extraction of live Fasciola hepatica during endoscopic retrograde cholangiopancreatography (ERCP). In all patients, clinical and laboratory response to treatment was assessed monthly. In patients with hepatic phase fascioliasis, radiological improvement was assessed at a 3-mo interval. All patients were followed until complete clinical and laboratory response or until 6 mo after treatment discontinuation.

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This prospective study was approved by Institutional Review Board, and all patients provided informed written consent for participation.

RESULTS

Fasciola hepatica infection was detected in 30 patients (24 females, mean age: 42.6 years, range: 19-79 years). In 22 (73%) patients, the diagnosis of fascioliasis was based on radiological findings on abdominal CT examination and positive IHA test (≥ 1/620). We did not perform ERCP in these patients because they did not have clinical or laboratory findings compatible with extrahepatic biliary obstruction; therefore, these patients were accepted as hepatic phase fascioliasis. In the remaining 8 (27%) patients, the diagnosis of fascioliasis was confirmed by extraction of live, mobile Fasciola hepatica from extrahepatic biliary ducts during ERCP; therefore, these patients were accepted as biliary phase fascioliasis. Microscopic examination of fecal specimens for Fasciola hepatica eggs revealed a positive result for only 2 (7%) of the 30 patients, one with biliary and one with hepatic phase.

Patients with hepatic phase fascioliasis

The mean antibody titer in the IHA was 1/2720 ± 1/549 (range: 1/640-1/5120) in the 22 patients with hepatic phase fascioliasis. Three patients were sisters and were admitted to the hospital on the same day. All patients were admitted at least five (mean: 7 ± 2) d before diagnosis. The mean duration of symptoms was 25 ± 36.6 (range: 3-144) wk. Abdominal pain was reported by all patients (100%), fever in 13 (59%), nausea in 3 (14%), chills in 4 (18%), weight loss in 4 (18%), pruritus and urticaria in 1 (5%), and recurrent oral aft and asthenia in 1 (5%) patient. On physical examination, there was mild right upper quadrant tenderness in 15 (68%) patients and hepatomegaly in 6 (27%) patients. Although 13 patients had a history of intermittent fever, only 3 (14%) patients had fever > 38 ℃ (2 of them were sisters), during clinical follow-up.

Table 1 shows the laboratory results for patients before treatment. Anemia was present in 6 (27%) patients, leukocytosis in 11 (50%), eosinophilia in 22 (100%) and elevations in the erythrocyte sedimentation rate (ESR) in 18 (82%) patients, alanine aminotransferase (ALT) in 6 (27%), aspartate aminotransferase (AST) in 2 (9%), alkaline phosphatase (ALP) in 13 (59%) , γ glutamyl transferase (GGT)

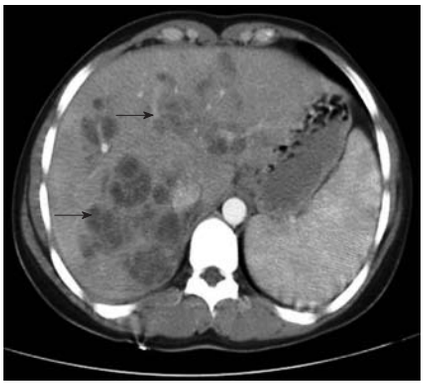
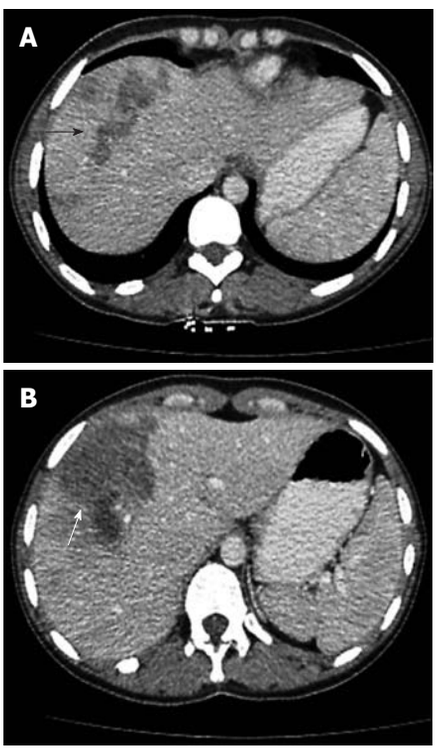


Figure 1 A 19-year-old female patient presented with right upper abdominal pain and fever lasting 3 wk. Abdominal computerized tomographic examination showed enlargement of the liver and extensive micro-abscesses (arrows).



Figures 2 A 30-year-old female patient presented with right upper abdominal pain lasting 8 wk. A: Tubular branching lesions in the right lobe (arrow); B: Abdominal computerized tomographic examination showed a sub-capsular low density area surrounded by a rim of parenchyma (arrow).

in 13 (59%), and total bilirubin in 1 (5%) patient. Platelet counts were normal in all patients.

On abdominal CT examination, the main abnormalities were multiple nodular lesions like micro-abscesses (Figure 1) in 21 (95%) patients, tubular branching lesions (Figure 2A) in 11 (50%), subcapsular low density areas surrounded by a rim of parenchyma (Figure 2B) in 7 (32%), solitary nodular lesions with hazy margins (Figure 3) in 5 (23%), lymph node enlargement in the portal area in 4 (18%), and localized perihepatic fluid accumulation in 2 (9%) patients.

After diagnosis of fascioliasis, triclabendazole was administered at a dose of 10-12 mg/kg for 1 d to all patients. We did not observe any side-effects related to triclabendazole administration. None of the patients with hepatic phase was administered antibiotics. Three months after treatment, we observed complete clinical and laboratory recovery in 18 (82%) patients and complete improvement on abdominal CT examination in 12 (55%) patients. Six months after treatment, there was complete clinical and laboratory recovery in all patients, but abdominal CT examination showed complete improvement in only 16 (73%) patients and residual hypo-dense lesions (Figure 4) in 6 (27%) patients.

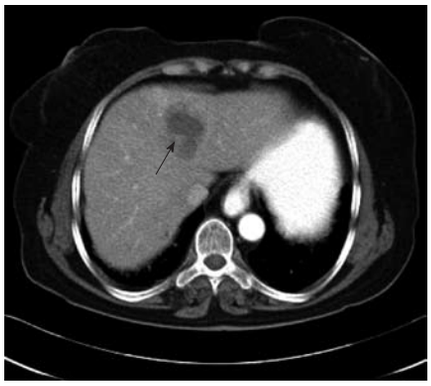


Figure 3 A 70-year-old female patient presented with right upper abdominal pain lasting 16 wk. Abdominal computerized tomographic examination showed low density masses with hazy margins located to medial segment of the left lobe (arrow).

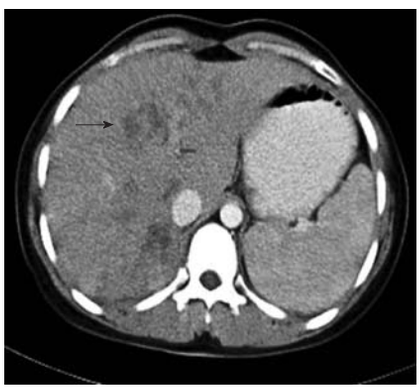


Figure 4 In the patient whose pre-treatment computerized tomographic image is shown in Figure 1, abdominal computerized tomographic examination showed residual lesions (arrow) and minimally enlarged spleen 6 mo after treatment with triclabendazole.

Patients with biliary phase fascioliasis

The mean duration of symptoms was 63.5 ± 80.6 (range: 1-208) wk in the 8 patients with biliary phase fascioliasis. One female patient underwent a cholecystectomy 6 mo ago after developing acute cholecystitis of unknown etiology. Abdominal pain was reported by all patients, fever in 2 (25%), nausea in 3 (38%), and weight loss in 1 (13%) patient. On physical examination, there was right upper quadrant tenderness in 5 (63%) and scleral icterus in 1 (13%) patient.

Table 1 Demographic features and laboratory results for patients with hepatic phase and biliary phase fascioliasis

Variables	Hepatic phase (mean ± SD)	Biliary phase (mean ± SD)
Age (yr)	40 (19-79)[1]	41 (29-49)[1]
Gender (M/F)	(5/17)	(1/7)
Hb (g/dL)	12.5 ± 1.4	12.7 ± 1.57
WBC (n/mm^3)	11862 ± 2829	8765 ± 1307
Eosinophil (% of total WBC count)	34.2 ± 16.2	14 ± 13.5
Plt (n/mm^3)	290 ± 62	273 ± 70
ESR (mm/h)	48 ± 26	17 ± 12
ALT (U/dL)	44 ± 49	220 ± 217
AST (U/dL)	26 ± 9	260 ± 357
ALP (U/dL)	157 ± 65	166 ± 85
GGT (U/dL)	64 ± 40	226 ± 128
Total bilirubin (U/dL)	0.57 ± 0.32	1.65 ± 2.02

[1]Age is represented using the median and range. Hb: Hemoglobin; WBC: White blood cell; Plt: Platelet; ESR: Erythrocyte sedimentation rate; ALT: Alanine aminotransferase (range, 10-40 U/L); AST: Aspartate aminotransferase (range, 10-35 U/L); GGT: γ glutamyl transferase (range, 9-64 U/L); ALP: Alkaline phosphatase (range, 40-150 U/L); Total bilirubin: range, 0.2-1.2 mg/dL; M: Male; F: Female.

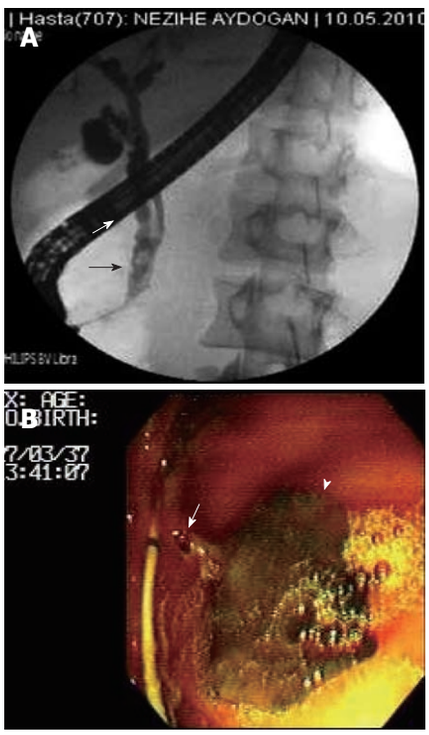


Figure 5 Live and mobil Fasciola hepatica removed from the choledochus by balloon catheter during endoscopic retrograde cholangiopancreatography. A: A 35-year-old female patient presented with acute pancreatitis associated with elevated liver enzymes. Endoscopic retrograde cholangiopancreatography image showed a radiolucent, roughly crescent-shaped shadow in the common bile duct (arrows); B: A 49-year-old female patient presented with acute cholangitis associated with eosinophilia. Arrow: Head of Fasciola hepatica; Arrowhead: Body of Fasciola hepatica.

The laboratory findings before treatment (Table 1) showed that mild anemia was present in 2 (25%) patients, eosinophilia in 4 (50%), and elevations in the ESR in 4 (50%), ALT in 7 (87%), AST in 6 (75%), ALP in 4 (50%), GGT in 7 (87%), and total bilirubin in 3 (38%) patients. Total white blood cell (WBC) and platelet counts were normal in all patients. One patient had a normal initial eosinophil count but elevated eosinophil count after the ERCP procedure (2.7% of total WBC before ERCP *vs* 23% after ERCP).

Abdominal CT examination showed no abnormalities in 6 (75%) and subcapsular low density areas surrounded by a rim of parenchyma in 2 (25%) patients. Although these 2 patients had clinical findings consistent with biliary phase fasciolisis, they had radiological findings consistent with hepatic phase fascioliasis. One of the patients was a 46-year-old woman who had cholangitis with a normal eosinophil count and live Fasciola hepatica extracted from the extrahepatic bile ducts. The other patient was a 29-year-old man who had acute pancreatitis, cholangitis, and eosinophilia (25% of total WBC); this patient also was one of the 3 patients who had biliary phase fascioliasis associated with acute pancreatitis.

Three (38%) patients (two females) with biliary phase fascioliasis also had acute pancreatitis. Two of these patients had elevated eosinophil counts, and all 3 patients had elevated liver enzymes and amylase (> 1275 U/dL). They also had acute edematous pancreatitis and were treated by extraction of live Fasciola hepatica by balloon during ERCP and conservative management.

ERCP was performed in all patients because there were clinical and laboratory findings of extrahepatic biliary obstruction. Before ERCP, we considered the diagnosis of fascioliasis in 4 (50%) patients with eosinophilia. Cholangiography showed slight extrahepatic and intrahepatic biliary dilatation in 1 (13%) patient with acute pancreatitis. In the remaining 7 (87%) patients, the intrahepatic and extrahepatic biliary systems were within normal diameter. ERCP demonstrated a radiolucent, roughly crescent-shaped shadow in the common bile duct in all patients (Figure 5A). After standard sphincterotomy, live Fasciola hepatica (3-5 Fasciola hepatica per patient) were removed using a balloon catheter from the extrahepatic bile ducts (Figure 5B).

We routinely administered 1 *g* ceftriaxone at least one hour before ERCP to prevent post-ERCP cholangitis. We did not administer antibiotics to any patient after ERCP. Triclabendazole was administered at a dose of 10-12 mg/kg for 1 d to all patients. Complete clinical and laboratory recovery was observed in all patients 3 mo after treatment. There was complete resolution on abdominal CT examination in the 2 patients who had initial lesions.

DISCUSSION

Fascioliasis is an emerging disease in humans. The epidemiological and transmission characteristics of fascioliasis suggest that the disease has a patchy distribution, with foci related to the local distribution of the intermediate snail host population in freshwater bodies as well as climatic conditions[2,3]. Epidemiological studies on the incidence of fascioliasis in our region have not been reported previously, but the results from this prospective single-center study suggest that Fasciola hepatica infection is not very rare in our region. Our hospital is a tertiary care cen-

ter in the southeast of Turkey that serves approximately 3 million people. All of the patients in this study resided in rural areas and had a history of consuming watercress grown in areas where sheep were raised. Twenty-four (80%) patients were female, and all of them were homeworking, suggesting that the females had more contact with watercress than the men.

Fascioliasis has a hepatic phase and a biliary phase, each displaying different clinical signs and symptoms. The acute stage of fascioliasis (hepatic phase) begins with the slow migration of Fasciola hepatica through the liver parenchyma; the mature flukes digest and consume hepatocytes, dig tunnels and caves, and reside in the liver for months[1,7,13]. The hepatic phase is characterized by fever with chills, upper abdominal pain, hepatomegaly, mild hepatitis, weight loss and prominent eosinophilia[6-8,14]. Reports suggest that the clinical presentation of hepatic phase fascioliasis is similar to that of liver abscesses of other etiology[1,8]. In our patients with hepatic phase fascioliasis, the common clinical signs and symptoms were right upper abdominal pain, intermittent fever, right upper quadrant tenderness and hepatomegaly. Based on our clinical experience with pyogenic liver abscesses[15], patients with Fasciola hepatica infection had a longer duration of symptoms (25 ± 36.6 wk *vs* 5.7 ± 1.6 wk), a healthier condition, and less upper abdominal tenderness than patients with pyogenic liver abscesses. Although 13 (59%) of 22 patients with hepatic phase fascioliasis had a history of intermittent fever, we recorded fever in only 3 patients. These findings suggest that fever in hepatic phase fascioliasis is not a prominent finding.

In the biliary phase of the disease, patients often present with biliary colic, epigastric pain, jaundice and abdominal tenderness due to the obstruction of the bile ducts by adult worms and the resultant inflammatory response. In this stage, the main laboratory findings are cholestasis including predominantly elevated serum ALP, GGT and total bilirubin[9-11]. Adult flukes in the extrahepatic bile ducts are visualized as a filling defect on cholangiogram[1,6,9,10]. Because of the chronic inflammation, the thickened walls of the extrahepatic ducts and gallbladder are visible on abdominal CT examination[1,5,6]. Although our patients with biliary phase fascioliasis had clinical signs and symptoms of biliary obstruction, we did not find the typical cholestatic biochemical abnormalities in these patients. We also found no specific abnormalities on abdominal CT examination in our patients with biliary phase except for slight dilatation of intrahepatic and extrahepatic bile ducts in 1 patient and subcapsular low density areas surrounded by a rim of parenchyma in 2 patients. The absence of bile duct wall thickness on CT examination and the absence of biochemical findings indicative of cholestasis in our patients may reflect intermittent rather than chronic biliary obstruction or our patients may have had early stage biliary duct involvement. The 2 patients with parenchymal lesions probably had overlapping hepatic and biliary phase fascioliasis. The most specific cholangiographic finding in our patients with biliary phase fascioliasis was a radiolucent, roughly crescent-shaped shadow in the extrahepatic bile ducts without dilatation.

Major causes of acute pancreatitis include alcohol ingestion and gallstones[16-18]. A small number of patients who have Fasciola hepatica infection complicated with acute pancreatitis have been reported. The pathogenesis of acute pancreatitis secondary to fascioliasis is unknown. Intermittent biliary obstruction and cholangitis caused by adult Fasciola hepatica may be the principle mechanism involved in the development of acute pancreatitis[19-21]. In our case series, 3 (38%) of 8 patients (2 with eosinophilia) with biliary phase fascioliasis also had acute edematous pancreatitis. One of the 3 patients, had both hepatic and biliary phase fascioliasis. Although our number of cases is small, we suggest that Fasciola hepatica infection should be considered during differential diagnosis in patients with acute pancreatitis associated with cholangitis and eosinophilia.

Diagnosis of fascioliasis may be delayed because of the wide spectrum of the differential diagnosis and the low incidence of Fasciola hepatica infection[3]. The abnormal laboratory and radiological findings in Fasciola hepatic infection may represent viral hepatitis, liver abscess, malignancy, cholecystitis, sclerosant cholangitis, AIDS-related cholangitis, ruptured hydatic cyst, and infection with parasites such as ascariasis and clonorchiasis[1,5,8]. The specificity of the indirect hemagglutination test (*IHA*) using purified adult Fasciola hepatica antigen F1 is 96.9% for serological diagnosis of Fasciola hepatica infection[12]. Diagnosis is confirmed only by demonstrating live parasites or eggs in the bile or feces[1,5,8]. The disease cannot be ruled out by a negative stool examination[3,5,8]. A high index of suspicion and specific radiological findings are very helpful in the diagnosis. We suspected of the possibility of fascioliasis in all patients with hepatic phase because of the presence of eosinophilia, characteristic abdominal CT findings, and typical clinical sign and symptoms. We found eggs in stool samples in 1 of 22 patients with hepatic phase fascioliasis and 1 of 8 patients with biliary phase fascioliasis. Diagnosis in patients with hepatic phase was confirmed by the clinical, laboratory and radiological responses to triclabendazole treatment and the high titer in the IHA. Diagnosis in patients with biliary phase fascioliasis was confirmed by extraction of live Fasciola hepatica form bile ducts. We suggest that stool examination for eggs is not a reliable method and that both serological testing and extraction of live parasites form bile ducts are very reliable methods for the diagnosis of fascioliasis.

Treatment of human fascioliasis has been difficult for a long time. Today, triclabendazole is the drug of choice for its effectiveness against both adult and immature worms[7,13,21]. Its anti-parasitic effect is derived from the inhibition by an active sulfoxide metabolite of the synthesis of the tegumental ultra-structure of Fasciola hepatica[22]. Triclabendazole at a dose of 10 mg/kg body weight (single or split postprandial dose) reportedly is effective in about 80%-90% of patients and is well tolerated. The most common drug-related side-effects are nausea, vomiting and abdominal pain[23]. All of our patients with hepatic phase were treated with triclabendazole alone, and those patients with biliary phase were treated with endoscopic

sphincterotomy, extraction of live parasite from bile ducts, and administration of triclabendazole. We observed that triclabendazole improved both clinical and laboratory findings in a few weeks; radiological improvement, however, required a longer period.

In conclusion, in addition to classically defined hepatic phase and biliary phase fascioliasis, some cases may have overlap of these two phases with or without acute pancreatitis. In cases of right abdominal pain, elevated eosinophil count, and multiple micro-abscesses and/or tunnel-like hypo-dense lesions on abdominal CT examination, hepatic phase fascioliasis should be considered, and a serological test for Fasciola hepatica should be used for diagnosis. In cases of biliary colic and/or acute pancreatitis associated with eosinophilia, we suggest that biliary phase fascioliasis should be considered, and ERCP should be used for both diagnosis and treatment.

COMMENTS

Background

In non-endemic areas, diagnosis of fascioliasis is difficult and usually is delayed because the disease is relatively rare and the symptoms may be confused with other hepatic or biliary disorders. Confirmation of the diagnosis is necessary and patients should be followed for response to treatment.

Research frontiers

Fascioliasis has a hepatic phase and a biliary phase, each displaying different clinical signs and symptoms. In addition to classically defined hepatic phase and biliary phase fascioliasis, some cases may have overlap of these two phases with or without acute pancreatitis.

Innovations and breakthroughs

Fascioliasis may have different clinical presentations. In cases of abdominal pain and elevated eosinophil count, fascioliasis should be considered in differential diagnosis. Serological tests and abdominal computerized tomographic (CT) examination are the methods of choice for diagnosis. But, fecal examination for Fasciola hepatica eggs is not a reliable diagnostic method. It may take a long time for complete clinical and radiological improvement after triclabendazole administration.

Applications

Fasciola hepatica infection should be considered in the differential diagnosis of patients with hepatic or biliary disease and/or acute pancreatitis associated with eosinophilia.

Terminology

Fascioliasis is an infection caused by a trematode of the liver. Fasciola hepatica, particularly affects sheep, goats and cattle. The flukes are leak-like, flat worms, measuring 2-4 cm. In humans, the infection begins with the ingestion of watercress or contaminated water containing encysted larva.

Peer review

The authors investigated the characteristic clinical, laboratory, and tomographic findings and response to treatment during follow-up in patients with fascioliasis. They revealed that fascioliasis has different clinical presentations and in cases of right abdominal pain, elevated eosinophil count, and multiple micro-abscesses and/or tunnel-like hypo-dense lesions on abdominal CT examination, the diagnosis of hepatic phase fascioliasis should be considered, and a serological test for Fasciola hepatica should be used for diagnosis.

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BRIEF ARTICLE

Prognostic role of sensitive-to-apoptosis gene expression in rectal cancer

Sevgi A Ozden, Hazan Ozyurt, Zerrin Ozgen, Olca Kilinc, Mustafa Oncel, Aylin E Gul, Nimet Karadayi, Nedime Serakinci, Beki Kan, Oya Orun

Sevgi A Ozden, Hazan Ozyurt, Clinic of Radiation Oncology, Dr. Lütfi Kırdar Kartal Education and Research Hospital, 34890 Istanbul, Turkey
Zerrin Ozgen, Clinic of Radiation Oncology, Medical Park, 81060 Istanbul, Turkey
Olca Kilinc, Beki Kan, Oya Orun, Department of Biophysics, Marmara University School of Medicine, 34668 Istanbul, Turkey
Mustafa Oncel, Clinic of Surgery, Dr. Lütfi Kırdar Kartal Education and Research Hospital, 34890 Istanbul, Turkey
Aylin E Gul, Nimet Karadayi, Department of Pathology, Dr. Lütfi Kırdar Kartal Education and Research Hospital, 34890 Istanbul, Turkey
Nedime Serakinci, Telomere Aging Group, Center for IRS, Southern Denmark University, 7100 Vejle, Denmark
Nedime Serakinci, Faculty of Medicine, Near East University, 922022 Lefkosa, Mersin 10, Turkey
Beki Kan, Department of Biophysics, Acıbadem University School of Medicine, 34848 Istanbul, Turkey
Author contributions: Ozden SA designed the study, treated the patients, analyzed the data and wrote the manuscript; Ozyurt H and Ozgen Z assisted Ozden SA in the clinical follow-up and treatment evaluations of the patients; Oncel M provided the biopsy tissues and Gul AE and Karadayi N conducted the pathological investigations; Serakinci N and Kan B gave technical support and conceptual advice and edited the manuscript; Ozden SA and Kilinc O performed the molecular biology experiments and collected the data; Orun O gave technical support, analyzed data, supervised the project and wrote the manuscript.
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Correspondence to: Oya Orun, PhD, Assist Professor, Department of Biophysics, Marmara University School of Medicine, 34668 Istanbul, Turkey. oakalin@marmara.edu.tr
Telephone: +9-216-3480585 Fax: +9-216-3480585
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Abstract

AIM: To investigate the association between prognosis of rectal cancer treated with chemoradiotherapy (CRT) and expression of sensitive-to-apoptosis (SAG), B-cell lymphoma-extra large (Bcl-X_L) and Bcl-2 homologous antagonist/killer (Bak).

METHODS: Real-time quantitative polymerase chain reaction was used to determine the expression of proteins of interest, namely SAG, Bcl-X_L, Bak and β-actin, in rectal carcinoma patients who had a follow-up period of 3 years after CRT. Biopsy specimens were excised from the rectal tumor preceding CRT.

RESULTS: SAG, Bcl-X_L and Bak proteins showed significant correlations with each other. In multivariate analysis, patients with high *vs* low SAG expression showed a statistically significant difference in 2-year survival rates: 56% *vs* 73%, respectively (P = 0.056). On the other hand, there were no significant correlations between the expression levels of all three genes and metastatic rates or tumor responses to CRT. Mean overall survival in the patients with elevated SAG expression was 27.1 mo ± 3.9 mo [95% confidence interval (CI): 19.3-34.9], and in patients with reduced expression, it was 32.1 mo ± 2.5 mo (95% CI: 27.3-36.9). The corresponding values for Bcl-X_L were 28.0 mo ± 4.1 mo (95% CI: 19.9-36.1) and 31.7 mo ± 2.9 mo (95% CI: 26.0-37.5), and those for Bak were 29.8 mo ± 3.7 mo (95% CI: 22.5-37.2) and 30.6 mo ± 2.4 mo (95% CI: 25.5-35.0), respectively.

CONCLUSION: Two-year survival rates significantly correlated with low SAG expression, and SAG may be a candidate gene for good prognosis, independent of therapeutic response of different individuals.

Key words: Sensitive-to-apoptosis gene; Sensitive-to-apoptosis; Rectal cancer; B-cell lymphoma-extra large; Bcl-2 homologous antagonist/killer; Apoptosis

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INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide, and 30% of patients experience local recurrence, thus posing a serious problem in treatment[1,2]. Currently, preoperative radiotherapy alone or in combination with chemotherapy is widely accepted to improve local control and overall survival.

The most frequently chosen treatment methods are either short-course irradiation (25 Gy in five fractions) followed by surgery after 1 wk, or conventional fractionated chemoradiotherapy (CRT) with delayed surgery[3]. Multidrug- and/or radiation resistance is one of the main causes of treatment failure in rectal carcinoma, as in other types of cancer. Predictors of treatment response are highly valuable, and they help to reduce the occurrence of undesirable treatment side effects, improve efficacy, and reduce costs, especially in long treatment schedules. Therefore, it is very important to identify those patients who will not show a good response to CRT. The success of CRT is strongly dependent on the molecular and cellular characteristics of the cells, therefore, identification of candidate molecular markers with significant prognostic power to estimate the response to CRT is crucial. Despite accumulation of data on prognostic markers, reliable markers for satisfactory clinical outcomes remain limited in number.

Sensitive-to-apoptosis gene (SAG)/regulator of cullins (ROC) 2/RING box protein (Rbx) 2/Hrt2 is a recently identified component of Skp/Cullin/F-box containing complex (SCF) E3 ubiquitin ligase, which controls cell-cycle progression by promoting ubiquitination and degradation of cell-cycle inhibitors. It has also been reported that SAG protects cells from apoptosis induced by redox agents such as hydroxyl radicals and radiation. The prognostic value of apoptotic activity in different cancer types has been previously described[4-7]. SAG overexpression has been shown in 60% of primary colon carcinomas; furthermore, significant correlation between SAG overexpression and poor survival has been demonstrated in non-small cell lung carcinoma[8,9]. Thus, it is proposed that SAG may regulate carcinogenesis *via* modulating both cell proliferation and apoptosis.

Ionizing radiation causes DNA damage that can lead cell to apoptosis and thus eradication of cancer cells. Antiapoptotic proteins such as SAG may have a major impact on the progress of cancer cell formation and proliferation. The present study investigated SAG expression as a potential molecular marker of ionizing radiation effect in rectal cancer. Two members of the Bcl-2 family, B-cell lymphoma-extra large (Bcl-Xl) and Bcl-2 homologous antagonist/killer (Bak), which are suggested as the most probable candidate biomarkers, were also analyzed. Our results indicate that SAG expression is a useful marker for early prognosis, regardless of local response to CRT, and that targeting SAG may have a potential role in the treatment of rectal cancer.

MATERIALS AND METHODS

Patients and tissue collection

This prospective study included 31 patients referred to the Kartal Education and Research Hospital with a diagnosis of stage Ⅱ and Ⅲ rectal cancer, according to the conventional tumor, node and metastases (TNM) classification[10]. The appropriate ethics committees related to the institution approved the study and all patients provided written informed consent before undergoing diagnostic colon biopsy. Prior to the start of treatment, all patients underwent examinations, including complete blood counts, liver and renal function tests, and tumor markers. Additionally, lung X-rays were evaluated before the start of treatment. Abdominal-pelvic magnetic resonance imaging or computed tomography was used for clinical staging and supplemented with transrectal ultrasound when needed. Biopsy specimens from the tumor and adjacent normal rectal tissues were obtained during colonoscopy. Freshly removed specimens were immediately immersed in RNAlater solution (Qiagen, Germany) and stored at -20 ℃ for RNA extraction.

Therapy

All patients received preoperative CRT. Patients were irradiated using the four-box-field technique and high-energy photon radiotherapy beams (15 mV), with a daily exposure of 1.8-2 Gy for five consecutive days. A cumulative dose of 45-50 Gy was administered during 5 wk and an additional 5.4-Gy boost was administered in three fractions. A short infusion of fluorouracil (320-400 mg/m^2) and of calcium folinate (20 mg/m^2) was administered on the first and last weeks, concomitantly[11,12].

Patients who were histopathologically diagnosed with rectal cancer underwent CRT prior to surgery. After a 4-6-wk interval, the patients underwent surgery. Patients were staged according to the TNM classification system[10], based on routine histopathological reports following surgery.

Real-time polymerase chain reaction

SAG, Bcl-Xl and Bak mRNA expression levels in tumor tissues and adjacent normal tissues were quantified by real-time polymerase chain reaction (PCR) analysis. Total RNA was extracted from RNAlater-conserved tissues using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's guidelines. RNA samples were treated with DNase I (MBI Fermentas, Burlington, Canada) to

Table 1 Clinicopathological factors and gene expression

		Survival (%)			Multivariate significance
	n	12 mo	24 mo	36 mo	*P* value
Age (yr)					
< 50	10	80	80	66	0.135
> 50	21	76	57	47	
Sex					
Male	17	64	52	45	0.073
Female	14	92	78	62	
preT					
T2	1	100	100	100	0.052
T3	24	79	70	59	
T4	6	66	33	16	
preN					
N0	18	77	66	66	0.036
N+	13	76	61	0	
Grade					
Low	4	100	75	75	0.0337
Moderate	22	77	68	58	
High	4	50	25	0	
pN					
N0	17	70	64	58	0.044
N1	6	83	83	56	
N2	3	66	83	67	
pT					
T0	6	83	66.7	66.7	0.067
T1	2	100	100	100	
T2	4	75	75	75	
T3	19	73	63	57	
Vascular invasion					
Negative	7	71	57	57	0.019
Positive	24	79	66.7	52.7	
Perineural invasion					
Negative	17	76.5	64.7	64.7	0.068
Positive	13	76.9	61.5	35.9	
Metastasis					
Negative	23	78	73	63	0.009
Positive	8	75	50	25	
SAG expression					
Increase	16	62	56	56	0.056
Decrease	15	93	73	49	
Bak expression					
Increase	14	73	62	31	0.731
Decrease	17	68	56	56	
Bcl-XL expression					
Increase	12	66	58	43	0.336
Decrease	19	84	68	61	

Clinical features of the 31 patients who received preoperative chemoradiotherapy and a summary of the relationship between protein expression level and patient survival after 1, 2 and 3 years of follow-up. SAG: Sensitive-to-apoptosis; CRT: Chemoradiotherapy.

remove possible genomic DNA contamination. RNA was quantified by measuring A_{260} using a conventional spectrophotometer. cDNA was synthesized from RNA using the 1st Strand cDNA Synthesis Kit for RT-PCR according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Following synthesis, 5 μg of cDNA was amplified using the appropriate primers. SAG expression was analyzed using following primers: forward (5'-CGGGATCCATGGCCGACGTGGAAG-3') and reverse (5'-CGAAGCTTTCATTTGCCGATTCTTTG-GAC-3'). Expression of two other apoptotic pathway genes (Bcl-X_L and Bak) was analyzed using the following primers: Bcl-X_L forward (5'-CCAGAAGGGACT-GAATCG-3') and Bcl-X_L reverse (5'-CCTTGTCTAC-GCTTTCCAC-3'); Bak forward (5'-GACCCAGAGA-TGGTCACCTT-3') and Bak reverse (5'-TCATAGC-GTCGGTTGATGT-3'). β-actin gene expression was used in parallel reactions as an internal PCR control. The β-actin primers were as follows: β-actin forward (5'-CT-GTGCTGTCCCTGTATGCC-3') and β-actin reverse (5' -GTGGTGGTGAAGCTGTAGCC-3'). Amplification products were 341, 361, 103 and 203 bp, respectively. Real-time PCR was performed using a Light-Cycler 480 system (Roche) and amplification conditions were set according to the instructions supplied with the Light Cycler FastStart DNA Master SYBR Green kit (Roche). The annealing temperature for amplifications was 56 ℃ for β-actin, 50 ℃ for Bcl-X_L and 60 ℃ for SAG and Bak proteins.

Statistical analysis

Multivariate analysis was performed using the logistic regression test. $P < 0.05$ was considered statistically significant. The relationship between protein regulations and disease-free survival up to 3 years was assessed by a log-rank comparison of Kaplan-Meier survival curves. All statistical analyses were conducted using SPSS 13 statistical software (SPSS, Chicago, IL, United States).

RESULTS

The study included 31 patients (17 males and 14 females) diagnosed with locally advanced rectal cancer. RNA was extracted from both normal and tumor tissues. Mean age of the patients was 59.9 years (range: 35-85 years). Survival, according to patient sex and age, was not significantly different (Table 1). Median follow-up was 3 years. In all, 8 of the 31 patients (34.8%) developed distant metastases. Table 1 summarizes the patients' 1-, 2- and 3-year survival rates, the corresponding data for some known prognostic factors, and the levels of SAG, Bcl-X_L and Bak protein expression.

There was an association between protein expression and survival following CRT. The 1- and 2-year survival rates were 93% and 73%, respectively, in patients with low SAG expression (fold change > 0.99), *vs* 62% and 56%, respectively, in patients with high SAG expression (fold change < 0.99); the correlation between SAG expression and survival was moderate ($P = 0.056$). A similar trend was observed in the expression of antiapoptotic protein Bcl-X_L; the corresponding survival values were 84% and 68%, respectively, in patients with low Bcl-X_L expression, *vs* 66% and 58%, respectively in patients with high Bcl-$X_L$expression. The levels of Bak were also in agreement with its apoptotic function. In accordance with our expectations, patients with high Bak expression had a higher 2-year survival rate (62%) than those with low Bak expression (56%). The expression patterns of SAG, Bcl-X_L and Bak proteins exhibited good correlations with the 1- and 2-year survival rates, in accordance with their anti- and pro-apoptotic roles, even though statistical

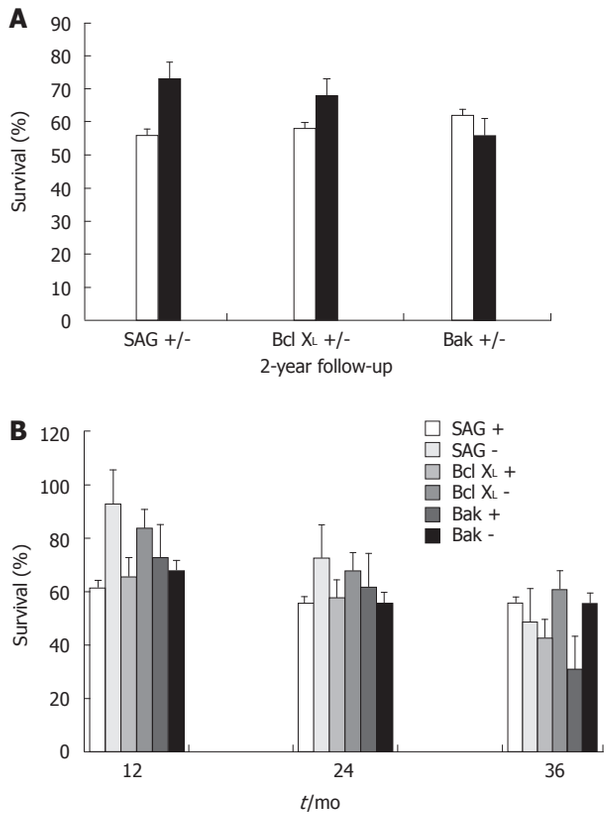

Figure 1 Dependence between gene expression and outcome of patients. A: Comparison for 2-year survival; B: Comparison after 1, 2 and 3 years of follow-up. All values were normalized with respect to β-actin expression. Calculations were based on the Pfaff method. +/- signs correspond to the subjects with increased or decreased expression with respect to their non-tumor tissues, respectively. SAG: Sensitive-to-apoptosis; Bcl-XL: B-cell lymphoma-extra large; Bak: Bcl-2 homologous antagonist/killer.

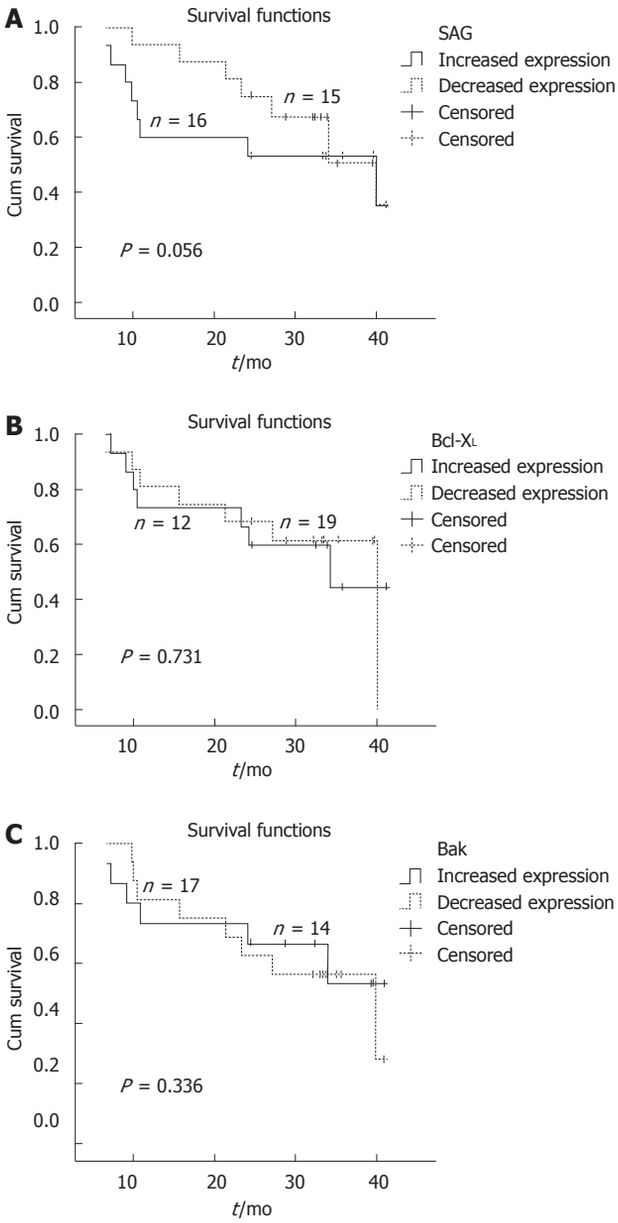

Figure 2 Kaplan-Meier survival curves with univariate log-rank comparisons at the end of 3 years for the three genes of interest. A: Sensitive-to-apoptosis; B: B-cell lymphoma-extra large; C: Bcl-2 homologous antagonist/killer. Metastasis was the major cause of death (seven cases). Information was not available for four of the patients. Other reasons of death were variable, but included advanced age, Alzheimer's disease and stroke. The median values for high- and low-level SAG expression in the study population were 24.5 and 30.4 mo, respectively. SAG: Sensitive-to-apoptosis gene, Bcl-XL: B-cell lymphoma-extra large, Bak: Bcl-2 homologous antagonist/killer.

significance was low, possibly due to the small number of patients included in the study (Figure 1A). There was no significant association in the expression of any gene with better survival at the end of the 3-year follow-up (Figure 1B).

Kaplan-Meier survival curves for SAG, Bcl-XL and Bak proteins are shown in Figure 2A-C, respectively. Mean overall survival in the patients with elevated SAG expression was 27.1 mo ± 3.9 mo [95% confidence interval (CI): 19.3-34.9]; and in patients with reduced expression, it was 32.1 mo ± 2.5 mo (95% CI: 27.3-36.9). The corresponding values for Bcl-XL were 28.0 mo ± 4.1 mo (95% CI: 19.9-36.1) and 31.7 mo ± 2.9 mo (95% CI: 26.0-37.5), and those for Bak were 29.8 mo ± 3.7 mo (95% CI: 22.5-37.2) and 30.6 mo ± 2.4 mo (95% CI: 25.5-35.0), respectively.

Tumor progression caused by distant metastases occurred only in eight patients (25.8%), of whom, seven had liver metastasis and one, who was alive at the time of evaluation, had bone metastasis. Mean overall survival was 41 mo. When the levels of SAG in tumor tissue were compared to the corresponding normal tissue, half of the patients expressed higher levels of SAG, whereas the other half had lower expression. Interestingly, patients with high SAG expression had a higher mean survival (28 mo) than did patients with low expression (18 mo). These results contradict those obtained in the non-metastatic patients. Among the non-metastatic patients, those with low SAG expression showed a higher survival (36 mo) and those with high SAG expression had lower survival (26 mo) (Table 1).

Pathological complete response was observed in five patients (5/31); three of whom (60%) had lower SAG expression and two (40%) had higher SAG expression.

DISCUSSION

Almost 50% of patients with rectal carcinoma who undergo potentially curative resection die from the disease, due to high recurrence rates. There is mounting evidence indicating that local control of disease and survival rates can be significantly improved with neoadjuvant CRT; however, responses to therapy vary widely among individuals and, as such, the ability to predict the response to CRT in patients with rectal cancer significantly improves therapeutic efficacy.

One of the immediate damaging effects of ionizing irradiation is the induction of cell-cycle arrest to provide time for DNA repair or apoptosis. Spontaneous apoptosis has been reported to be an important predictor of tumor regression in rectal cancer[13]. Reactive oxygen species, such as hydroxyl radical radicals, produced by ionizing radiation are highly reactive and easily trigger apoptosis, possibly by an indirect action on redox-sensitive molecules such as *p53* and *p27*[14,15]. Recently, SAG was identified as a redox-sensitive protein that protects cells from apoptosis, either by scavenging oxygen radicals or by acting on apoptosis-related proteins as part of the ubiquitin ligase complex SCF, thereby affecting the apoptotic sensitivity of cells. Moreover, SAG was identified as a potential regulator of *p27*, through inhibition of *p27* accumulation[16]. SAG overexpression has also been detected in a subset of human colon cancers and non-small lung carcinomas and has been shown to be associated with poor prognosis[8,9,17].

The present results, which are in agreement with above-mentioned studies, showed that low SAG expression in tumor tissue, as compared to normal tissue, had a positive effect on survival. The patients with low SAG expression had a median disease-free survival of 31.7 mo, as compared to 28 mo in those with high SAG expression. The clinical outcomes of the 31 patients revealed a strong discrepancy at the 2-year follow-up period between the groups with high *vs* low SAG expression, but this profile seemed to vanish at 3 years of observation.

Previously, Bak and Bcl-Xl, which are members of the Bcl-2 family, were also identified as candidate proteins for regulating chemotherapy-induced apoptosis[18]. Bcl-2 was shown to block γ-radiation-induced cell death[19,20]. Bak can form heterogenous dimers with Bcl-2 or Bcl-Xl to inhibit their antiapoptotic functions. Strong interaction between endogenous Bak and Bcl-Xl has been reported in hepatocytes as well as in other cells[21]. Furthermore, quantitative expression levels of Bcl-Xl and Bak have also been determined to correlate with apoptotic sensitivity of tissues in different carcinomas. Both genes have previously been shown to play a major role in colorectal carcinogenesis and tumor progression. Bcl-Xl, on the other hand, is suggested to be more crucial than Bcl-2 for regulation of apoptotic cell death in colon cancer[22]. Significant overexpression of Bcl-Xl mRNA has been observed in the majority of colorectal carcinomas as compared to the corresponding normal tissues. Krajewska *et al*[23] have also reported elevated Bcl-Xl and reduced Bak expression in colorectal adenocarcinoma. These studies reinforce the major roles of Bcl-Xl and Bak in colorectal carcinogenesis and tumor progression; furthermore, they also imply that expression levels of Bak and Bcl-Xl can be used as prognostic factors in rectal carcinoma, as pointed out by some other studies[24,25].

Thus, in addition to SAG, we also evaluated the expression of two other Bcl-2 family proteins, Bcl-Xl and Bak. The expression levels of antiapoptotic Bcl-Xl and proapoptotic Bak were well correlated with those of SAG, which lends further support to the role of apoptotic factors in the malignant potential of tumors. Higher survival rates for 2 years were recorded for increased expression of proapoptotic Bak, as opposed to SAG and Bcl-Xl levels.

We did not observe any correlation between development of metastases and expression of apoptotic factors; however, in patients who did not develop metastases, gene regulation, especially that of SAG, was closely associated with survival. In non-metastatic tumors, tissues with low-level SAG expression (n = 12) were associated with longer mean survival (36 mo), as opposed to those with high expression levels (27.9 mo, n = 11).

In conclusion, the present study shows that the level of expression of apoptosis-related genes may be associated with the degree of resistance to radiation exposure and may significantly affect therapeutic outcome. We observed an inverse correlation between SAG expression and survival. Furthermore, the data obtained in this study imply that SAG and Bak expression may serve as predictive parameters of disease progression. On the other hand, SAG and Bak expression levels did not significantly affect overall survival. This pilot study included a rather small number of patients, therefore, prospective studies with larger cohorts that include other proteins in the molecular apoptotic pathway are required to assess the roles of individual regulators in anticancer therapy.

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COMMENTS

Background

Apoptotic proteins have been reported to be important prognostic factors in various cancers. Sensitive-to-apoptosis gene (SAG) is a recently identified apoptotic protein, which may be a new candidate to estimate the outcome of treatment in rectal cancers.

Research frontiers

SAG was identified as a redox-sensitive protein that protects cells from apoptosis, either by scavenging oxygen radicals or by acting on apoptosis-related proteins as part of the ubiquitin ligase Skp/Cullin/F-box containing complex (SCF), thereby affecting the radiation sensitivity of cells. This study investigated the correlation between expression levels of SAG and survival rates of patients, who have advanced rectal carcinoma. In addition to SAG, this study also examined two other proteins, B-cell lymphoma-extra large (Bcl-XL) and Bcl-2 homologous antagonist/killer (Bak) proteins, which are important members of the mitochondrial apoptotic pathway.

Innovations and breakthroughs

The present study showed that the level of expression of some apoptosis-related genes may be associated with the degree of resistance to radiation ex-

posure and may significantly affect therapeutic outcome. The present research observed an inverse correlation between SAG expression and 2-year survival, although the overall survival rate was not affected significantly. There were no significant correlations between the expression levels of all three genes and metastatic rates or tumor responses to chemoradiotherapy (CRT).

Applications

The data obtained in this study imply that SAG and Bak expression may serve as predictive parameters of disease progression. This pilot study included a rather small number of patients, therefore, prospective studies with larger cohorts that include other proteins in the molecular apoptotic pathway are required to assess the roles of individual regulators in anticancer therapy.

Terminology

SAG/regulator of cullins 2/Rbx/Hrt2 is a recently identified component of SCF E3 ubiquitin ligase, which controls cell-cycle progression by promoting ubiquitination and degradation of cell-cycle inhibitors. It has also been reported that SAG protects cells from apoptosis induced by redox agents such as hydroxyl radicals and radiation. The Bcl-2 family proteins are members of the intrinsic apoptotic pathway. Bak is a proapoptotic member, while Bcl-X_L blocks apoptosis in many systems.

Peer review

This was an interesting and well conducted study which should be published because it confirms the usefulness of SAG in the prognostication of rectal cancer patients.

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BRIEF ARTICLE

Continuous regional arterial infusion and laparotomic decompression for severe acute pancreatitis with abdominal compartment syndrome

Zhi-Gang Deng, Jian-Yin Zhou, Zhen-Yu Yin, You-Yuan Peng, Fu-Qiang Wang, Xiao-Min Wang

Zhi-Gang Deng, Jian-Yin Zhou, Zhen-Yu Yin, You-Yuan Peng, Fu-Qiang Wang, Xiao-Min Wang, Department of Hepatobiliary Surgery, Xiamen University Zhongshan Hospital, Xiamen 361004, Fujian Province, China

Author contributions: Deng ZG and Zhou JY contributed equally to this work; Yin ZY designed the research and edited the manuscript; Deng ZG and Zhou JY performed the majority of research and analyzed the data; Peng YY, Wang FQ and Wang XM performed the research; Deng ZG wrote the paper.
Supported by the National Natural Science Foundation of China, No. 30872484
Correspondence to: Zhen-Yu Yin, MD, PhD, Professor, Department of Hepatobiliary Surgery, Xiamen University Zhongshan Hospital, 209 South Hubin Road, Xiamen 361004, Fujian Province, China. yinzhenyumd@gmail.com
Telephone: +86-592-2993130 Fax: + 86-592-2212328
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Abstract

AIM: To evaluate the therapeutic effects of abdominal decompression plus continuous regional arterial infusion (CRAI) *via* a drug delivery system (DDS) in severe acute pancreatitis (SAP) patients with abdominal compartment syndrome (ACS).

METHODS: We presented our recent experience in 8 patients with SAP. The patients developed clinical ACS, which required abdominal decompression. During the operation, a DDS was inserted into the peripancreatic artery (the catheter was inserted from the right gastroepiploic artery until it reached the junction between the pancreaticoduodenal and gastroduodenal artery). Through this DDS, a protease inhibitor, antibiotics and octreotide were infused continuously. The duration of the regional artery infusion ranged from 8 to 41 d. The outcomes and the changes in the APACHE Ⅱ score, computed tomography (CT) severity index and intra-abdominal pressure (IAP) of the patients were retrospectively evaluated.

RESULTS: Eight patients with an initial APACHE Ⅱ score of 18.9 (range, 13-27) and a Balthazar CT severity index of 9.1 (range, 7-10) developed severe local and systemic complications. These patients underwent subsequent surgical decompression and CRAI therapy because of intra-abdominal hypertension (IAH). After a mean interval of 131.9 ± 72.3 d hospitalization, 7 patients recovered with decreased APACHE Ⅱ scores, CT severity indexes and IAP. The mean APACHE Ⅱ score was 5.4 (range, 4-8), the CT severity index was 2.3 (range, 1-3), and IAP decreased to 7.7 mmHg (range, 6-11 mmHg) 60 d after operation. One patient died of multiple organ failure 1 wk after surgery.

CONCLUSION: CRAI and laparotomic decompression might be a therapeutic option for SAP patients with ACS.

Key words: Severe acute pancreatitis; Arterial infusion; Laparotomy; Abdominal compartment syndrome

Peer reviewer: Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma-Sapienza, Viale del Policlinico 155, Roma 00161, Italy

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INTRODUCTION

Acute pancreatitis is an inflammatory disease that is self-

limited in the majority of patients and resolves within 48-72 h. However, approximately 20% of the patients develop a more severe form of the disease with evidence of organ dysfunction and complications such as pancreatic necrosis, abscess or pseudocyst. This acute form is classified as severe acute pancreatitis (SAP) according to the Atlanta classification and has a mortality approaching 30%[1]. Previous studies have shown that 60% of the patients developed organ failure on admission, and those with persistent organ failure had the worst outcomes[2].

The current treatment paradigm calls for non-operative management of SAP as long as there is no evidence of infection. However, there is a subset of patients with acute pancreatitis who may need an urgent laparotomy in the absence of an infection to decompress the clinically significant abdominal compartment syndrome (ACS)[3].

SAP with ACS, which is defined as a sustained intra-abdominal pressure (IAP) greater than 20 mmHg that is associated with the development of organ dysfunction or failure[4], is the most severe form of acute pancreatitis and has a high morbidity and mortality. SAP with ACS injures not only the pancreas itself but also the surrounding organs. Despite various treatment protocols, including intensive care therapy and blood filtration, the mortality rate of SAP with ACS is still reported to be 30%-60%[2].

In a number of recent studies, continuous regional arterial infusion therapy (CRAI) for SAP using protease inhibitors and antibiotics has been shown to control the inflammation of the pancreas and to prevent the extension of the inflammatory process, which would decrease the rate of infection and mortality[5-11].

Accordingly, in this study, we used a new system during our decompressing surgical procedure in 8 patients suffering from SAP with ACS, and the therapeutic effects of continuous regional intra-arterial infusion were retrospectively evaluated.

MATERIALS AND METHODS

Eight patients, 6 men and 2 women, with a mean age of 51.5 year (35-66 year) were diagnosed with SAP at Xiamen University Zhongshan Hospital from April 2009 to July 2010. SAP was diagnosed based on clinical manifestation, biological findings and contrast-enhanced abdominal computed tomography (CT) within 3 d after admission. These patients presented with multiple organ dysfunction (MOD) or multiple organ failure (MOF) within 3 d of admission, and they continued to deteriorate under intensive medical support (Table 1).

Laparotomy was performed 3-9 d after the onset of SAP in these patients because of clinical deterioration despite intensive medical care and persistent abdominal hypertension. The abdominal pressure was measured by the bladder technique[4]. In contrast to the traditional surgical procedure for SAP, necronectomy (debridement) was not performed. For abdominal decompression and placement of wide-bore drains for continuous postoperative irrigation, the catheter of a drug delivery system (DDS) was inserted into the peripancreatic artery. Because the pancreas has a multiple-sourced blood supply, an appropriate drug distribution in the pancreas can not be achieved unless the catheter is properly placed. The proper position of the catheter tip was decided based on the methylthionine chloride injection. By injecting methylthionine chloride from the head of DDS, we could find the dyeing area, and adjust the position of catheter tip. In cases which it was difficult to find the right gastroepiploic artery, an intraoperative ultrasound was used for guiding. The injection head of the DDS was subcutaneously placed for postoperative drug delivery (Figure 1), and the patients subsequently received an arterial infusion with protease inhibitor, antibiotics and octreotide. In addition, patients with biliary stones had emergency surgery such as endoscopic sphincterotomy, endoscopic nasobiliary drainage, cholecystectomy or common bile duct lithotomy. If we had difficulties with the closure after laparotomy, we left the abdomen open and applied a temporary closure device until suturing again.

Table 1 Multiple organ dysfunction or multiple organ failure within 3 d after admission

Dysfunction (failure)	*n* (%)
Pulmonary insufficiency	8 (100.0)
Requiring mechanical ventilation	8 (100.0)
Renal insufficiency	7 (87.5)
Requiring dialysis	6 (75.0)
Shock	7 (87.5)
Requiring catecholamines	8 (100.0)
Sepsis (or SIRS)	8 (100.0)
Coagulopathy	7 (87.5)
Hepatic dysfunction (failure)	7 (87.5)
Cardiovascular disable	7 (87.5)
Gastrointestinal bleeding	7 (87.5)
Center nerve system problem	5 (62.5)

Ulinastatin (100 000 U), antibiotics (imipenem/cilastatin 0.5 g) and octreotide (0.3 mg) were dissolved into the saline (48 mL) and continually infused through the DDS twice a day. To ensure the therapeutic effects, the infusion was continued even after the patients' conditions had improved and until the Balthazar CT severity index had decreased to 3 or less.

All patients were treated in the intensive care unit (ICU). Fluid, electrolytes, albumin, and insulin were replaced dependent on central venous pressure (6-10 mm H_2O), hematocrit (30%-35%), urinary excretion, and blood glucose measurement. Assisted ventilation was begun if the partial pressure of oxygen could not be maintained at a level of > 60 mmHg with an oxygen mask. In patients with progressive renal failure (serum creatinine > 3.0 mg/dL), hemodialysis or continuous venovenous hemodialysis (CVVHD) was performed.

The clinical data of patients, transvesical measurement of IAP, CT severity index[12], APACHE Ⅱ score[13], presence of MOD, local-regional complications and outcome were examined and reported.

RESULTS

The severity of the pancreatitis was judged according to

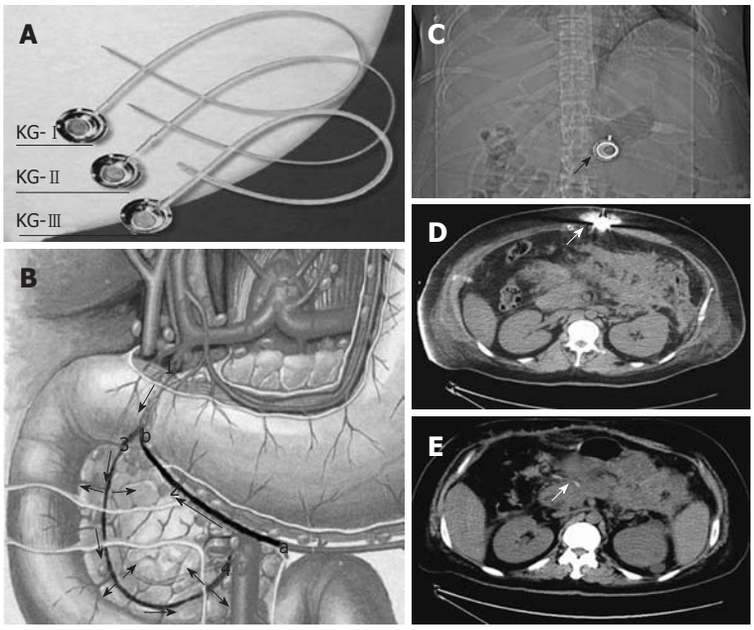

Figure 1 Procedure of placing drug delivery system and imaging after drug delivery system was inserted. A: Drug delivery system; B: Anatomy and how it is inserted; 1: Gastroduodenal artery; 2: Right gastroepiploic artery; 3: Superior pancreatico-duodenal artery; and 4: Inferior pancreatico-duodenal artery (arrows indicate the direction of drug delivery and blood flow); a: The inserting point; and b: The end in which the catheter should be inserted; C: Abdominal X-ray film showed the subcutaneous drug delivery system (DDS) head (arrow); D: Computed tomography (CT) scan showed the subcutaneous DDS and the drug delivery needle inside the DDS (arrow); E: DDS catheter around the pancreas (arrow) in the CT scan.

Table 2 Clinical data of 8 patients with severe acute pancreatitis treated by postoperative peripancreatic artery infusion

Patients	Sex/age (yr)	Area of pancreatitis	Operation	AI (d)	IAP		Apache Ⅱ		CT-SI (d)		ICU (d)	Hosp. (d)	Outcome
					Pre	Post	Pre	Post	Pre	Post			
1	M/35	Entire	3	31	35	11	27	6	10	3	45	259	Recovered
2	F/42	Entire	4	8	38	18	24	18	10	8	11	11	Dead
3	M/64	Entire	5	25	26	8	13	4	7	1	34	68	Recovered
4	M/53	Entire	8	18	30	7	16	5	9	3	64	101	Recovered
5	M/49	Entire	6	41	31	9	21	8	10	3	58	159	Recovered
6	F/66	Entire	3	35	24	6	19	4	10	3	44	186	Recovered
7	M/57	Entire	4	19	26	6	16	5	9	1	33	74	Recovered
8	M/46	Entire	5	33	23	7	15	6	8	2	40	76	Recovered

AI: Artery infusion; AP: Abdominal pressure (mmHg); CT-SI: CT severity index by Balthazar *et al*; ICU: Intensive care unit; Pre: Preoperation; Post: 60 d after operation or before the patient died (No. 2 patient).

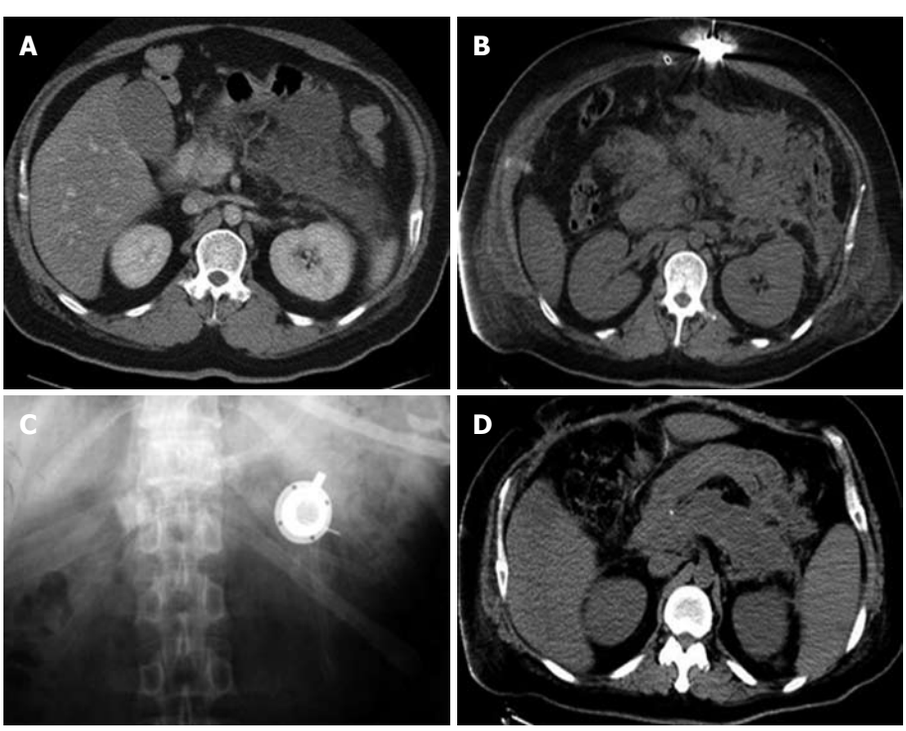

Figure 2 Imaging comparison between preoperation and postoperation. A: Abdominal computed tomography (CT) scan before the operation; B: Abdominal CT scan after the operation with the drug delivery system (DDS) inserted; C: Abdominal X-ray film showing the subcutaneous DDS head (white); D: CT scan showing the recovered pancreas. No more fluid could be seen around the pancreas (CT scan before discharge).

the APACHE Ⅱ score[1,14,15] , with a score of > 8 points considered indicative of severe disease[16]. In our patients, the APACHE Ⅱ score ranged from 13 to 27 points, with a mean of 18.9 points. The Balthazar CT severity index was evaluated at each CT study[12]. The CT severity index of the 8 patients ranged from 7 to 10 (average 9.1) before arterial infusion. The abdominal pressure of the patients was measured before operation, and the results suggested that every patient had high abdominal pressure and was diagnosed as having ACS. The average abdominal pressure was 29.1 mmHg (range, 23-38 mmHg). The cause of the pancreatitis was cholelithiasis in 4 cases, alcoholism in 1, hyperlipidemia in 2, and unknown in 1. The arterial infusion was started 3-9 d after the onset of SAP in these patients, and lasted 8-41 d (mean, 26.3 d).

The clinical conditions of the patients improved within 3 d after decompression and the initiation of the arterial infusion. The APACHE Ⅱ score was decreased from 13-27 (mean, 18.1) to 4-8 (mean, 5.4), and the CT severity index from 7-10 (mean, 9.0) to 1-3 (mean, 2.3), respectively, 60 d after the operation. IAP decreased to 7.7 mmHg (range, 6-11 mmHg). One patient died of MOF, and all other patients were discharged from the hospital within 68-259 d (mean, 131.9 d). The patients are all in good health now. The catheter was removed percutaneously 1 year after discharge, and there were no complications related to the procedure or drug delivery after long-term catheter placement. The clinical data are shown in Table 2 and Figure 2.

DISCUSSION

SAP injures not only the pancreas itself but also the surrounding organs, culminating in ACS and MOF in many cases. Despite the various treatment protocols, including intensive care therapy and blood filtration, SAP is still has a high mortality. The basic principles of the initial management of acute pancreatitis are adequate monitoring of vital signs, fluid replacement, correction of any electrolyte imbalance, nutritional support, and prevention of local and systemic complications.

Infected necrosis is generally accepted as an indication for surgery, but there is a subset of acute pancreatitis which may need an urgent laparotomy in the absence of infection to decompress abdominal pressure, which is unique as a compartment syndrome and virtually affects all organ systems within the body. Pathophysiologically, it deranges cardiovascular hemodynamics, respiratory and renal functions and may eventually lead to multi-organ failure. In addition, the gold standard for the treatment of established ACS is surgical decompression of the abdomen[17].

Our main purpose was to decompress the intra-abdominal hypertension (IAH). A double drainage tube was placed in peritoneal cavity around the region of pancreas, through which the patient's cavity was persistently douched using a large amount of saline solution. This can help drain the intra-abdominal hemorrhagic ascites, alleviate IAH, dilute the inflammatory mediators and activated amylase, and reduce toxin absorption through the peritoneum. In addition, we placed a DDS for postoperative treatment based on the positive results of regional infusion reported by other authors[5-11]. In contrast to their methods of vascular intervention using an angiocatheter, we inserted the DDS catheter into the pancreaticoduodenal artery from the right gastroepiploic artery to the gastroduodenal artery during the procedure. The DDS was subcutaneously fixed and did not result in any discomfort after drug delivery. In the ICU, the DDS could be easily used for the continuous injection of protease inhibitors, antibiotics and octreotide. This device was simple, safe and did not hinder the patient's movement. No complications related to the procedure or the device setting were observed, even after long-term placement.

Regarding the drug infusion, because acute pancreatitis is an autodigestive disease, protease inhibition has been the focus of experimental and clinical research. However, in clinical settings, the effect of protease inhibitors in the treatment of acute pancreatitis is still controversial. Some randomized, controlled trials failed to demonstrate any significant benefits[18,19]. For this reason, in Europe and the United States, protease inhibitors are not usually applied in the treatment of acute pancreatitis. In Japan, however, protease inhibitors are often applied, and in particular, it has been demonstrated that the CRAI of protease inhibitors and antibiotics are beneficial for severe acute necrotizing pancreatitis[5-11]. They believed that there were many reasons why the protease inhibitors were not as effective as expected in the experimental studies, such as the timing of administration, the concentration of the protease inhibitor in the pancreatic tissue, the diminution of the vasculature of the pancreas and so on. With intravenous administration, the concentration of the agent reaching the pancreas eventually becomes insufficient for controlling the inflammation. As a result of drug dilution as well as serum and hepatic metabolism, most of the aprotinin administered intravenously tended to go into the liver and thereafter accumulate in the kidneys, with only a small amount of aprotinin distributed in the pancreas. In contrast, with intra-arterial administration in experimental studies, the local concentration in the pancreas was high enough to improve the biochemical indices of inflammation and survival[20,21].

Because pancreatic and extrapancreatic infections are determining factors leading to death in patients with SAP, much attention has been paid to the potential role for antibacterial prophylaxis, especially in those patients with pancreatic necrosis. Many studies on the prophylactic effect of antibiotics have demonstrated that broad-spectrum antibiotics with good pancreatic tissue penetration decreased the incidence of infectious complications and mortality[22,23]. The antibacterial agent of first choice is likely to be imipenem because it reaches a higher distribution in the pancreatic tissue and provides higher bactericidal activity against most of the bacteria present in pancreatic infection compared with other types of antibiotics. An alternative antibiotic regimen is either ciprofloxacin

or ofloxacin in combination with metronidazole[24]. It also has been suggested that the effect was induced markedly by intra-arterial administration.

Octreotide reduces exocrine pancreatic secretion in acute pancreatitis, which would decrease pancreatic autodigestion, and it may also significantly prevent the bacterial translocation by preventing mucosal damage[25]. As a treatment guideline for acute pancreatitis, octreotide has been widely used in clinical practice, although the results of clinical investigations using somatostatin or its analogue are controversial. In a multicenter randomized controlled study with a large number of patients (n = 302) with an adequate level of disease severity, no benefit of octreotide on progression or outcome was found[26], but other research suggests that octreotide may have a beneficial effect in the treatment of SAP[27]. One study suggested that octreotide seemed to have a dose- and time-dependent effect on histopathology and lipid peroxidation: an early bolus application of octreotide reduced the severity of histopathological changes in acute pancreatitis and decreased lipid peroxidation in the pancreatic tissue samples. However, a late bolus application and continuous intravenous infusion did not influence pancreatitis or lipid peroxidation[28].

According to the above-mentioned clinical and experimental results, in this study, we used a combination of these three kinds of drugs *via* DDS as regional therapy in the 8 patients. Their clinical conditions improved after the treatment and the overall mortality was 12.5%, which is much lower than that reported in the literature. These results suggest that CRAI plus laparotomic decompression might be a therapeutic choice for SAP with ACS.

COMMENTS

Background

Severe acute pancreatitis (SAP), characterized by intricate mechanism, variant symptoms, poor prognosis and multiple complications, seriously threatens the life of patients. About 11% of SAP patients suffer from abdominal compartment syndrome (ACS). SAP complicated by ACS has a mortality rate of that is 30%-60%.

Research frontiers

ACS has been recognized as a contributing factor for the multiple organ failure commonly seen in SAP. Surgical decompression is the preferred method of treatment for ACS. Although decompression has a significant effect in lowering IAP, the mortality still remains high in SAP patients with ACS. Some recent studies showed that continuous regional arterial infusion of protease inhibitors and antibiotics may reduce the mortality rate and incidence of infectious complications in SAP.

Innovations and breakthroughs

SAP with ACS is the most severe form of acute pancreatitis and has a high morbidity and mortality which need an urgent laparotomy. In this study, a new system was applied during the decompressing surgical procedure in 8 patients. In addition to abdominal decompression and the placement of wide-bore drains for continuous postoperative irrigation, the catheter of a drug delivery system (DDS) was inserted into the peripancreatic artery for postoperative continuous regional arterial infusion, which increased the tissue concentration of the drugs in the inflamed pancreas and improved the biochemical indices of inflammation and survival.

Applications

The DDS applied for continuous regional arterial infusion (CRAI) in this study is simple, safe and easy to implement. These positive results suggest that CRAI plus laparotomic decompression might be a therapeutic choice for SAP with ACS.

Peer review

Even if it is not accepted by all, the presence of an ACS in severe pancreatitis is a very difficult complication to deal with. The paper reports a small group of patients with such a complication treated with a multidisciplinary approach (surgery, antibiotics, anti protease, *etc.*). It is difficult to understand the real value of each therapy, but the utility of the paper lies in reporting the complexity of such a disease.

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BRIEF ARTICLE

Plasma DNA methylation of Wnt antagonists predicts recurrence of esophageal squamous cell carcinoma

Ji-Bin Liu, Fu-Lin Qiang, Jing Dong, Jin Cai, Shu-Hui Zhou, Min-Xin Shi, Ke-Ping Chen, Zhi-Bin Hu

Ji-Bin Liu, Ke-Ping Chen, Institute of Life Science, Jiangsu University, Zhenjiang 212013, Jiangsu Province, China
Ji-Bin Liu, Fu-Lin Qiang, Jin Cai, Min-Xin Shi, Nantong Tumor Hospital, Nantong 226361, Jiangsu Province, China
Jing Dong, Zhi-Bin Hu, Department of Epidemiology and Biostatistics, Ministry of Education Key Lab for Cancer, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Shu-Hui Zhou, School of Radiology and Public Health, Soochow University, Suzhou 215123, Jiangsu Province, China
Zhi-Bin Hu, Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Cancer Center, Nanjing 210029, Jiangsu Province, China
Author contributions: Liu JB and Qiang FL contributed equally to this work; Liu JB and Qiang FJ performed the majority of experiments; Liu JB, Qiang FJ, and Dong J provided vital reagents and analytical tools and edited the manuscript; Liu JB, Qiang FJ, Cai J, Zhou SH and Shi MX collected all the specimens for this work; Liu JB, Dong J, Chen KP, and Hu Z designed the study and wrote the manuscript.
Supported by Jiangsu University and Nantong Tumour Hospital
Correspondence to: Ke-Ping Chen, Professor, Institute of Life Science, Jiangsu University, Zhenjiang 212013, Jiangsu Province, China. kpchen@ujs.edu.cn
Telephone: +86-511-88791923 Fax: +86-511-88791923
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Abstract

AIM: To detect the effects of plasma DNA methylation of Wnt antagonists/inhibitors on recurrence of esophageal squamous cell carcinoma (ESCC).

METHODS: We used methylation-specific polymerase chain reaction to detect hypermethylation of the promoter of four Wnt antagonists/inhibitors (*SFRP-1*, *WIF-1*, *DKK-3* and *RUNX3*) using DNA from the plasma of ESCC patients (n = 81) and analyzed the association between promoter hypermethylation of Wnt pathway modulator genes and the two-year recurrence of ESCC.

RESULTS: Hypermethylation of *SFRP-1*, *DKK-3* and *RUNX-3* was significantly associated with an increased risk of ESCC recurrence (P = 0.001, 0.003 and 0.001 for *SFRP-1*, *DKK-3* and *RUNX3*, respectively). Patients carrying two to three methylated genes had a significantly elevated risk of recurrence compared with those not carrying methylated genes (odds ratio = 15.69, 95% confidential interval: 2.97-83). The area under the receiver operating characteristic curve (AUC) was 77.1 for ESCC recurrence prediction (sensitivity = 66.67 and specificity = 83.3). When combining methylated genes and the clinical stage, the AUC was 83.69, with a sensitivity of 76.19 and a specificity of 83.3.

CONCLUSION: The status of promoter hypermethylation of Wnt antagonists/inhibitors in plasma may serve as a non-invasive prognostic biomarker for ESCC.

Key words: Plasma; Methylation; Esophageal Cancer; Recurrence

Peer reviewer: Dr. Yukie Sato-Kuwabara, Hospital A. C. Camargo, Teaching and Research International Center, Sao Paulo 01508-010, Brazil

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INTRODUCTION

Esophageal cancer, predominantly esophageal squamous cell carcinoma (ESCC), is one of the most common malignancies in the world, with 482 300 incident cases and

406 800 deaths estimated in 2008[1]. Most patients with ESCC are diagnosed at an advanced stage. Although therapeutic advances have been designed to improve treatment outcome, the prognosis of patients with ESCC is still poor, with long-term survival rates between 5% and 20%[2]. The poor prognosis is partially due to the high rate of recurrence, which occurs in approximately half of patients after curative surgical resection. Therefore, development of accurate prognostic biomarkers for ESCC is imperative and crucial for improving ESCC prognosis and for guiding treatment.

DNA methylation is one of the most common epigenetic modifications[3]. One of the major changes caused by DNA methylation is transcriptional silencing of tumor suppressor genes resulting from hypermethylation of CpG islands in promoter regions[4]. This change occurs frequently during tumor pathogenesis and progression and has become widely recognized as a mechanism of gene inactivation in cancer[5-7]. The methylation profile may also help predict the response to a chemo-/radio-therapeutic agent and thus the prognosis of cancer[8,9]. In addition, detection of promoter CpG methylation in body fluid DNA is feasible and nearly non-invasive[10].

Wnt signaling operates across cell boundaries *via* secretion by cells of one tissue type, which results in activation of surface receptors on neighboring cells and tissues, leading to activation of transcription factors that regulate cell proliferation, survival, and differentiation[11]. Dysregulation of these processes in cancer results in aberrant activation of the Wnt pathway[11,12]. Several antagonists of Wnt signaling have been identified, including the secreted frizzled-related protein-1 (SFRP-1) and Wnt inhibitory factor-1 (WIF-1), which bind directly to Wnt proteins, and Dickkopf-3 (DKK-3), which binds to the LDL-receptor-related protein5 (LRP5)/LRP6 component of the Wnt receptor complex[13]. In addition, another Wnt inhibitor, runt-related transcription factor-3 (RUNX3), reportedly forms a ternary complex with β-catenin/transcription factor-4 (TCF4) to attenuate Wnt signaling, which regulates cell proliferation, apoptosis, and invasion[14,15]. Given the important roles of Wnt antagonists/inhibitors in cancer progression and prognosis, we evaluated the association between methylation of promoter CpG islands of the four tumor suppressor genes, *SFRP-1*, *WIF-1*, *DKK-3* and *RUNX3*, in the Wnt signaling pathway and ESCC recurrence using plasma DNA from 81 Chinese patients with ESCC.

MATERIALS AND METHODS

Study population

This study was approved by the institutional reviewer board of Nantong Cancer Hospital, and written informed consent was obtained from each patient or from the patient's representative. Briefly, patients with histopathologically diagnosed incident ESCC were recruited from Nantong Cancer Hospital from June to December 2008. Exclusion criteria included self-reported previous cancer history, metastasis from other organs, and surgical section, radiotherapy, or chemotherapy before blood collection. Patients were followed up at the 24th month after recruitment by personal or family contacts. All patients were genetically unrelated, ethnic Han Chinese, and each patient donated 5 mL of venous blood at their first admission to the hospital. As a result, 81 ESCC patients who had complete clinical information, adequate DNA samples, and successful follow-up were included.

Table 1 Primers for methylation-specific polymerase chain reaction

Gene		Primer sequence (5'–3')
WIF-1		
U	F	GGGTGTTTTATTGGGTGTATTGT
	R	AAAAAAACTAACACAAACAAAATACAAAC
M	F	CGTTTTATTGGGCGTATCGT
	R	ACTAACGCGAACGAAATACGA
RUNX-3		
U	F	TTATGAGGGGTGGTTGTATGTGGG
	R	AAAACAACCAACACAAACACCTCC
M	F	TTACGAGGGGCGGTCGTACGCGGG
	R	AAAACGACCGACGCGAACGCCTCC
SFRP-1		
U	F	GAGTTAGTGTTGTGTGTTTGTTGTTTTGT
	R	CCCAACATTACCCAACTCCACAACCA
M	F	GTGTCGCGCGTTCGTCGTTTCGC
	R	AACGTTACCCGACTCCGCGACCG
DKK-3		
U	F	TTAGGGGTGGGTGGTGGGGT
	R	CTACATCTCCACTCTACACCCA
M	F	GGGGCGGGCGGCGGGGC
	R	ACATCTCCGCTCTACGCCCG

M: Methylated sequence; U: Unmethylated sequence; F: Forward primer; R: Reverse primer.

DNA extraction and methylation-specific polymerase chain reaction

DNA was extracted from 200 μL plasma from each patient using an SG Spin Column Clinical Sample Genomic DNA MiniPreps kit (Shanghai ShineGene Molecular Biotech, Inc., Shanghai, China). The plasma DNA was modified with sodium bisulfite using the EZ DNA methylation™ kit (Zymo Research, Orange, CA, United States). The methylation status of CpG islands in the promoter region of *SFRP-1*, *WIF-1*, *DKK-3* and *RUNX-3* was determined by methylation-specific polymerase chain reaction (PCR)[16]. In brief, the first universal primer set covered no CpG sites in either the forward or the reverse primer but amplified a DNA fragment of the promoter region containing several sites. Then, a second round of nested methylation-specific PCR or unmethylation-specific PCR was performed using the universal PCR products as templates. Primer sequences are shown in Table 1.

Statistical analysis

Differences in demographic and clinical characteristics were evaluated with χ^2 tests (or the Fisher's exact test). The association between methylation and ESCC recurrence was

Table 2 Characteristics of patients

Variables	Recurrence		*P* value
	No	Yes	
Age (yr)			0.804
< 63	31	10	
⩾ 63	29	11	
Gender			0.569
Female	16	4	
Male	44	17	
Smoking			0.614
No	31	9	
Yes	29	12	
Drinking			0.799
No	26	8	
Yes	34	13	
Operation			0.614
No	38	12	
Yes	22	9	
Chemo-/radio-therapy			0.751
No	11	5	
Yes	49	16	
Stage			< 0.001
Ⅰ	25	3	
Ⅱ	22	3	
Ⅲ	13	15	

Table 3 Correlation between methylation and recurrence in esophageal squamous cell carcinoma patients

Gene	*n*	Recurrence		Crude OR (95% CI)	*P* value	Adjusted OR[1] (95% CI)	*P* value[1]
		No	Yes				
SFRP-1							
Negative	57	49	8	1.00	< 0.001	1.00	< 0.001
Positive	24	11	13	7.24 (2.42-21.68)		10.8 (2.54-46)	
WIF-1							
Negative	52	42	10	1.00	0.069	1.00	0.107
Positive	29	18	11	2.57 (0.93-7.11)		2.61 (0.8-8.47)	
DKK-3							
Negative	51	44	7	1.00	0.001	1.00	0.003
Positive	30	16	14	5.50 (1.88-16.08)		6.07 (1.73-21.28)	
RUNX-3							
Negative	52	45	7	1.00	< 0.001	1.00	0.001
Positive	29	15	14	6.00 (2.04-17.65)		7.81 (2.30-47)	

[1]Adjusted for age, gender, smoking, drinking, stage, surgical operation and chemo-/radio-therapy. CI: Confidence intervals; OR: Odds ratios.

Table 4 Combined analysis of methylation and recurrence in esophageal squamous cell carcinoma patients

No. of M gene	*n*	Recurrence		Crude OR (95% CI)	*P* value	Adjusted OR[1] (95% CI)	*P* value[1]
		No	Yes				
0	32	29	3	Ref.		Ref.	
1	25	21	4	1.84 (0.37-9.11)	0.454	1.71 (0.27-10.73)	0.570
2-3	24	10	14	13.53 (3.21-57.07)	< 0.001	15.69 (2.97-83.00)	0.001
Locus Trend				4.10 (1.92-8.75)	< 0.001	4.10 (1.73-9.72)	0.001

[1]Adjusted for age, gender, smoking, drinking, stage, surgical operation and chemo-/radio-therapy. CI: Confidence intervals; OR: Odds ratios.

estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from logistic regression analyses for adjusted ORs and crude ORs, with or without adjustments for age, gender, smoking, drinking, clinical stage, surgical operation and chemo-/radio-therapy status. Receiver operating characteristic (ROC) curve analysis was conducted by using the "pROC" package in R. All the statistical analyses were performed with R software (version 2.11.1; The R Foundation for Statistical Computing).

RESULTS

Characteristics of patients

The clinicopathologic features of the patients are summarized in Table 2. A total of 81 ESCC patients were included in our current study. The mean age was 63 years (range, 46-80 years), and 61 patients were male (75.3%). Sixty-five (80.2%) patients underwent chemo-/radio-therapy, and 31 (38.3%) patients underwent surgical operation after blood collection. Among the 81 patients, there were 21 recurrences (26%) at the 24th month after recruitment. As expected, advanced stage was a risk factor for ESCC recurrence ($P < 0.001$). However, there were no significant differences in the recurrence rates among the subgroups of age, gender, smoking status, drinking status, with or without surgical operation, and/or chemo-/radio-therapy.

Single-gene analysis

The percentage of promoter methylation in the genes we analyzed was as follows: 29.6% for *SFRP-1*, 35.8% for *WIF-1*, 37.4% for *DKK-3*, and 35.8% for *RUNX-3*. Three genes showed significant associations with ESCC recurrence after adjusting for age, gender, smoking, drinking, stage, surgical operation, and chemo-/radio-therapy. As shown in Table 3, patients with methylated *SFRP-1* had a 10.8-fold increased risk of recurrence compared with those with unmethylated *SFRP-1* (95% CI: 2.54-46, $P < 0.001$). Similarly, methylated *DKK-3* was associated with a 6.07-fold increased risk of recurrence (95% CI: 1.73-21.28, $P = 0.003$), and methylated *RUNX-3* was associated with a 7.81-fold increased recurrence risk (95% CI: 2.30-47, $P = 0.001$). Methylation of *WIF-1*, however, was not significantly associated with an increased risk of recurrence ($P = 0.107$).

Combined analysis

We then performed combined analysis to assess the effect of methylation of the three genes (*SFRP-1*, *DKK-3* and *RUNX-3*) on ESCC recurrence. As shown in Table 4, compared with patients without methylated genes, those with two to three methylated genes had a significantly increased risk of recurrence (OR = 15.69, 95% CI: 2.97-83).

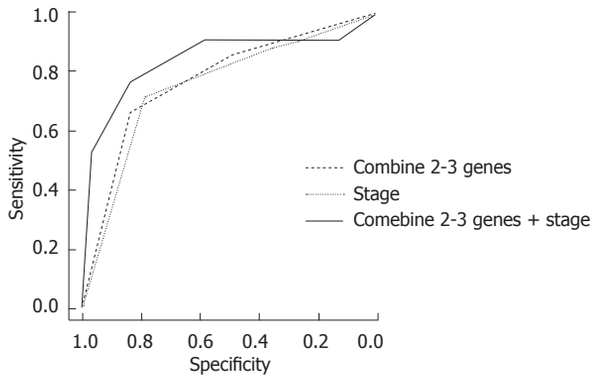

Figure 1 Receiver operating characteristic curve analysis to assess the sensitivity and specificity of the status of methylated genes in combination with the stage for predicting esophageal squamous cell carcinoma recurrence using the "pROC" package in R. Combination of 2-3 genes, area under curve (AUC) = 77.1, sensitivity = 66.67, specificity = 83.3, stage, AUC = 75.24, sensitivity = 71.43, specificity = 78.3, combination of 2-3 genes + stage, AUC = 83.69, sensitivity = 76.19, specificity = 83.3.

Furthermore, there was a dose-response effect between plasma DNA methylation status and ESCC recurrence (OR = 4.1, 95% CI: 1.73-9.72, *P* for trend: 0.001).

AUC analysis

We also conducted a ROC curve analysis and area under curve (AUC) analyses to assess the sensitivity and specificity of the status of methylated genes individually and in combination for predicting ESCC recurrence. For single genes, the AUC were 71.79 (*SFRP-1*), 70 (*DKK-3*) and 70.83 (*RUNX3*). When we considered two to three methylated genes, the AUC increased to 77.1, with a sensitivity of 66.67 and a specificity of 83.3. As a risk factor for ESCC recurrence, the AUC for stage was 75.24 (sensitivity = 71.43, specificity = 78.3). For the combination of methylated genes and clinical stage, the AUC was 83.69 (sensitivity = 76.19, specificity = 83.3) (Figure 1).

DISCUSSION

Wnt antagonists/inhibitors function as tumor suppressors, and thus hypermethylation of their promoters and subsequent silencing of these genes may be implicated in the pathogenesis or progression of a broad spectrum of human malignancies[17-19]. In our current study, we found that the methylation status of *SFRP-1*, *DKK-3* and *RUNX-3* promoters in plasma DNA can individually and jointly predict ESCC recurrence.

Most published studies on the association between hypermethylation of Wnt antagonist/inhibitor gene promoters and cancer development have focused on tumor tissues. Yu *et al*[17] found that epigenetic silencing of *DKK-3* is a common event in gastric cancer and is associated with a poor disease outcome. Urakami *et al*[18] used a methylation score to analyze the combined effects of hypermethylated Wnt antagonist family genes on bladder cancer detection, and they found that the score was significantly higher in bladder tumors than in bladder mucosa. Hamilton *et al*[9] assessed the methylation status of nine genes, including *RUNX-3*, in esophageal cancer patients and found that increased methylation of this gene correlated with poor responsiveness to therapy, suggesting potential clinical application of these biomarkers in guiding prognosis and management. A growing body of evidence suggests that the methylation signature is consistent between DNA derived from tissue and DNA derived from serum/plasma[19,20]. However, serum/plasma DNA has obvious advantages compared with DNA derived from tissue samples, i.e., with respect to the ease to obtain, non-invasiveness, and relative reproducibility. Therefore, serum/plasma DNA may be an excellent source of samples for assessing biomarkers, especially for patients who have not undergone surgery.

Previous studies have shown that methylation of various genes in serum/plasma can be a highly specific biomarker for human cancers[19,21-26]. Herbst *et al*[24] reported that *NEUROG1* is frequently methylated in sera of patients with colorectal cancers independent of the tumor stage and is a suitable non-invasive screening approach for detecting asymptomatic colorectal cancer. Göbel *et al*[25] found that methylated *PITX2* and *RASSF1A* in plasma are therapy-independent prognostic factors in breast cancer patients. Salazar *et al*[26] showed that the methylation status of *CHFR* in serum can influence the outcome of chemotherapy in stage Ⅳ non-small-cell lung cancer patients and that unmethylated *CHFR* predicts increased survival to EGFR TKIs. Urakami *et al*[19] reported that hypermethylation of Wnt antagonists can serve as an excellent epigenetic biomarker panel for detection, staging and prognosis of renal cell carcinoma using serum DNA. These studies all indicate the available and feasibility of using plasma DNA to establish the methylation status of gene promoters as a biomarker for cancer.

Given that ESCC is one of the most aggressive cancers in the world and that no non-invasive test is available, our preliminary study yielded a promising result. Limitations of our study include the lack of detection of DNA methylation in tissue and the unpredictability of the link between methylation status and gene expression. Nevertheless, the methylation status of Wnt antagonists/inhibitors may serve as a valuable biomarker for predicting ESCC recurrence.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies in the world. The prognosis of patients with ESCC is poor, which is partially due to the high rate of recurrence. Therefore, development of accurate prognostic biomarkers for ESCC is imperative and crucial for improving ESCC prognosis and for guiding treatment.

Research frontiers

Wnt antagonist/inhibitor genes function as tumor suppressors, and hypermethylation of their promoters and subsequent silencing of the genes, which can be detected in plasma DNA, may serve as a valuable non-invasive biomarker for predicting ESCC recurrence.

Innovations and breakthroughs

The association between methylation of promoter CpG islands of four tumor suppressor genes (*SFRP-1*, *WIF-1*, *DKK-3* and *RUNX3*) in the Wnt signaling pathway and ESCC recurrence was evaluated using plasma DNA from ESCC patients.

Applications

This result offers great potential for using plasma DNA to analyze methylation as a non-invasive biomarker for predicting ESCC recurrence.

Terminology

Epigenetic changes: Heritable changes in the gene structure that do not change the gene sequence. CpG islands: CpG-rich areas located in the promoter regions of many genes. CpG island methylation: The addition of a methyl group to a cytosine residue that is next to guanine.

Peer review

The study aimed to verify the promoter hypermethylation status of four Wnt [antagonist genes odds ratio (OR) antagonists] (using methylation-specific polymerase chain reaction with OR with the methylation-specific polymerase chain reaction technique using) DNA from plasma of 81 ESCC patients. The article reports interesting results that are important to the field.

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BRIEF ARTICLE

SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer cells

Xin Zhou, Shu-Yan Huang, Jing-Xin Feng, Yan-Yan Gao, Li Zhao, Jun Lu, Bai-Qu Huang, Yu Zhang

Xin Zhou, Jing-Xin Feng, Yan-Yan Gao, Li Zhao, Jun Lu, Bai-Qu Huang, Yu Zhang, The Institute of Genetics and Cytology, School of Life Science, Northeast Normal University, Changchun 130024, Jilin Province, China
Shu-Yan Huang, Department of Biology, Zhounan High School of Hunan Province, Changsha 410081, Hunan Province, China
Author contributions: Zhou X, Huang SY, Gao YY, Zhao L and Zhang Y designed the research; Zhou X and Huang SY performed the research; Huang SY, Zhou X, Feng JX, Lu J and Huang BQ analyzed the data; Zhou X, Huang SY and Zhang Y wrote the paper; Zhou X and Huang SY contributed equally to the work.
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Correspondence to: Dr. Yu Zhang, The Institute of Genetics and Cytology, School of Life Science, Northeast Normal University, Changchun 130024, Jilin Province, China. zhangy288@nenu.edu.cn
Telephone: +86-431-85099362 Fax: +86-431-85099768
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Abstract

AIM: To confirm the role of sex-determining region Y-box 7 (Sox7) in aspirin-mediated growth inhibition of COX-independent human colorectal cancer cells.

METHODS: The cell survival percentage was examined by MTT (Moto-nuclear cell direc cytotoxicity) assay. SOX7 expression was assessed by using reverse transcription-polymerase chain reaction and Western blotting. SB203580 was used to inhibit the p38MAPK signal pathway. SOX7 promoter activity was detected by Luciferase reporter assay.

RESULTS: SOX7 was upregulated by aspirin and was involved in aspirin-mediated growth inhibition of SW480 human colorectal cancer cells. The p38MAPK pathway played a role in aspirin-induced SOX7 expression, during which the AP1 transcription factors c-Jun and c-Fos upregulated SOX7 promoter activities.

RESULTS: SOX7 is upregulated by aspirin and is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells.

Key words: SOX7; Aspirin; p38MAPK; Colorectal cancer; SB203580

Peer reviewer: Dr. Ondrej Slaby, Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Zlutykopec 7, Brno 65653, Czech Republic

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INTRODUCTION

In Western Europe and the United States, colorectal cancer is the second most common fatal cancer next to lung cancer. Aspirin is believed to have a chemopreventive role in colorectal cancer based on considerable observational data, which show that the rates of colorectal cancers and adenomas are 40%-50% lower in aspirin users[1,2]. Aspirin is a nonsteroidal anti-inflammatory drug (NSAID). There have been indications that use of NSAIDs can lead to the regression of colorectal adenomas[3]. In several rodent models of colorectal cancer, the NSAIDs indomethacin, aspirin, sulindac and piroxicam were shown to be able to reduce tumor growth[4]. However, the molecular mechanisms underlying the cancer preventive effects of NSAIDs are not well understood, and this has been an active issue of research interest.

Commonly, prevention of cancer may be implemented

through several means, including cell cycle arrest, induction of apoptosis and inhibition of angiogenesis. One most widely accepted mechanism for the anticancer effect of NSAIDs is the reduction of prostaglandin synthesis by inhibiting COX activity[5-7]. However, the importance of COX inhibition for the anti-proliferative effects of NSAIDs is controversial at the present time, since NSAIDs also manifest growth inhibitory effects against colon cancer cell lines that do not express COX-1 or COX-2 enzymes[8-11]. A common mechanism of NSAID action appears to be the induction of apoptosis, although several *in vitro* studies in CRC cells have proposed that different molecular pathways may be affected by distinct types of NSAIDs[9,12-14]. To date, little is known about the COX-independent molecular targets of NSAID action in cancer cells.

The mitogen-activated protein kinase (MAPK) superfamily, including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, is involved in mediating the processes of cell growth and death[15,16]. JNK and p38 MAPK pathways are activated in response to chemicals and environmental stress[17], while the ERK cascade, activated by mitogenic stimuli, is critical for proliferation and survival[18]. Recent evidence indicates that the MAPK family proteins are important mediators of apoptosis induced by stressful stimuli[18,19]. JNK and p38 MAPK are collectively termed stress-activated protein kinases because they are activated by a variety of stress-related stimuli and also activated by chemotherapy drugs[20,21]. Activation of p38 MAPK distorts mitochondrial function *via* an increase in the ratio of Bax and anti-apoptotic (Bcl-2) members leading to an increased mitochondrial membrane permeability, the release of cytochrome c, and the activation of caspases[22]. Once activated, p38 regulates multiple cellular processes, including transcription, translation, cell cycle progression, and apoptosis. Schwenger *et al*[23] previously showed that sodium salicylate, the active component of aspirin, activated p38 to induce apoptosis. Also, aspirin activates the p38MAPK pathway, leading to the degradation of cyclin D1, nucleolar translocation of RelA, and apoptosis[24].

Sox genes encode transcription factors that possess strong homology to the high-mobility group (HMG box), which are homologous to sex-determining region of Y-chromosome in the HMG box. There are at least 30 Sox members expressed in many different cell types and tissues, and at multiple stages during development[25]. Sex-determining region Y-box 7 (Sox7), together with Sox17 and Sox18, belongs to the Sox group F subfamily. Sox7 encodes an HMG box transcription factor and has been implicated in parietal endoderm differentiation[26]. Our previous study demonstrated that the expression of SOX7 mRNA was frequently down-regulated in human colorectal cancer cell lines and in primary colorectal tumor tissues, and restoration of SOX7 induced colorectal cancer cell apoptosis, inhibited cell proliferation and colony formation[27].

Despite these available data, the mechanistic function of aspirin in inhibiting COX2 negative colorectal cancer cells awaits further investigation. In this study, we have examined the role of SOX7 in aspirin-mediated growth inhibition of COX2 negative SW480 cells, and we found that SOX7 is regulated by aspirin and the p38 MAPK pathway in SW480 cells. Our study has disclosed the involvement of SOX7 in aspirin-mediated growth inhibition of COX2 negative cancer cells, providing a new insight into the mechanism by which aspirin inhibits COX2 negative colorectal cancer.

MATERIALS AND METHODS

Cell lines and reagents

Human colorectal cancer cell line SW480 and human embryonic kidney HEK-293T cells were cultured in appropriate media with 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin, and kept in a humidified atmosphere of 20 mL/L CO_2. Genomic DNA was extracted using the standard Proteinase-K method. Total RNA was extracted by using the Trizol reagent (TAKARA).

Drug treatment

Aspirin (Sigma, St. Louis, MO) was dissolved in 1 mol/L Tris-HCl (pH 7.5) to a stock concentration of 1 mol/L and the pH adjusted to 7.2 with 4 mol/L HCl. SW480 cells were treated for 24, 48 and 72 h by adding various volumes of stock to obtain final concentrations of 1, 2 and 5 mmol/L of aspirin. Control cells were treated with an equivalent volume of Tris-HCl (pH 7.2).

The p38 inhibitor SB203580 (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mmol/L. SW480 cells were treated with 10 μmol/L SB203580 for 30 h. Control cells were treated with an equivalent volume of DMSO.

MTT assay

Cell proliferation was assessed by the MTT (Moto-nuclear cell direc cytotoxicity assay) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay. SW480 cells were plated at 1×10^3 cells/well on 96-well plates. After treatment with aspirin or transfection, 20 μL of MTT (5 mg/mL) was added to each well; the samples were incubated for 4 h at 37 ℃ and then subcultured in the medium with 100 μL DMSO. The absorbance of each well was determined at 492 nm. Survival percentage (%) was calculated relative to the control.

Plasmid constructs and transfection

SOX7 gene promoter (GenBank accession NM_031439) was cloned by PCR from the genome derived from normal human colorectal tissue using the following primers: 5'-CCCAAGCTTCTGCCGACTTTCATTCAGTAGGTG-3' (sense) and 5'-CCGCTCGAGGTAGGCTCCCAGCAGCGAAG-3' (antisense). To generate SOX7 promoter-luciferase (pGL3-SOX7-luc) construct, the PCR production was subcloned into the pGL3 enhancer vector *via* *Hin*dⅢ and *Xho*l Ⅰ sites. Short interfering RNA (siRNA) targeting SOX7 sequence (ACGCCGAGCTGTCGGATGG) was synthesized[27]. An oligonucleotide that represents the siRNA was cloned into the pSliencer4.1-CMV neo-vector (Ambion) between *Bam*H Ⅰ and *Hin*d Ⅲ sites following the manufacturer's instructions. c-Fos

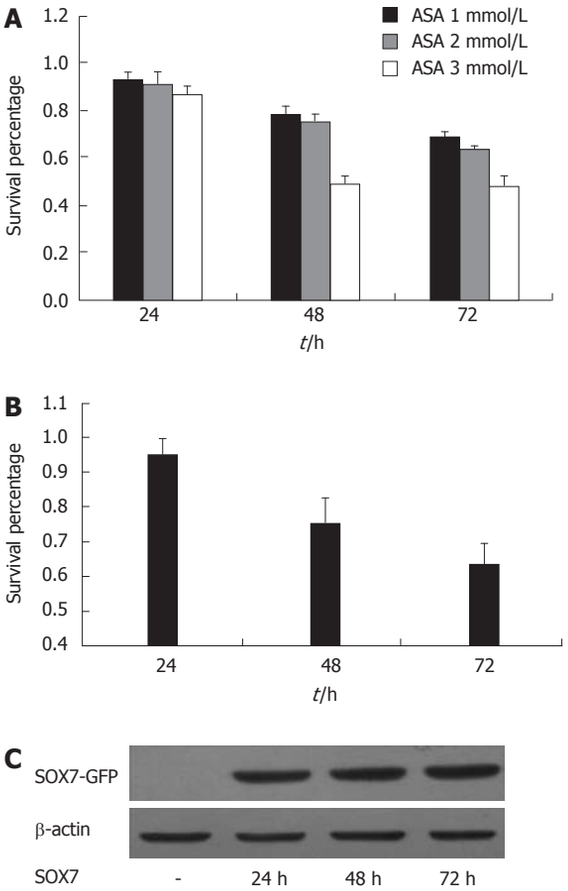

Figure 1 Aspirin and SOX7 inhibit the growth of SW480 cells. A: Dose- and time-dependent effects of aspirin on the growth rates of SW480 human colon cancer cells. SW480 cells were treated for 24, 48 or 72 h in aspirin-containing culture medium; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to estimate the survival percentage compared with untreated cells; B: Effect of SOX7 expression on cell proliferation. MTT assay was used to estimate the proliferation at different time points after transfection of SOX7 expression vector; C: Western blotting was used to verify the expression of SOX7 after transfection of SOX7 expression vector at indicated time points. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. ASA: Aspirin.

and c-Jun were gifts from Dr. Shizuo Akira (Osaka University, Japan); p38α was a gift from Dr. Roger David (University of Mass. Medical School); and SOX7 expression plasmid was previously constructed in this laboratory[27]. Plasmids were transfected using Lipofectamine™ 2000 (Invitrogen) follow the manufacturer's instructions.

Luciferase reporter assay

Reporter gene assays were done as previously described[27]. Briefly, 5×10^4 cells were seeded in 24-well tissue culture plates 24 h before transfection. The pGL3-SOX7-luc reporter vector was transfected at 500 ng/well and the *Renilla* luciferase control plasmid pREP7-RLuc was cotransfected at 50 ng/well as an internal control reporter. After treatment with indicated doses of aspirin or cotransfection with c-Fos and c-Jun expression vectors for 24 h, cells were washed and lysed in passive lysis buffer (Promega) and the transfection efficiency was normalized to the paired *Renilla* luciferase activity by using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction

For cDNA synthesis, 1 μg of total RNA was reverse transcribed using the RT-Systems supplied by Promega. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), and SYBR Green (TOYOBO) was used as a double-stranded DNA-specific fluorescent dye. The PCR primer sequences were as follows: SOX7: 5'-ACCAACGGGTCCCACAGA-3'(sense) and 5'-CCACTCAAGGCACAAGAAGG-3' (antisense)[27]; β-actin: 5'-TCGTGCGTGACATTAAGGAG-3' (sense) and 5'-ATGCCAGGGTACATGGTGGT-3' (antisense)[28].

Western blotting assay

Western blotting was performed as described previously[29]. The primary antibodies used were the mouse anti-SOX7 (1:1 000, R&D system) and mouse anti-β-actin (1:10 000, Sigma).

RESULTS

Aspirin and SOX7 inhibit the growth of SW480 human colorectal cancer cells

We first demonstrated that aspirin treatment results in a profound concentration- and time-dependent reduction in the proliferation rate of SW480 cells (Figure 1A). Our previous experiments showed that SOX7 was frequently down-regulated in human colorectal cancer cell lines including SW480[27]. In order to investigate the relationship between SOX7 and aspirin, we tested the effect of SOX7 on the growth of SW480 cells, and we found that restoration of SOX7 inhibits SW480 cell proliferation (Figure 1B and C).

SOX7 is induced by aspirin in SW480 colorectal cancer cells

Since both aspirin and SOX7 were able to inhibit the growth of SW480 cells, we sought to examine whether SOX7 can be induced by aspirin in SW480 cells. Our reporter gene experiments demonstrated that aspirin upregulated the activities of SOX7 promoter, and a dose of 1 mmol/L aspirin was sufficient to induce SOX7 promoter activity (Figure 2A). This dose was then used in the later experiments throughout the study. RT-PCR and Western blot assays showed that the mRNA (Figure 2B) and protein (Figure 2C) levels of SOX7 were both upregulated by 1 mmol/L aspirin. These data indicate that the SOX7 expression is induced by aspirin in SW480 cells.

SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer cells

The above data indicate that both aspirin and SOX7 inhibit the growth of SW480 cells, and aspirin upregulates the expression of SOX7 in SW480 cells. We next intended to determine whether SOX7 played a role in aspirin-inhibited growth of SW480 cells. First, we constructed a SOX7 siRNA plasmid and tested the interference efficiency of this plasmid. As shown in Figure 3A, transfection of SW480 cells with SOX7 siRNA plasmid resulted

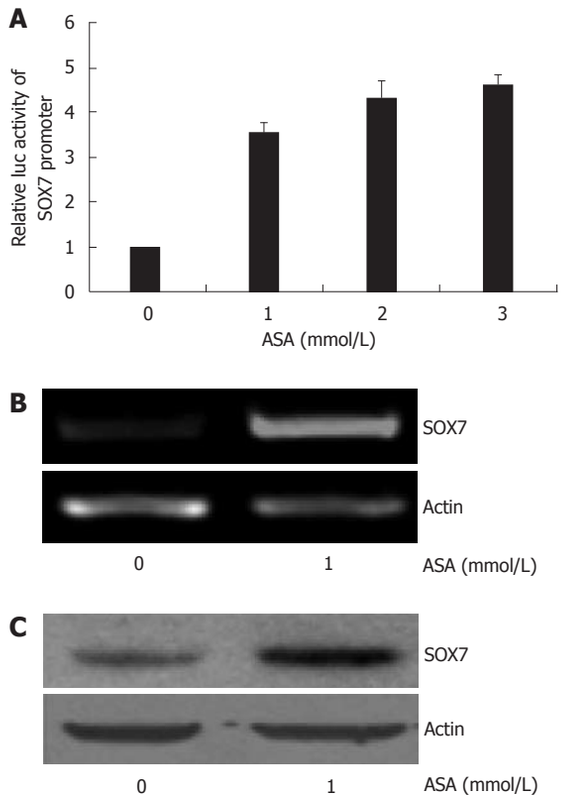

Figure 2 Aspirin upregulates SOX7 expression in SW480 cells. A: SW480 cells were transfected with pGL3-SOX7-luc plasmid, and relative luciferase activity was determined after treatment with different doses of aspirin for 24 h; B and C: Reverse transcription-polymerase chain reaction (B) and Western blotting (C) detected the expression of SOX7 after treatment with aspirin for 30 h; β-actin was used as the internal reference. ASA: Aspirin.

in a significant reduction in the SOX7 protein expression induced by aspirin, confirming the effective interference of this SOX7-siRNA. We then transfected SOX7 siRNA plasmid into SW480 cells, and we found that interference of SOX7 expression restored the growth rate of the aspirin-inhibited SW480 cells (Figure 3B). This indicated that SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells.

Aspirin induces SOX7 expression through the p38MAPK pathway in SW480 colorectal cancer cells

We and others have shown that the p38 MAPK pathway can be activated by aspirin (data not shown)[24]. We thus sought to examine whether the p38 MAPK pathway is involved in aspirin-induced SOX7 expression. We demonstrated that SOX7 mRNA is significantly upregulated upon the overexpression of p38α in SW480 cells (Figure 4A). In contrast, in HEK-293T cells that express relatively high levels of SOX7, inhibition of p38 by SB203580 reduced the mRNA level of SOX7 (Figure 4B). We further examined the effects of the p38 inhibitor SB203580 on aspirin-induced SOX7 expression, and we discovered that chemical inhibition of p38 substantially abrogates the aspirin-induced upregulation of SOX7. We also found that upon the inhibition of the p38 MAPK pathway by SB203580, the upregulation of SOX7 by aspirin is counteracted at

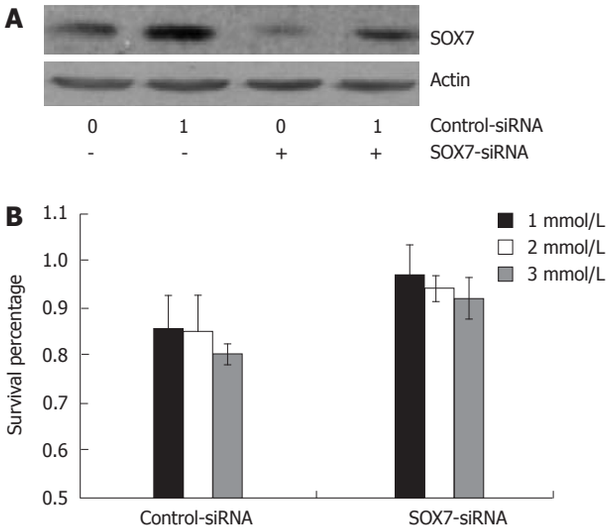

Figure 3 Knockdown of SOX7 counteracts the growth inhibitory effect of aspirin in SW480 cells. A: Western blotting verification of the interfering efficiency of SOX7 Short interfering RNA in aspirin-treated SW480 cells. β-actin was used as the internal reference; B: Effect of SOX7 knockdown on SW480 cell proliferation after treatment with different doses of aspirin for 48 h by moto-nuclear cell direc cytotoxicity assay assay. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

both mRNA and protein levels (Figure 4C and D). In addition, our data show that the AP1 transcription factors c-Jun and c-Fos upregulate SOX7 promoter activities in SW480 cells (Figure 4E).

DISCUSSION

The primary purpose of this study was to shed insights into the complex mechanistic action of aspirin in colorectal cancer inhibition and prevention. The results of the study show that SOX7 is upregulated by aspirin, and that SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells. Moreover, we provided evidence that aspirin induces SOX7 expression through the p38 MAPK pathway.

Recently, aspirin and related NSAIDs have attracted considerable research attention as the compounds that might be of potential benefit in the chemoprevention of cancer[2]. However, the molecular mechanisms by which aspirin and related NSAIDs inhibit tumor formation and growth have largely remained unsolved; and hence the utilization of these compounds in cancer therapeutics is still a substantially disputed issue. One potential mechanism underlying the action of NSAIDs involves the inhibition of COX activity[5]; but there is evidence that in COX negative colorectal cancer SW480 cells, aspirin is also able to inhibit the growth of the cells[8]. Goel *et al*[30] reported that aspirin could act through COX-independent mechanisms that resulted in an increased expression of DNA mismatch repair proteins and subsequent apoptosis in SW480 cells. Our previous data suggested that the *SOX7* gene may play a crucial role in colorectal cancer development as a tumor suppressor[27]. The data from the present study show that SOX7 is upregulated by aspirin in SW480

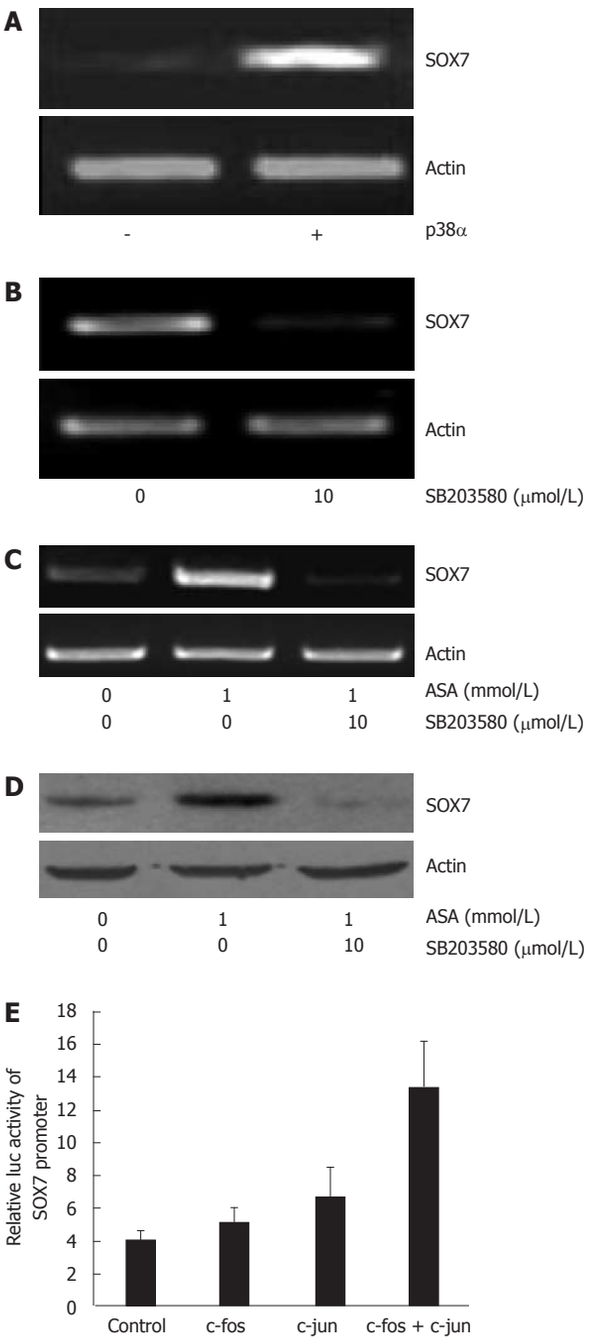

Figure 4 Role of the p38MAPK pathway in aspirin-induced SOX7 expression. A: HEK 293T cells were transfected with p38α expression vector; reverse transcription-polymerase chain reaction (RT-PCR) was used to detect SOX7 mRNA level 30 h after transfection; B: SW480 cells were treated with 10 μmol/L SB203580 for 30 h and SOX7 mRNA level was measured by RT-PCR; C: SW480 cells were treated with aspirin or aspirin combined with SB203580 for 30 h and SOX7 mRNA level was measured by RT-PCR; D: Cells were treated as in Figure 4 and SOX7 protein level was detected by Western blot; E: SW480 cells were transfected with pGL3-SOX7-luc plasmid and various expression vectors as indicated for 24 h and relative luciferase activity was examined. ASA: Aspirin.

cells and that it is involved in aspirin-mediated growth inhibition of SW480 cells (Figures 2 and 3), implying that SOX7 may be a target for aspirin in COX-independent cancer cells.

The p38 MAPK pathway plays pivotal roles in regulation of inflammation, proliferation, and cell death. Activation of the p38 pathway is generally mediated by conditions of cell stress and culminates in phosphorylation of p38 on a conserved regulatory domain by the upstream kinases MKK3 and MKK6[31]. In COX negative colorectal cancer SW480 cells, aspirin can activate the p38 MAPK pathway[24]. However, the mechanisms that are involved in this process need to be future investigated. Moreover, in the process of mouse preimplantation development, inhibition of the p38 MAP kinase pathway resulted in the suppression of SOX7 expression, implying that SOX7 may be a target gene of the p38 MAPK pathway[32]. Our experiments in this study indeed prove that the chemical inhibition of p38 substantially abrogates the upregulation of SOX7 upon aspirin treatment (Figure 4). These results indicate that SOX7 is regulated by aspirin and the p38 MAPK pathway in COX negative colorectal cancer cells.

The AP1 transcription complex consists of homodimers and heterodimers of the members from the fos (c-fos, fosB, fra-1 and fra-2) and jun (c-jun, junB and junD) families, and the complex activates target genes by binding with high affinity to particular DNA cis-elements, i.e., the 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE)[33]. The AP1 activity is regulated at the transcriptional and post-translational levels by several external stimuli, mainly involving MAPK cascades[34]. Our Luciferase reporter assays showed that SOX7 promoter activity was moderately upregulated upon the overexpression of c-fos or c-jun, but markedly upregulated upon the simultaneous overexpression of both c-fos and c-jun (Figure 4E), indicating that c-fos and c-jun may form a dimer to regulate SOX7 promoter, though the details need to be further studied.

In summary, the experimental data presented in this report demonstrate that SOX7 is upregulated by aspirin and is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells. The regulation of SOX7 by aspirin is implemented through the p38MAPK pathway. Overall, this study will help to advance our understanding of the mechanisms of action of aspirin in inhibiting COX-independent colorectal cancer cells.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide. Aspirin has been implicated to prevent CRC. The p38MAPK pathway is activated in aspirin-mediated apoptosis in a number of cancer cells. The sex-determining region Y-box 7 (Sox7) is a member of the high mobility group (HMG) transcription factor family, essential for embryonic development and endoderm differentiation.

Research frontiers

Aspirin is a nonsteroidal anti-inflammatory drug (NSAID), which has been implicated in preventing human colorectal cancer (CRC). However, the molecular mechanisms underlying the cancer preventive effects of NSAIDs are not well understood, and this has been an active issue of research interest. One most widely accepted mechanism for the anticancer effect of NSAIDs is the reduction of prostaglandin synthesis by inhibiting COX activity. To date, little is known about the COX-independent molecular targets of NSAID action in cancer cells.

Innovations and breakthroughs

The experimental data presented in this report demonstrate that SOX7 is upregulated by aspirin and is involved in aspirin-mediated growth inhibition of COX-independent human colorectal cancer SW480 cells. The regulation of SOX7 by aspirin is implemented through the p38MAPK pathway. Overall, this study will help to advance our understanding towards the mechanisms of action of aspirin in inhibiting COX-independent colorectal cancer cells.

Applications

By understanding how SOX7 is involved in aspirin-mediated growth inhibition of COX-independent human colorectal cancer cells, this study may represent a future gene target strategy for patients with COX-independent colorectal cancer.

Terminology

SOX7 is an HMG box transcription factor and has been implicated in parietal endoderm differentiation; recent reports have referred to the role of SOX7 in tumor suppression. mitogen-activated protein kinase (MAPK) is the mitogen-activated protein kinase superfamily, which includes the extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 MAPK, and is involved in mediating the processes of cell growth and death. Recent evidence indicates that the MAPK family proteins are important mediators of apoptosis induced by stressful stimuli.

Peer review

The authors examined the role of SOX7 in aspirin-mediated growth inhibition of COX2 negative SW480 cells, and found that SOX7 was regulated by aspirin and the p38 MAPK pathway in SW480 cells. The present results disclose the involvement of SOX7 in aspirin-mediated growth inhibition of COX2 negative cancer cells, providing a new insight to the mechanism as to how aspirin inhibits COX2 negative colorectal cancer.

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CASE REPORT

Acute liver failure caused by drug-induced hypersensitivity syndrome associated with hyperferritinemia

Masayuki Miyazaki, Masatake Tanaka, Akihiro Ueda, Tsuyoshi Yoshimoto, Masaki Kato, Makoto Nakamuta, Kazuhiro Kotoh, Ryoichi Takayanagi

Masayuki Miyazaki, Masatake Tanaka, Akihiro Ueda, Masaki Kato, Kazuhiko Kotoh, Ryoichi Takayanagi, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, 812-8582 Fukuoka, Japan
Tsuyoshi Yoshimoto, Makoto Nakamuta, Department of Gastroenterology, Kyushu Medical Center, National Hospital Organization, 812-8582 Fukuoka, Japan
Author contributions: Miyazaki M wrote the paper; Tanaka M, Ueda A, Yoshimoto T and Nakamuta M managed medical treatment of this case; Kato M and Kotoh K analyzed the data; Takayanagi R supervised the paper.
Correspondence to: Masayuki Miyazaki, MD, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. mmasayuk@intmed3.med.kyushu-u.ac.jp
Telephone: +81-92-6425282 Fax: +81-92-6425287
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Abstract

Drug-induced hypersensitivity syndrome (DIHS) is a severe reaction usually characterized by fever, rash, and multiorgan failure, occurring 2-6 wk after drug introduction. It is an immune-mediated reaction involving macrophage and T-lymphocyte activation and cytokine release. A 54-year-old woman was diagnosed with rheumatic arthritis and initiated salazosulfapyridine by mouth. About 10 d later, she had a high fever, skin rash and liver dysfunction. She was admitted to hospital and diagnosed with a drug eruption. She was treated with oral prednisolone 30 mg/d; however, she developed high fever again and her blood tests showed acute liver failure and cytopenia associated with hyperferritinemia. She was diagnosed with acute liver failure and hemophagocytosis caused by DIHS. She was transferred to the Department of Medicine and Bioregulatory Science, Kyushu University, where she was treated with arterial steroid injection therapy. Following this treatment, her liver function improved and serum ferritin immediately decreased. We hypothesized that an immune-mediated reaction in DIHS may have generated over-activation of macrophages and T-lymphocytes, followed by a cytokine storm that affected various organs. The measurement of serum ferritin might be a useful marker of the severity of DIHS.

Key words: Acute liver failure; Drug-induced hypersensitivity syndrome; Ferritin; Human herpes virus 6; Macrophage

Peer reviewers: Marko Duvnjak, MD, Department of Gastroenterology and Hepatology, Sestre Milosrdnice University Hospital, Vinogradska cesta 29, 10000 Zagreb, Croatia; Satoru Kakizaki, MD, PhD, Assistant Professor, Department of Medicine and Molecular Science, GunmaUniversity, Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan

Miyazaki M, Tanaka M, Ueda A, Yoshimoto T, Kato M, Nakamuta M, Kotoh K, Takayanagi R. Acute liver failure caused by drug-induced hypersensitivity syndrome associated with hyperferritinemia. *World J Gastroenterol* 2011; 17(44): 4928-4931 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4928.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4928

INTRODUCTION

Drug-induced hypersensitivity syndrome (DIHS) is one of the most severe drug eruptions, and is similar to toxic epidermal necrolysis and Stevens-Johnson syndrome[1]. It is characterized by maculopapular rash, high fever (≥ 38 ℃), hepatic dysfunction, leukocytosis with eosinophilia, and an increased number of atypical lymphocytes usually appearing 2-6 wk after exposure to certain drugs[2]. Although the pathology of DIHS remains unknown, in-

volvement of human herpes virus 6 (HHV-6) has recently been suggested[3,4]. DIHS is an immune-mediated reaction involving macrophage and T-lymphocyte activation and cytokine release associated with HHV-6 reactivation. However, there is no consensus on its etiology[5,6]. On the other hand, overactivation of macrophages is crucial for the development of other diseases, including hemophagocytic syndrome[7]. In patients with hemophagocytic syndrome, increased serum ferritin levels are thought to be secreted by activated macrophages[8]. However, to our knowledge, there are few reports that examine the correlation between the severity of DIHS and serum ferritin levels. In this report, we describe a case of acute liver failure (ALF) caused by DIHS associated with hyperferritinemia.

CASE REPORT

In January 2009, a 54-year-old woman presented to a clinic with morning stiffness in her hands. She was diagnosed with rheumatic arthritis and started treatment with oral salazosulfapyridine at 1 g/d on January 21. She developed a rash on her upper and lower extremities on February 2, with a fever of 37.6 ℃ on February 5. She presented to the clinic and discontinued salazosulfapyridine. Her blood test showed liver enzyme elevation [aspartate aminotransferase (AST), 50 U/L; alanine aminotransferase (ALT), 65 U/L]. However, her skin rash worsened. She was admitted to the Department of Dermatology, Kyushu Medical Center, National Hospital Organization on February 13. On admission, she had multiple areas of exudative erythema on her trunk and limbs. Laboratory data showed a white blood cell count of 14 700/μL with 9.0% eosinophils. AST and ALT levels were 59 U/L and 112 U/L, respectively.

She was diagnosed with drug eruption on the basis of clinical symptoms and the results of laboratory data, and treated with oral prednisolone 30 mg/d. After treatment, her liver function and rash improved gradually. However, she developed a high fever again on February 17. Her blood test showed ALF and cytopenia. Laboratory data on February 20 were as follows: white blood cell count of 6200/μL with 2.0% eosinophils; hemoglobin, 10.8 g/dL; platelet counts, 63 000/mm^3; AST, 1849 IU/L; ALT, 1623 IU/L; PT-INR, 1.79. Ultrasonography showed moderate ascites. A bone marrow aspiration demonstrated an increased number of macrophages with hemophagocytosis. The patient was diagnosed with ALF and hemophagocytosis caused by DIHS. She was transferred to the Department of Medicine and Bioregulatory Science, Kyushu University, for treatment of ALF.

Laboratory data on admission were as follows: total bilirubin, 5.8 mg/dL; AST, 2121 IU/L; ALT, 2231 IU/L; alkaline phosphatase, 1283 IU/L; γ-glutamyl transpeptidase, 736 IU/L; ferritin, 14 270 ng/mL; PT-INR, 1.79. Viral markers were negative for hepatitis A-C, Epstein-Barr virus, and cytomegalovirus. Serum anti-HHV-6 immunoglobulin G (IgG) titer was normal, but HHV-6 DNA was positive. She had multiple areas of exudative erythema on her trunk and limbs (Figure 1) and swelling of cervical lymph nodes. Abdominal computed tomography showed edematous change of the portal vein and moderate ascites (Figure 2).

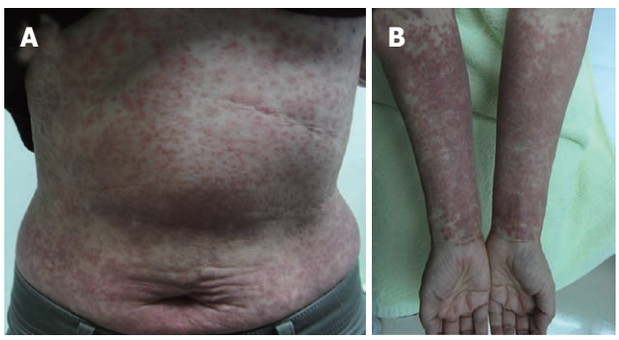

Figure 1 Multiple areas of exudative erythema were seen on admission. A: Body trunk; B: Forearm.

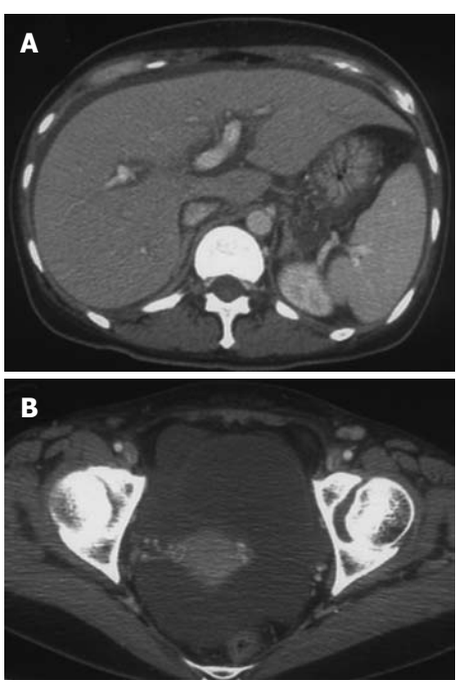

Figure 2 Abdominal computed tomography. A: Edema of portal vein; B: Moderate ascites.

Our diagnosis was DIHS based on high fever, rash, liver dysfunction, swelling of cervical lymph nodes, and eosinophilia. Hyperferritinemia in her blood samples led us to think that activated macrophages in the liver might play a key role in ALF. Therefore, we decided to use arterial steroid injection therapy to suppress macrophage activation seen in ALF[9].

A 5-frame catheter was inserted from the right femoral artery to the common hepatic artery and the tip of the catheter was set in the proper hepatic artery. After insertion of the catheter, 1000 mg of methylprednisolone was infused for 2 h per day. We also performed plasma exchange therapy on the first day of admission, because

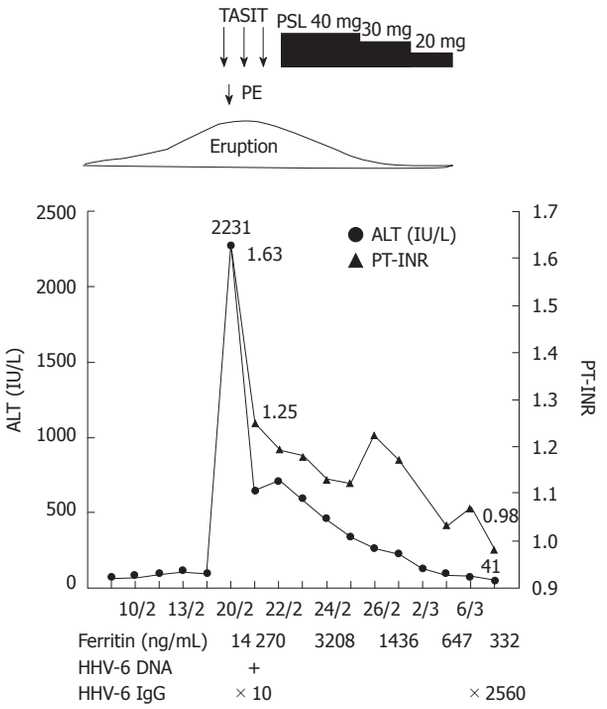

Figure 3 Clinical course of the patient. PE: Plasma exchange, TASIT: Transarterial steroid injection therapy.

the level of PT-INR was decreased. The arterial steroid injections were continued for 3 d, and the catheter was removed just after injection on the third day. Following arterial steroid injection therapy, her liver function improved immediately (Figure 3). Laboratory data on the fourth day of admission were as follows: AST 180 IU/L; ALT 597 IU/L; PT-INR, 1.18. After arterial steroid injection therapy, we started treatment with oral prednisolone at 40 mg/d and gradually decreased the dose. When liver dysfunction resolved, she was discharged from hospital (on March 3). In a serum sample taken 2 wk after hospitalization, anti-HHV-6 IgG titer had increased from 1:10 to 1:2560. She was diagnosed with typical DIHS according to criteria proposed by the Japanese Consensus Group; the patient had 7 items.

DISCUSSION

It is well-known that many patients with DIHS suffer liver injury and some progress to fatal liver failure. Until now, however, there has been no explanation of what causes ALF in some patients. Recently, it has been widely accepted that reactivation of herpes viruses such as HHV-6 and Epstein-Barr virus play a key role in the development of DIHS[3,4]. The question then becomes whether the reactivation of these viruses also contributes to the progression to ALF.

Hashimoto *et al*[10] evaluated clinical symptoms in 100 patients with DIHS and found that those with increasing serum anti-HHV-6 IgG titers suffered from severe organ involvement and a prolonged course of illness. In addition, they noted that flaring of symptoms, such as fever and hepatitis, was closely related to HHV-6 reactivation. However, none of the subjects in the study had ALF. In an investigation of case reports on the progression to ALF from DIHS, none indicated a correlation between viral reactivation and the development of ALF.

Since DIHS is triggered by some drugs, we should evaluate the possibility of drug-induced reactions that might directly harm hepatocytes and cause ALF. To our knowledge, there is no evidence that ALF is easily caused by specific drugs or in a dose-dependent manner. Therefore, we believe that the drugs causing DIHS do not directly injure numerous hepatocytes.

Some authors, speculating on the mechanism of progression to ALF, have indicated that DIHS develops through an immune-mediated reaction involving macrophages and T-lymphocytes[11]. In the last decade, several studies have suggested that activated intrahepatic macrophages play a key role in the development of ALF[12,13]. We also observed over-activation of intrahepatic macrophages in most of the patients with ALF in Japan[14]. One of the findings supporting this hypothesis is the markedly elevated serum ferritin concentration in those patients.

The patient presented in this report also had a markedly elevated serum ferritin concentration of over 20 000 ng/mL. One hypothesis is that intrahepatic over-activation of macrophages contributes to the development of ALF from DIHS as well as other etiologies. However, none of the past reports describing ALF from DIHS have referred to the elevation of serum ferritin concentration. To evaluate this hypothesis, cohort studies have focused on the correlation between the clinical course of patients with ALF from DIHS and the factors reflecting immune-mediated reactions.

Patients with DIHS can suffer not only from liver failure, but also various patterns of multiple organ failure, however, it remains unclear what induces these complications. We have hypothesized that an immune-mediated reaction in DIHS might generate over-activation of macrophages and T-lymphocytes, followed by a cytokine storm that affects various organs. In fact, this patient had elevated serum cytokines on admission (IL-6 10.8 pg/mL; IL-10 21 pg/mL). Such an immune reaction in bone marrow would result in pancytopenia, and liver involvement would cause liver failure. Both of these were seen in our patient.

It is well known that corticosteroids can suppress the activity of macrophages[15,16]. Therefore, it seems appropriate to use high-dose corticosteroids for DIHS. However, past reports show that corticosteroids are not always effective for ALF from DIHS. We speculate that intravenous injection of corticosteroids could be ineffective because the disturbance of hepatic microcirculation would prevent the drug from diffusing to the whole liver. Even in such a situation, injection *via* hepatic artery might be a useful option.

In this case report, we described a patient with ALF caused by DIHS associated with hyperferritinemia. In conclusion, we speculate that activated macrophages and

a cytokine storm were associated with DIHS. The measurement of serum ferritin might be a useful marker of the severity of DIHS.

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CASE REPORT

Pneumatosis cystoides intestinalis

Francesco Azzaroli, Laura Turco, Liza Ceroni, Stefania Sartoni Galloni, Federica Buonfiglioli, Claudio Calvanese, Giuseppe Mazzella

Francesco Azzaroli, Laura Turco, Liza Ceroni, Federica Buonfiglioli, Claudio Calvanese, Giuseppe Mazzella, Department of Clinical Medicine, Division of Gastroenterology, S.Orsola-Malpighi Hospital, University of Bologna, 40138 Bologna, Italy
Stefania Sartoni Galloni, Department of Emergency, General Surgery and Transplantation, S.Orsola-Malpighi Hospital, University of Bologna, 40138 Bologna, Italy
Author contributions: Azzaroli F, Turco L, Ceroni L, Buonfiglioli F, Calvanese C and Mazzella G all contributed to perform the research and to write the paper; Sartoni Galloni S performed the radiological study and contributed to data discussion.
Correspondence to: Francesco Azzaroli, Professor, Department of Clinical Medicine, S.Orsola-Malpighi Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy. francesco.azzaroli@unibo.it
Telephone: +39-51-6363888 Fax: +39-51-6364120
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Abstract

Pneumatosis cystoides intestinalis (PCI) is a rare condition that may be associated with a variety of diseases. The presenting clinical picture may be very heterogeneous and represent a challenge for the clinician. In the present paper we describe both a common and an uncommon clinical presentation of PCI and review the pertaining literature. Our cases confirm that, apart from asymptomatic cases, the clinical presentation of PCI may be widely different and suggest that a new onset of stipsis might be the presenting symptom. Diagnosis might be suggested by a simple X-ray of the digestive tract showing a change in the characteristics of the intestinal wall in two-thirds of these patients. However, one third of the patients do not have a suggestive X-ray and require a computed tomography (CT) scan/nuclear magnetic resonance that may reveal a thickened bowel wall containing gas to confirm the diagnosis and distinguish PCI from intraluminal air or submucosal fat. CT also allows the detection of additional findings that may suggest an underlying, potentially worrisome cause of PCI such as bowel wall thickening, altered contrast mucosal enhancement, dilated bowel, soft tissue stranding, ascites and the presence of portal air. Our results also point out that clinicians and endoscopists should be aware of the possible presentations of PCI in order to correctly manage the patients affected with this disease and avoid unnecessary surgeries. The increasing number of colonoscopies performed for colon cancer screening makes PCI more frequently casually encountered and/or provoked, therefore the possible endoscopic appearances of this disease should be well known by endoscopists.

Key words: Pneumatosis cystoides intestinalis; Pneumoperitoneum; Treatment; Hyperbaric oxygen; Endoscopy

Peer reviewer: Nikolaus Gassler, Professor, Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

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INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a rare disease characterized by the presence of gaseous cysts containing nitrogen, hydrogen and carbon dioxide[1] in the intestinal wall that may be iatrogenic[2-5] or associated with a wide variety of conditions[6-9].

In particular, the cysts are located beneath the serosa and mucosa of the intestine with an increase, in recent years, of cases of colonic localization due to an increase in the number of examinations with barium and colonoscopies[10].

Jamart[11] in a study of 919 cases in 1979 found a prevalence of 42% for ileal localization, and 36% for the colon, in the remaining 22% of cases both the small and the large intestine were involved.

The exact etiology of the disease is still unknown. PCI may appear in association with ileal surgery[12], colonoscopies[5], chronic pulmonary disease[13], connective tissue disorders[14] and ingestion of sorbitol[15] or lactulose[16].

Various theories have been proposed: mechanical, bacterial and pulmonary. According to the mechanical theory, the bowel gas is pushed through a mucosal defect into lymphatic channels and is then distributed distally by peristalsis[17]. This may happen secondarily to a bowel obstruction that may be caused by trauma, surgery and colonoscopy leading to increased intraluminal pressure[18] and this could explain the association between these maneuvers and PCI. However this theory does not explain the high content of hydrogen present in the cysts[19].

The bacterial theory proposes that submucosal localization of fermenting *Clostridia* and *Escherichia Coli* leads to the production of gas which is retained by the submucosa and lymphatic channels. In fact, in animal experiments the introduction of bacteria in the gut wall by injection causes the pneumatosis and these cysts have a high content of hydrogen[13]. This theory is also supported by the resolution of pneumatosis with the use of metronidazole for bacterial overgrowth[20].

The pulmonary theory is demonstrated in patients with asthma and chronic bronchitis and argues that the gas freed by the rupture of the alveoli, travels through the mediastinum into the retroperitoneal space and then comes through the perivascular spaces in the intestinal wall[21].

Some recent reports[22] show an association between PCI and treatment with alpha-glucosidase inhibitor. The explanation would be the fermentation of carbohydrates by the intestinal bacterial flora with production of intestinal gas. The absorption of these carbohydrates is inhibited by αGI. Here we describe two cases of pneumatosis cystoides intestinalis.

CASE REPORT

Case 1

A 49 years old male (S.A.) presented to the gastroenterology outpatient clinic for abdominal pain. The pain was crampy and diffuse with no clear localization in the abdomen and had no clear relationship with meals or evacuation. Bowel habit was characterized by chronic constipation; the abdomen showed no relevant physical findings, in particular there was no palpable mass. The patient also suffered from a chronic obstructive pulmonary disease and had finger clubbing. Routine biochemical examinations were within normal values as well as inflammation indices. The physician prescribed a colonoscopy revealing melanosis coli, a sessile polyp 2 cm in diameter and two sessile formations with a large base and a reddened but regular overlying mucosa (Figure 1). The formations appeared soft when touched with a closed biopsy forceps and collapsed when biopsied suggesting the presence of air. The endoscopist performed multiple biopsies along the colon. The pathologist described the presence of optically empty spaces in the biopsies confirming the hypothesis of pneumatosis cystoides intestinalis. The patient was then referred to the pneumologist for oxygen therapy. At a subsequent colonoscopy the air cysts appeared reduced in volume and the patient referred pain reduction to the endoscopist. The patient did not present to subsequent control visits.

Case 2

N.F. 44 years old presented to the GI Unit for the re-evaluation of a known celiac disease. Despite adequate gluten free diet the patient complained of a worsening of his symptoms dominated by stipsis and abdominal distension. The patient suffered from recurrent subocclusive episodes and referred recent tetanic crisis from hypocalcemia on treatment with calcium and vitamin D. Biochemical analysis on admission revealed a megaloblastic anemia related to a Vitamin B12 deficiency and hypomagnesemia. Physical examination was consistent with abdominal distension and revealed hyperreflexia of the extremities. The patient had no fever nor any other remarkable finding.

The first postulated hypothesis, based on patient history was the complication of a celiac disease with intestinal lymphoma. A plain X ray of the abdomen revealed distension caused by dilatation of colonic loops leading to diaphragm elevation. The patient was then prescribed an upper GI endoscopy and an abdomen computed tomography (CT). The esophagogastroduodenoscopy revealed a normal endoscopic appearance of the mucosa and orientated biopsies were done in the distal duodenal mucosa. Histological examination revealed normal villi with a mild, non significant, lymphocytic infiltrate (below 25%) consistent with a celiac disease in remission responding to gluten free diet. The abdomen CT scan showed a complex picture including fecal stasis in the colon, severe distension of the large bowel with the presence of free air under the diaphragm (Figure 2A), and small air bubbles in the rectum wall (Figure 2B). Despite the radiologic picture, the patient was feeling well, with distended but tractable abdomen and no Blumberg sign. Therefore, the patient was kept fasting and received liquids and antibiotics iv. Subsequent radiologic evaluation of the small and large bowel, using gastrografin as contrast media, revealed no perforation suggesting that the free air might have come from the rupture of a subserosal air cyst. The patient then started hyperbaric therapy (5 treatments of 90 min: 2.5 atmospheres, 75 min of oxygen respiration divided in 3 cycles) with prompt amelioration of bowel movements and subjective feeling. The patient became asymptomatic and apyretic with normal bowel movements and was dismissed with antibiotic therapy.

DISCUSSION

There is no characteristic clinical presentation of pneumatosis. Patients may be asymptomatic or complain of

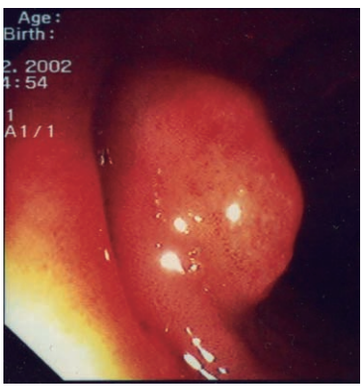

Figure 1 This photograph shows a typical endoscopic appearance of a larger cysts with a reddened overlying mucosa.

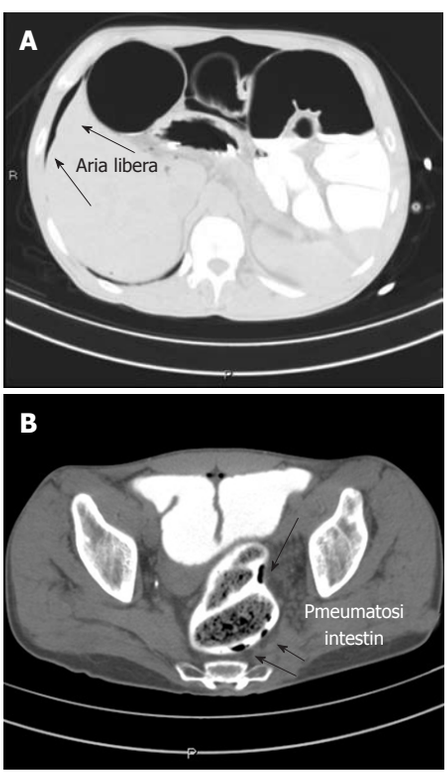

Figure 2 Computed tomography scan image. A: Presence of free air (arrows) in the abdomen; B: Presence of air in the bowel wall (arrows).

pain and abdominal distension, diarrhea and rectal blood loss with a mortality rate that may reach 75%[23]. Apart from the cases associated with chronic intestinal pseudo-obstruction[20,24,25], the majority of cases reported in the literature present with diarrhea; in the present manuscript we described two cases of PCI that presented with stipsis. The first was diagnosed after a colonoscopy that put the suspect of PCI, while the second required a CT scan because of the atypical presentation and the misleading anamnesis. In fact, the history of celiac disease lead the clinicians to hypothesize a complication of the pre-existent disease more than the onset of a new pathology. It is difficult to say whether the motility defects are a cause or a result of the pathologic condition and we are not aware of any longitudinal study evaluating such a question. However, on a purely hypothetical basis, it seems more reasonable to think that motility defects are secondary to PCI or to underlying pathological process that may have lead to PCI (i.e., ischemia, diverticular disease, *etc.*).

Colonoscopy is frequently requested to exclude colonic lesions. The endoscopic appearance of PCI is typically dual: multiple white small cysts coupled to a sub-atrophic mucosa or larger cysts (up to 3 cm) with a reddened overlying mucosa[26]. The cysts usually collapse when biopsied. Nowadays, given the increasing number of colonoscopies performed because of the colon cancer screening programs, the endoscopists should be aware of the endoscopic appearance of this rare pathology. In fact, some patients may be asymptomatic and in such cases the clinical suspect may rely on the endoscopist performing the procedure. In our case 1, the endoscopist cautiously biopsied the cyst because of his unusual appearance that was not suggestive of a typical polyp. This allowed a confirmation of the suspect and avoided an unnecessary snare polypectomy with the related costs and complications.

A simple X-ray of the digestive tract may show a change in the characteristics of the intestinal wall in two-thirds of these patients leading to further investigations. However, one third of the patients do not have a suggestive X-ray and require a CT scan/magnetic resonance imaging, showing a thickened bowel wall containing gas to confirm the diagnosis[27]. Suggestive images on plain radiography comprise different pattern of radiolucency: linear, small bubbles or collection of larger cysts[27]. CT is more sensitive than plain radiography in distinguishing PCI from intraluminal air or submucosal fat. In fact, CT more easily visualizes the presence of air in the bowel wall. Furthermore, CT allows the detection of additional findings that may suggest an underlying, potentially worrisome cause of PCI, i.e., bowel wall thickening, altered contrast mucosal enhancement, dilated bowel, soft tissue stranding, ascites, and the presence of portal air[28].

The intestinal pneumatosis may experience various complications, in particular, Goel *et al*[29] described the complications of pneumatosis of the small intestine which may be intestinal or extra-intestinal. Intestinal complications are obstruction caused by the cysts (i.e., fecal impaction) and perforation from stercoral ulceration. The extra-intestinal complications are adhesions or compression of adjacent structures by large masses of cysts.

For the resolution of these complications surgical treatment is often required because sometimes we have a picture of pneumoperitoneum due to rupture of cysts.

To determine the need for surgical therapy Knechtle *et al*[23] found a correlation between the clinical presentation, the need for surgery and the final outcome. It is necessary to evaluate six physical parameters, like pain, diarrhea, fever, tenderness, rectal blood loss and hypotension, and their severity coupled to clinical laboratory tests including white blood cell count, aspartate aminotransferases, alanine aminotransferases, alkaline phosphatase, pH, bicarbonate, lactic acid and amylase.

Surgical therapy is still a second-line therapy, chosen especially for complications, the first approach is oxygen therapy. It is also our opinion that the clinical decisions should not rely only on the radiologic picture of pneumoperitoneum, but should be coupled to the clinical symptoms (i.e., the positivity of the Blumberg sign). In fact, when the mucosa is intact surgery may be avoidable as in our case 2.

The rationale of oxygen treatment is based on increasing partial pressure of oxygen in blood and thus increasing the pressure gradient of the gas in the cysts. Cysts release gases contained within them and refills with oxygen which is then metabolized leading to resolution[26].

Oxygen therapy can be made through humidified oxygen administered by Venturi mask (6 L/min) or nasal cannula (4 L/min). However, treatment with oxygen at high doses can be toxic. The patient may experience a narcotic effect and therefore lung function should be monitored closely (during therapy) by measuring the vital capacity, daily blood gas estimations and chest radiography. A decrease in lung vital capacity can be a early parameter of oxygen toxicity[30].

To reduce the duration of oxygen administration hyperbaric oxygen can be used at a pressure of 2.5 atmosphere for up to 2 h a day [31]. To decrease the recurrence rate oxygen therapy should be continued until two days after the disappearance of cysts[32].

In conclusion, our cases confirm that the clinical presentation of PCI may be very heterogeneous and suggest that a new onset of stipsis might be the presenting symptom. Furthermore, it should be taken into account that the patients may also be totally asymptomatic. The clinicians and the endoscopist should be aware of the possible presentations of PCI in order to correctly manage the patients affected with this disease and avoid unnecessary surgeries. It is possible that with the increasing number of colonoscopies performed for colon cancer screening PCI is casually encountered and/or provoked, therefore the possible endoscopic appearances of this disease should be known.

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Satoshi Osawa, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan

Arturo Panduro, **MD, PhD, Head** of the Department of Molecular Biology in Medicine, Civil Hospital of Guadalajara Fray Antonio Alcalde/University of Guadalajara, Hospital No. 278 S.H., Guadalajara, Jalisco 44280, Mexico

Paul M Schneider, **MD , Professor** of Surgery, Department of Surgery, University Hospital Zurich, Raemistrasse 100, Zurich 8091, Switzerland

Andrada Seicean, MD, PhD, Third Medical Clinic Cluj Napoca, University of Medicine and Pharmacy Cluj Napoca, Romania, 15, Closca Street, Cluj-Napoca 400039, Romania

Eldon Shaffer, Professor of Medicine, Division of Gastroenterology, Department of Medicine, Health Science Centre, University of Calgary, 3330 Hospital Dr N.W., Calgary AB T2N4N1, Canada

Yoshitaka Takuma, **MD, PhD,** Department of Gastroenterology, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki, Okayama, 710-8602 Japan 0035407

Cesare Tosetti, **MD,** Department of Primary Care, Health Care Agency of Bologna, Via Rosselli 21, 40046 Porretta Terme (BO), Italy

Anthony T Yeung, BS, MS, PhD, Fox Chase Cancer Center, Room R404, 333 Cottman Avenue, Philadelphia, PA 19111-2497, United States

Cuong D Tran, **PhD, Research Fellow, Affiliate Lecturer,** University of Adelaide, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006, Australia

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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]

Books

Personal author(s)

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Chapter in a book (list all authors)

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Statistical data

Write as mean ± SD or mean ± SE.

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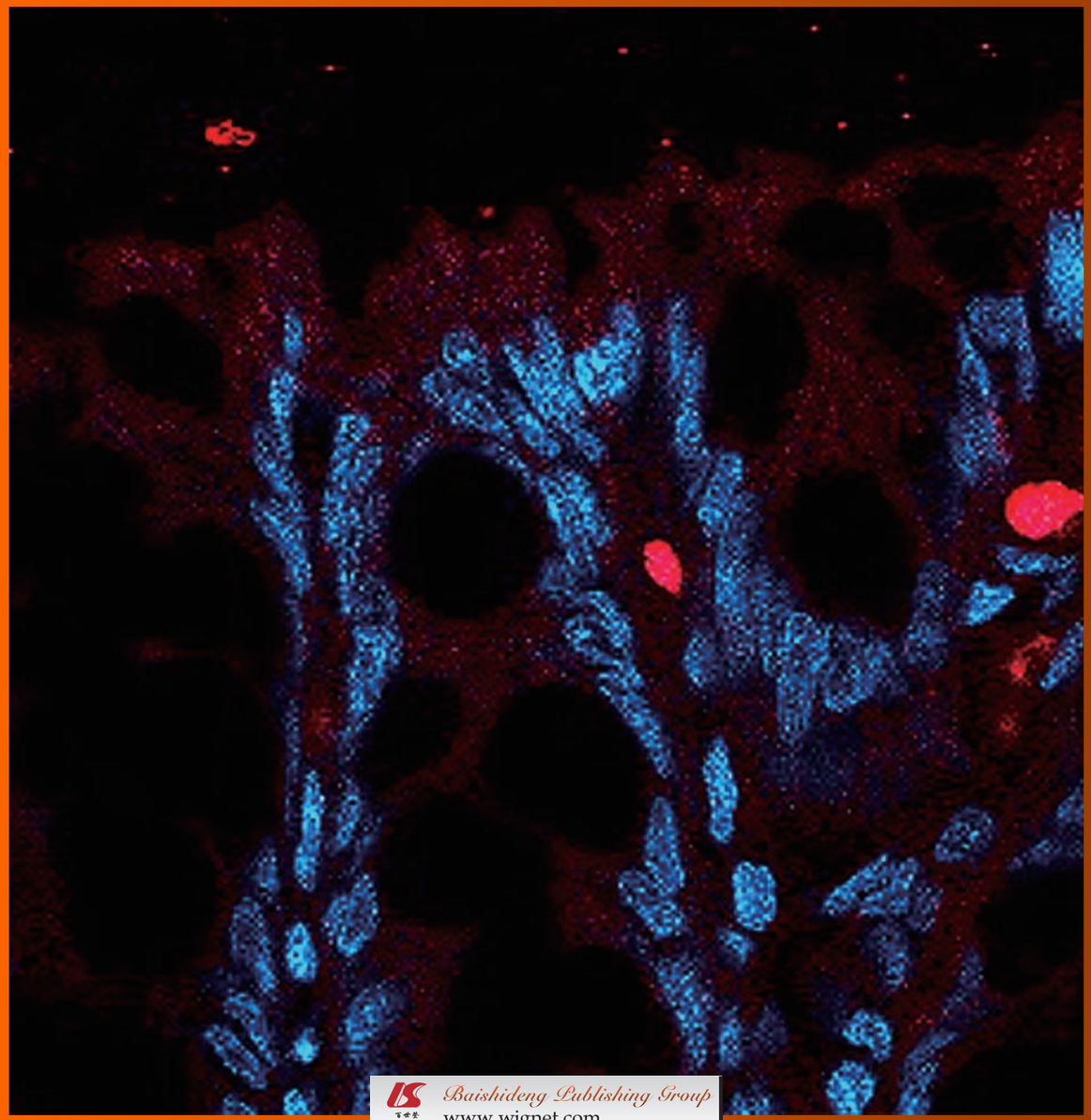

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EDITORIAL

Genetic interactions and modifier genes in Hirschsprung's disease

Adam S Wallace, Richard B Anderson

Adam S Wallace, Richard B Anderson, Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria 3010, Australia
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Correspondence to: Richard B Anderson, PhD, Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria 3010, Australia. rba@unimelb.edu.au
Telephone: +61-03-83445783 Fax: +61-03-93475219
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Abstract

Hirschsprung's disease is a congenital disorder that occurs in 1:5000 live births. It is characterised by an absence of enteric neurons along a variable region of the gastrointestinal tract. Hirschsprung's disease is classified as a multigenic disorder, because the same phenotype is associated with mutations in multiple distinct genes. Furthermore, the genetics of Hirschsprung's disease are highly complex and not strictly Mendelian. The phenotypic variability and incomplete penetrance observed in Hirschsprung's disease also suggests the involvement of modifier genes. Here, we summarise the current knowledge of the genetics underlying Hirschsprung's disease based on human and animal studies, focusing on the principal causative genes, their interactions, and the role of modifier genes.

Key words: Neural crest; Enteric nervous system; Hirschsprung's disease; Aganglionosis; Modifier genes

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INTRODUCTION

The enteric nervous system (ENS) comprises neurons and glial cells within the wall of the gastrointestinal tract. It is responsible for regulating intestinal motility, immune function, luminal secretions, and blood flow[1]. During development, the ENS arises from a highly migratory population of cells, called the neural crest[2-4]. The neural crest arises as a result of an epithelial to mesenchymal transition during the formation of the neural tube. Two separate populations of neural crest, arising from different axial levels, contribute to the ENS - the vagal level (defined as the post-otic hindbrain adjacent to somites 1-7) and the sacral level (caudal to somite 24 in mice and humans)[5,6]. Vagal neural crest cells migrate ventrally to the presumptive foregut, and then into and along the entire length of the gastrointestinal tract, a process that takes five days in mice (embryonic day E9.5-E14.5)[7] and three weeks in humans (during 4th-7th wk of gestation)[8].

The formation of a functional ENS requires the coordination of many processes, including survival, migration, proliferation, and differentiation of precursor cells within the gastrointestinal tract. Failure of neural crest cells to fully colonise the entire length of the gastrointestinal tract results in a region of gut that lacks enteric neurons, called an "aganglionic zone", which affects a variable length of the distal most bowel. As enteric neurons are essential for motility, the aganglionic zone remains

tonically constricted, preventing the passage of faecal material. In humans, this condition is known as Hirschsprung's disease (HSCR) and occurs in approximately 1:5000 live births[9]. HSCR can either be familial or sporadic, and is subdivided into short or long segment HSCR (S-HSCR and L-HSCR), which refers to the extent of the aganglionic zone[10]. The less severe S-HSCR (about 80% of cases) is more common than L-HSCR (about 20% of cases) and displays a more pronounced gender bias (4:1 male:female in S-HSCR compared to 1.2:1 male:female in L-HSCR)[11]. HSCR may present as an isolated condition (about 70% of cases) or as part of a syndrome, such as Mowatt-Wilson or Waardenburg Shah type 4 (Table 1). Although HSCR is normally detected soon after birth, there have been reports of HSCR being identified in patients after childhood[12]. Failure to treat HSCR is often fatal because of malnutrition or sepsis following rupture of the bowel. Present treatment involves surgery to remove the affected portion of bowel and re-anastomosis of the remaining gut to the anus. Although refinements to surgical techniques have improved patient outcome, post-operative complications persist in a large number of patients[13,14].

In the majority of cases, the genetics of HSCR are complex and non-Mendelian in nature[15]. To date, more than a dozen genes have been identified as being associated with HSCR[9,16]. However, mutations in these genes account for only about 50% of all HSCR cases[17]. The phenotypic variability and incomplete penetrance observed in HSCR also suggests the involvement of modifier genes. The aim of this review is to summarise the current knowledge of the genetics underlying HSCR. We first discuss the principal causative genes and detail the interactions between these genes that alter the severity or incidence of HSCR. Finally, we discuss the accumulating evidence for the role of modifier genes in the development of HSCR.

GENES INVOLVED IN ENS DEVELOPMENT

Many of the genes associated with HSCR encode members of the Glial cell line-derived neurotrophic factor (GDNF)/RET- and ET-3/EDNRB-signalling pathways or transcription factors, such as *SOX10, PHOX2B* or *ZFHX1B*. Mutations in these genes have been shown to result in Hirschsprung's disease in humans (Table 1) or aganglionosis in mice (Table 2).

GDNF/RET-GFRα1

GDNF is a secreted protein and a distant member of the TGF-β superfamily[18]. GDNF binds to the glycosylphosphatidylinsoitol-linked receptor, GFRα1. The GDNF-GFRα1 complex then binds to and activates the transmembrane receptor tyrosine kinase, RET[19]. Mutations in genes encoding members of the GDNF/RET-GFRα1 signalling pathway account for about 50% of familial cases and around 30% of sporadic cases of HSCR[17]. Non-coding mutations in RET have also been proposed to increase susceptibility to HSCR[20-23]. In mice, *Gdnf* is expressed by the gut mesenchyme prior to the entry of neural crest cells[24]. *Ret* is expressed exclusively by neural crest-derived cells and *Gfrα1* is expressed by both crest-derived cells and the gut mesenchyme[25]. *Gdnf*-, *Gfrα1*- or *Ret*-null mice die within 24 hours of birth, and lack enteric neurons along the entire length of the gastrointestinal tract caudal to the stomach[25-27]. $Gdnf^{+/-}$ and $Ret^{+/-}$ mice are viable and do not exhibit aganglionosis[28].

RET is subject to alternative splicing and translated into two functional isoforms, RET51 and RET9, which differ in the number of amino acids at their C terminal end[29]. These isoforms are highly conserved between human and mouse[30]. Mice lacking the Ret51 isoform ($Ret^{9/9}$ mice) have enteric neurons along the entire length of the gastrointestinal tract, while mice lacking the Ret9 isoform ($Ret^{51/51}$ mice) suffer colonic aganglionosis and kidney hypodysplasia[31]. The phenotype of the $Ret^{51/51}$ mice is highly reminiscent of the colonic aganglionosis observed in patients with HSCR. Interestingly, the developing ENS in humans appears to be more sensitive to reduced RET signalling than that of the ENS in mice. *RET* mutations in humans act dominantly to give rise to HSCR, whereas ENS development is normal in *Ret* heterozygous mice[28]. In fact, it has recently been shown that a loss of around 60%-70% of *Ret* expression in mice is required to mimic the aganglionic phenotype observed in humans[32].

Targeted mutations in RET have identified signalling sites that are required for ENS development. Mutation of a putative protein kinase A phosphorylation site, which changes serine to alanine (Ret^{S697A}), results in aganglionosis of the distal colon[33]. Mutation of an intracellular docking site, which converts tyrosine to phenylalanine (Ret^{Y1062F}), induces total intestinal aganglionosis[34]. The mutation of cysteine to arginine (Ret^{C620R}), which is observed in some MEN2A/HSCR patients, has also been shown to result in total intestinal aganglionosis[35].

ENDOTHELIN SIGNALLING PATHWAY

Endothelin 3 (ET-3) is a secreted peptide, which is expressed by the gut mesenchyme[36]. ET-3 is initially expressed in an immature form before being processed to an active peptide by the enzyme, endothelin converting enzyme 1 (ECE1)[37,38]. ET-3 signals through the receptor Endothelin receptor B (EDNRB), which is expressed on migrating enteric neural crest cells[39].

Mutations in *ET3* and *EDNRB* account for around 5% of HSCR cases, whilst only a single case of *ECE1*-associated HSCR has been reported[40]. *ET3*- and *EDNRB*-associated HSCR can present as both syndromic (such as Wardenburg-Shah syndrome) and non-syndromic forms of HSCR. In mice, *lethal spotted* (*ls*) and *piebald lethal* (*sl*) are naturally occurring mutants of *Et-3* and *Ednrb* respectively, and lack enteric neurons in the distal bowel[37,41]. Although enteric neurons are absent only from the distal colon of *Et-3* and *Ednrb*-null mice, the migra-

Table 1 Genes associated with Hirschsprung's disease

Locus	Gene	Associated syndrome	Incidence	Penetrance	Inheritance	Ref.
10q11	*RET*	Non-syndromic HSCR	50% familial 30% sporadic	70% male 50% female	Dominant	82-84
5p13	*GDNF*	Non-syndromic HSCR	5 cases	Low	Dominant	85-89
13q22	*EDNRB*	Shah-Waardenburg Non-syndromic HSCR	5%	Low	Dominant or recessive	44,90
20q13	*ET3*	Shah-Waardenburg Non-syndromic HSCR	1 case	N/A	Dominant or recessive	91
1p36	*ECE1*	Cardiac and autonomic nervous system defects with HSCR	1 case	N/A	Dominant	40
22q13	*SOX10*	Shah-Waardenburg Non-syndromic HSCR	> 5%	~80%	Dominant	47,49,50,92
2q22	*ZFHX1B*	Mowat-Wilson	< 5%	60%	Dominant	62,93-95
4p12	*PHOX2B*	CCHS–Ondines Curse	< 5%	20%	Dominant	96
19p13	*NTN*	Non-syndromic HSCR	1 case		Dominant	97
18q21	*TCF4*	Epileptic encephalopathy	1 case		Dominant	98
10q21.1	*KIAA1279*	Goldberg-Shprintzen	Rare		Recessive	21

HSCR: Hirschsprung's disease; CCHS: Congenital central hypoventilation syndrome.

Table 2 Phenotypes of mouse models of enteric nervous system defects

	Wild-type	Colonic aganglionosis	Total intestinal aganglionosis	Hypoganglionosis
				
Ret	+/- 9/9 Y162F/+	51/51 S697A/S697A	-/- Y1062F/Y1062F C620R/C620R	C620R/+
Ednb	sl/+	sl/sl $Ednrb^{tm1Ywa}/Ednrb^{tm1Ywa}$		
Et3	ls/+	ls/ls $Et3^{tm1Ywa}/Et3^{tm1Ywa}$		
Sox10	Dom/+ (~80%) LacZ/+ (~80%)	Dom/+ (~20%) LacZ/+ (~20%)	Dom/Dom LacZ/LacZ	
Interactions		$Ret^{+/-}$; $Ednrb^{s/s}$ $Ret^{51/+}$; $Et3l^{s/ls}$	$Ret^{51/51}$; $Et3l^{s/ls}$ $Sox10^{Dom/+}$; $Et3l^{s/ls}$ $Sox10^{Dom/+}$; $Edrnb^{sl/sl}$	
Other genotypes		$Sall4^{-/-}$ B1Intergrin$^{-/-}$ $Ece1^{-/-}$	$Gdnf^{-/-}$ $Gfra1^{-/-}$ $Phox2b^{-/-}$ $Pax3^{-/-}$	$Gdnf^{+/-}$ $Hlx1^{-/-}$

tion of neural crest cells through the small intestine is also delayed[42,43]. As with *RET*, the human ENS appears to be more sensitive to reduced *EDNRB* signalling than that in mice. Around 21% of patients heterozygous for the W276C mutation in *EDNRB* develop HSCR[44], while heterozygous *piebald lethal* (*sl*) mice do not develop any form of aganglionosis[45].

SOX10

SRY (*Sex determining region Y*)-*box 10* (*SOX10*) is a high mobility group transcription factor of the SRY (sex determining factor) family. Mutations in *SOX10* account for around 5% of HSCR cases[46-48], and comprise both syndromic [Waardenburg-Shah types 4 (WS4)] and non-syndromic forms[49]. Some WS4 patients with *SOX10* mutations also suffer dysmyelination of the central and peripheral nervous systems[47]. *Sox10* is expressed by migrating enteric neural crest cells[50]. *Dom* is a naturally occurring mouse mutant of *Sox10*, which carries a single base insertion in the *Sox10* locus that prematurely truncates the transcription factor downstream of the DNA binding domain, producing a dominant negative form of the protein[50]. Mice lacking *Sox10* are devoid of enteric neurons throughout the entire gastrointestinal tract[50,51]. Around 20% of $Sox10^{+/-}$ mice suffer colonic aganglionо-

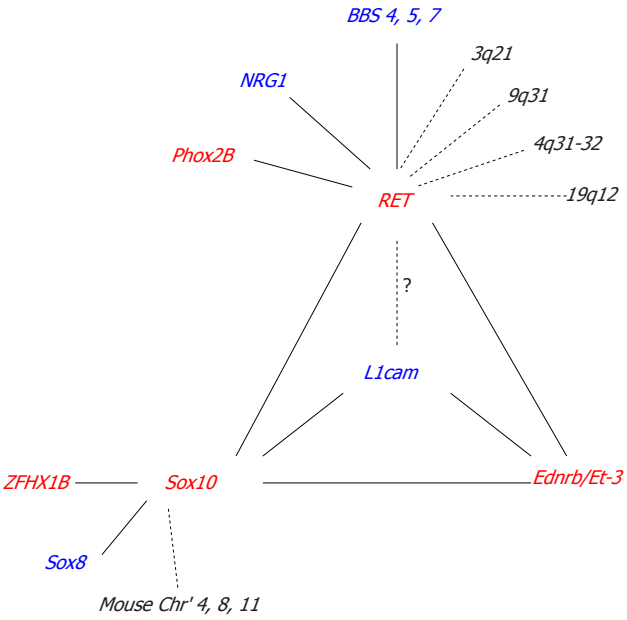

Figure 1 Interactions between known Hirschsprung's disease associated genes and their modifiers. Confirmed and putative interactions are shown by the solid and dotted lines, respectively. Red: HSCR associated gene; Blue: Known HSCR modifier gene; Black: Modifier loci with unknown gene. The question mark represents a disparity in the existing data obtained from human clinical and animal studies. HSCR: Hirschsprung's disease; BBS: Bardet-Biedel syndrome.

sis, although the incidence varies depending on the background strain[45,50,52,53]. These mice also exhibit coat colour defects analogous to the pigmentation defects seen in WS4 patients. *Sox10* has been shown to regulate the expression of both *Ret* and *Ednrb*[54-56].

ADDITIONAL TRANSCRIPTION FACTORS ASSOCIATED WITH HSCR

PHOX2B

Paired-like homeobox 2b (*PHOX2B*) is a transcription factor that is expressed by enteric neural crest cells[57]. Human studies have linked mutations in *PHOX2B* with HSCR associated with congenital central hypoventilation syndrome (CCHS)/Ondines curse in two thirds of patients[58]. The causative mutations in these patients are most commonly polyalanine expansions[59]. *Phox2b* null mice lack enteric neurons along the entire length of the gastrointestinal tract[60]. *Phox2b* has been shown to regulate the expression of *Ret*[60-61].

ZFHX1B

ZFHX1B, also known as SMAD interacting protein 1 (*SMADIP1*/*SIP1*), is a zinc finger homeodomain transcription factor. Mutations in *ZFHX1B* are associated with Mowat-Wilson syndrome, and have been shown to result in HSCR with a varying degree of penetrance[62]. In mice, *Zfhx1b* is expressed by vagal neural crest cells, which are absent in *Zfhx1b* null mutant mice[63].

INTERACTIONS BETWEEN KNOWN HSCR ASSOCIATED GENES

Interactions between known HSCR associated genes significantly influence the incidence and severity of intestinal aganglionosis (Table 2) (Figure 1).

Gdnf/Ret and Et-3/Ednrb signalling pathways

Genetic interactions were first proposed based on a human study of the genetically isolated Mennonite population, suggesting that the *RET* and *EDNRB* loci may interact to govern the susceptibility to Hirschsprung's disease[64]. Studies in mice, using a two-locus complementation approach, confirmed a genetic interaction between the *Ret* and *Ednrb* loci by showing that the generation of $Ret^{+/-}$; $Ednrb^{s/s}$ mice resulted in colonic aganglionosis; a phenotype not observed in $Ret^{+/-}$ or $Ednrb^{s/s}$ mice alone[64,65]. A similar genetic interaction was also reported using Ret^{51} and $Et\text{-}3^{ls}$ mice[42]. A significant increase in aganglionosis, extending all the way to the stomach, was observed in $Ret^{51/51}$; $Et\text{-}3^{ls/ls}$ mice compared to the colonic aganglionosis normally observed in $Ret^{51/51}$ or $Et\text{-}3^{ls/ls}$ mice alone[42]. The mechanism underlying these interactions is not yet known; however, it has been proposed that *Ret* and *Ednrb* may interact by activating common downstream signalling molecules, such as PKA[42].

Gdnf/Ret signalling pathway and the transcription factors Sox10 and Phox2b

Although genome-wide linkage studies failed to detect any genetic interaction between the *Sox10* and *Ret* loci[66], *Sox10* has been shown to form a transcriptional complex with the transcription factor, *Pax3*, to directly regulate the expression of RET[67]. In addition to *Sox10*, *Phox2B* has also been shown to bind the *RET* promoter and regulate transcription[61]. Although no genetic interactions were observed in double heterozygotic mice ($Ret^{+/-}$; $Phox2b^{+/-}$ mice), human clinical studies have reported interactions between *RET* and *PHOX2B* in CCHS patients[68].

Sox10 and Zfhx1b

Sox10 has been shown to interact with the transcription factor, *Zfhx1b*, in mice[69]. The generation of double heterozygotic progeny ($Sox10^{LacZ/+}$; $Zfhx1b^{\lrcorner/+}$ mice) resulted in a significant increase in the severity of aganglionosis compared to a mutation in $Sox10^{LacZ/+}$ or $Zfhx1b^{\lrcorner/+}$ mice alone[69]. The mechanism underlying this interaction is not known, but is likely to be mediated by the modulation of *Bmp* expression[69].

Et-3/Ednrb signalling pathway and Sox10

In mice, interactions between *Sox10* and members of the endothelin signalling pathway (*Et-3* and *Ednrb*) have been reported[45,52]. Using a two-locus complementation approach, mice carrying mutations in *Sox10* and *Et-3* ($Sox10^{Dom/+}$; $Et\text{-}3^{ls/ls}$) or *Ednrb* ($Sox10^{Dom/+}$;$Ednrb^{s/s}$ and *Sox-*

10$^{Dom/+}$; *Ednrb*$^{sl/sl}$) exhibited a significant increase in the severity of intestinal aganglionosis compared to mutations in *Sox10*, *Et-3*, or *Ednrb* alone[45,52]. In addition, the expression of Ednrb has been shown to be significantly reduced in *Sox10*$^{Dom/+}$ mice[50]. The mechanism underlying this interaction can be explained, at least in part, by the presence of SOX10 binding sites within a conserved enhancer region of the *Ednrb* promoter, which are required for the spatiotemporal expression of Ednrb in the ENS[56].

MODIFIER GENES

The incomplete penetrance and interfamilial variation commonly observed in HSCR strongly suggests the involvement of modifier genes. We define a modifier gene as a gene that, when mutated, is insufficient on its own to produce an effect, but, when coupled with another genetic mutation, it produces or enhances an effect[70]. To date, only a handful of modifier genes have been identified for HSCR (Figure 1).

Modifiers for RET

Linkage studies and genome-wide screens have identified a number of putative modifying loci for *RET*, such as 3q21, 4q31-32, 8p12, 9q31, and 19q12[71,72]. However, many of the genes responsible for interacting with *RET* at these loci are yet to be identified. One gene that has been identified is neuregulin 1 (*NRG1*)[72]. Association studies have shown that individuals that possess a specific *NRG1* haplotype have an increased risk of HSCR conferred by *RET*[72]. *NRG1* signals through ErbB2 and ErbB3 receptors to regulate neural crest cell development and in turn, *ErbB3* is regulated by the HSCR associated gene *Sox10*[73].

Although not detected in any of the genome-wide screens, three further modifier genes for *RET* were identified through the Bardet-Biedel syndrome (BBS). Subsets of patients with BBS, a genetically heterogeneous disorder with 14 identified causative loci, also present with HSCR. BBS patients with HSCR are more frequent carriers of a common *RET* intronic hypomorphic allele than the general population[74]. In zebrafish, suppression of *Ret* in conjunction with a loss of either *Bbs 4*, *5*, or *7* has been shown to significantly increase the severity of ENS defects compared to loss of these genes independently[75].

Human clinical studies have also suggested that the X-linked gene *L1CAM*, may act as a modifier gene for *RET*. Some individuals with *L1CAM* mutations who have HSCR, also possess a common *RET* polymorphism that is over-represented in HSCR populations[76]. However, animal model studies using a two-locus complementation approach failed to detect any genetic interaction between *L1cam* and *Ret*[70]. One reason for this discrepancy could be that humans are more sensitive to a reduction in RET levels than mice[28,32]. It is not yet known whether interactions with *L1cam* can be detected in *Ret*$^{51/51}$ and *Ret*$^{S697A/S697A}$ mice that exhibit colonic aganglionosis and more closely resemble human HSCR[31,33].

Modifiers for Sox10

A genome-wide screen in mice has identified five putative modifying loci for *Sox10* on chromosomes 3, 5, 8, 11, and 14[66]. Two of these loci have been identified as Ednrb and Phox2b[66], while the other three loci, on chromosomes 3, 8, and 11, are yet to be determined.

Although not identified in the *Sox10* genome-wide screen, one modifier gene that has been shown to significantly increase the penetrance and extent of aganglionosis in *Sox10* heterozygous mice, is *Sox8*[53]. *Sox8* is a transcription factor that is closely related to *Sox10*, and is expressed by all enteric neural crest cells[53]. *Sox8*$^{-/-}$ mice are viable and fertile and show no ENS phenotype[53]. Using a two-locus complementation approach, double heterozygotic progeny (*Sox8*$^{+/-}$; *Sox10*$^{+/-}$ mice) were shown to have a significant increase in the incidence and severity of aganglionosis compared to a mutation in *Sox8* or *Sox10* alone[53]. The most likely mechanism underlying this interaction is genetic redundancy, as Sox8 has been shown to have DNA binding and subcellular redistribution properties similar to that of Sox10[77-80] and is capable of activating Sox10 target genes[78].

The X-linked gene, *L1cam*, can also act as a modifier gene for *Sox10* in mice[70]. Loss or haploinsufficiency of *L1cam* in conjunction with a heterozygous loss of *Sox10* significantly increases the incidence of intestinal aganglionosis compared to a mutation in *Sox10* alone[70]. *Sox10* has been shown to directly regulate the expression of endogenous *L1cam*[70].

Modifiers of Et3/Ednrb

To date, only one modifier gene has been identified for members of the endothelin signalling pathway. Loss or haploinsufficiency of *L1cam* in conjunction with a null mutation in *Et-3* or *Ednrb* significantly increases the severity of intestinal aganglionosis compared to a loss of *Et-3* or *Ednrb* alone[81]. Although the mechanism underlying these interactions is not yet known, it is most likely mediated through the activation of common downstream targets, such as PI3K[81].

CONCLUSION

HSCR research has now entered a second phase. Having identified many of the key genes capable of independently inducing HSCR, we are now undertaking the difficult task of identifying the interactions that modulate the severity and penetrance of this disease. By combining human genetic data from patients, family pedigrees, and genome wide association screens with animal studies, we are beginning to assemble the pieces of the HSCR puzzle into a coherent picture of multigenetic inheritance and interactions. To further aid this goal, as the cost of genome sequencing becomes more affordable, the potential to sequence the entire genome of individual HSCR patients becomes viable, which is likely to provide significant advances into our understanding of the genetic basis of HSCR.

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TOPIC HIGHLIGHT

Dr. Marco Scarpa, PhD, *Series Editor*

Health-related quality of life outcomes after cholecystectomy

Amedeo Carraro, Dania El Mazloum, Florian Bihl

Amedeo Carraro, Department of Surgery, Veneto Oncological Institute, IRCCS, 35128 Padova, Italy
Dania El Mazloum, School of Medicine, University of Padova, 35128 Padova, Italy
Florian Bihl, Gastroenterology and Hepatology Unit, San Giovanni Hospital, 6500 Bellinzona, Switzerland
Florian Bihl, Gastroenterology and Hepatology Service, University Hospital of Geneva, 1201 Geneva, Switzerland
Author contributions: Bihl F, Carraro A and El Mazloum D designed the study; Carraro A and El Mazloum D performed the research; Bihl F and Carraro A contributed analytic tools; Bihl F, Carraro A and El Mazloum D analyzed the data and wrote the paper; all authors revised and approved the final version of the manuscript.
Correspondence to: Florian Bihl, MD, Gastroenterology and Hepatology Unit, San Giovanni Hospital, Via Ospedale 32, 6500 Bellinzona, Switzerland. florian.bihl@eoc.ch
Telephone: +41-91-8118708 Fax: +41-91-8118708
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Abstract

Gallbladder diseases are very common in developed countries. Complicated gallstone disease represents the most frequent of biliary disorders for which surgery is regularly advocated. As regards, cholecystectomy represents a common abdominal surgical intervention; it can be performed as either an elective intervention or emergency surgery, in the case of gangrene, perforation, peritonitis or sepsis. Nowadays, the laparoscopic approach is preferred over open laparotomy. Globally, numerous cholecystectomies are performed daily; however, little evidence exists regarding assessment of post-surgical quality of life (QOL) following these interventions. To assess post-cholecystectomy QOL, in fact, documentation of high quality care has been subject to extended discussions, and the use of patient-reported outcome satisfaction for quality improvement has been advocated for several years. However, there has been little research published regarding QOL outcomes following cholecystectomy; in addition, much of the current literature lacks systematic data on patient-centered outcomes. Then, although several tools have been used to measure QOL after cholecystectomy, difficulty remains in selecting meaningful parameters in order to obtain reproducible data to reflect postoperative QOL. The aim of this study was to review the impact of surgery for gallbladder diseases on QOL. This review includes Medline searches of current literature on QOL following cholecystectomy. Most studies demonstrated that symptomatic patients profited more from surgery than patients receiving an elective intervention. Thus, the gain in QOL depends on the general conditions before surgery, and patients without symptoms profit less or may even have a reduction in QOL.

Key words: Gallbladder disease; Gallstones; Quality of life; Laparoscopy

Peer reviewers: Bernabe Matias Quesada, MD, Department of Surgery, Hospital Cosme Argerich, Talcahuano 944 9 A, Buenos Aires 1013, Argentina; Hayrullah Derici, MD, Associate Professor, Department of General Surgery, Balikesir University Medical Faculty, Balikesir 10145, Turkey

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INTRODUCTION

Gallbladder diseases are very common in developed countries. They comprise a large spectrum of disorders caused by alterations in bile composition and biliary function, placing a substantial burden on inpatient and outpatient resources. Clinical manifestation of gall-

stone disease varies from attacks of intense biliary colic, prompting surgical intervention, to an absence of symptoms. Biliary colic is usually secondary to temporary obstruction of the cystic duct by a gallstone. When obstruction holds over, the gallbladder becomes inflamed and the patient may develop cholecystitis or other, potentially serious complications, such as cholangitis, gangrene, perforation, peritonitis, sepsis or pancreatitis. Complicated gallstone disease (e.g., symptomatic cholelithiasis) represents the most frequent of biliary disorders for which surgery is regularly advocated. In fact, patients with cholelithiasis account for about 10% to 15% of the total adult western population[1-4]; among them around 30% have surgery, and only 2% develop symptoms[4,5]. Today, cholecystectomy is a standard practice for cholelithiasis, and surgery for complicated gallstone diseases has a significant impact on quality of life (QOL) in developed countries[4]. QOL assessments are increasingly being recognized as an integral factor in surgical decision-making. However, considering the enormous number of cholecystectomies performed daily worldwide, surprisingly little data has been collected about QOL after biliary surgery. Laparoscopic cholecystectomy (LC) has become a very frequent surgical procedure, with over 500 000 operations annually in Western countries[5]. The laparoscopic technique, introduced in the 1990s, resulted in a significant reduction in the number of open cholecystectomies. As a consequence of this movement towards minimally invasive procedures, over the past 15 years the number of cholecystectomies increased, which may reflect a change in the threshold to perform surgery. This shift has also significantly impacted postoperative QOL. Today, estimates are that 86% of cholecystectomies are performed laparoscopically. This number continues to increase, especially in the treatment of acute cholecystitis and biliary colic; therefore, in recent years, the accumulating surgical experience and advances in technology have extended the indications for LC to include patients with complicated gallbladder disease[6,7]. On this basis, there is suggestive evidence that immediate postoperative health-related QOL (HRQOL) may be better after laparoscopic procedures. That being said, the introduction of LC has also increased the incidence of injuries to the biliary tree, along with an increasing number of serious vascular lesions[8-10]. In fact, 15%-20% of patients require conversion to open cholecystectomy for the safe completion of the procedure, countering the potential benefit of the laparoscopic approach[11].

To assess QOL, documentation of high quality care in cholecystectomy has been subject to extended discussions, and the use of patient-reported outcome satisfaction for quality improvement has been advocated for several years[12]. It would be ideal to consider the entire spectrum of gallbladder diseases that indicate surgery. Among them, for example, acalculous cholecystitis represents a controversial clinical indication for surgery, yet it accounts for 5%-20% of all cholecystectomies[13]. Furthermore, debate continues regarding the decision for elective surgery in patients following an acute episode of gallstone disease. Although several tools have been used to measure QOL after cholecystectomy, difficulty remains in selecting meaningful parameters in order to obtain reproducible data to reflect postoperative QOL. Classically, evaluations of surgical procedure outcomes have focused on perioperative complications, morbidity, recurrence rate, and long-term survival. However, much of the current literature lacks systematic data on patient-centered outcomes. Endpoints such as symptom resolution or duration of convalescence represent QOL measures that are at least as important as the classical outcomes. There has been little research done regarding QOL outcomes following cholecystectomy. Furthermore, laparoscopic surgery is usually perceived by patients as a routine procedure. Thus, the impact of LC on QOL, as well as the identification of predictors of subjective patients outcomes, remains undetermined in these patients[14].

Usually, the principal criterion guiding patients' acceptance of a treatment modality is their subjective condition prior to surgery. Additionally, those subjective reports become important criteria in a surgeon's decision-making process[15]. Thus, the aim of this review is to evaluate and summarize the published data on QOL after cholecystectomy in adults. A text word literature search was performed using the Medline databases. Although this was not a systematic review, the search terms used were as follows: gallstones, cholecystitis, surgery, gallbladder disease, and quality of life. The reference lists of identified articles were searched for further relevant publications. The databases were consulted from January 1993 to July 2010. The authors independently selected the studies, particularly those comparing different surgical approaches. Whenever there was discordance regarding study inclusion the authors negotiated an agreement.

GLOBAL QOL MEASURES FOR CHOLECYSTECTOMY: A LACK OF STANDARDIZED AND UNIVERSALLY VALIDATED INSTRUMENTS

HRQOL measures have been shown to be useful in predicting health care expenditure; different QOL indices exist and have been validated to determine the general subjective perceptions and expectations of individuals; in surgery in general, and in particular in the case of cholecystectomy, there is no clear, validated and standardized instrument for assessing QOL postoperatively. The development of well-validated and sensitive non-disease-specific questionnaires is useful for comparing different surgical approaches and techniques. Although specific, HRQOL instruments have been proposed for cholelithiasis and cholecystectomy, they have appeared

with only limited reproducibility, restricted psychometric aspects and with linguistic gaps when translated into different languages[16-18].

The most frequently used tool to assess QOL is the short form (SF)-36 questionnaire and the Gastrointestinal Quality-of-Life Index (GIQLI), each instrument having its own advantages and limitations. The generic SF-36 is a widely used instrument that allows comparison between different studies. However, it has a low discriminative ability and low specificity for identifying determinant changes related to a specific clinical factor. The GIQLI is an established tool for assessing QOL outcomes for patients with various gastrointestinal symptoms including domains of general health, but it is not specific for gallbladder disease.

Some studies used both SF-36 and GIQLI, combining a questionnaire for general well-being and another for more specific postoperative QOL. Quintana *et al*[19] used, for example, the SF-36 to validate the explicit appropriateness criteria in subjects after cholecystectomy. Their results indicated similar improvements in SF-36 QOL measures compared with GIQLI, indicating that both tools were adequate QOL measures and thus confirmed their validity. Recently Fledman *et al*[20] proposed a physical activity questionnaire (Community Health Activities Model Program for Seniors) as an indicator of postoperative recovery. Their aim was to specifically correlate physical activity caloric expenditure as an estimation of postoperative recovery after LC in older patients; evidence has been provided for the validity of this questionnaire as a measure of surgical recovery.

However, the most appropriate measures for identifying relevant changes in QOL after biliary surgery remain to be determined.

An important proposed concept of a questionnaire' s appropriateness is the accuracy of a measure over time in the same patient, assessing prospective changes in the patient's health status. In fact, a highly responsive QOL instrument has been considered able to detect significant treatment effects in a small sample size: an outstanding proposed tool is the "minimal clinically important difference" (MCID) that potentially can examine all significant differences at the individual patient level[21,22]. The MCID is one of the most effective and widely used methods of HRQOL assessment, and can be used to provide an indication of the minimal change that is of clinical relevance. An interesting work by Shi *et al*[23] aimed to estimate MCIDs for the GIQLI score of patients after cholecystectomy; they showed that this instrument can play a role in interpretation of scores and useful application in clinical practice. Thereafter, the same group clinically compared the responsiveness derived by the SF-36 and the GIQLI before and after cholecystectomy; correlation analyses revealed significant correlation between the SF-36 and GIQLI in the preoperative and 3-mo postoperative period[24].

In conclusion, there is an overall propensity to use both generic instruments, SF-36 and GIQLI, to assess the QOL after cholecystectomy; however in the case of limited time and resources, the GIQLI index may be used alone since it incorporates all domains of a QOL. The main issue is the choice of disease-specific outcome measures, adjusted for potential variables, that may act as confounders to identify the effective relevant changes after cholecystectomy.

IMPACT ON QOL OF LAPAROSCOPIC *VS* OPEN CHOLECYSTECTOMY

The literature offers positive and encouraging results in several reports comparing laparoscopic *vs* open surgery in the clinical setting. The development of the laparoscopic technique has drastically changed the protocols for treatment of gallstone disease and cholecystitis, and has been accompanied by evident clinical benefit for patients. Over the years since its introduction, reduced morbidity and mortality rates have confirmed LC as a safe and standard procedure in the treatment of some gallbladder diseases[25]. These results reinforce the feasibility of laparoscopy as a treatment modality for the biliary tract itself, and have provided reliable scientific material in support of an expanded role for laparoscopy in hepatobiliary surgery. Collected data seems to confirm a positive post-laparoscopic subjective satisfaction and perceived QOL[20,26]. Indeed, Harju *et al*[27] compared minilaparotomy with LC, demonstrating that the minilaparotomy procedure represents a good alternative to the LC procedure, when QOL is measured.

Although the rate of increase of QOL following LC is greater than that after open surgery, long-term overall QOL has proven to be only slightly better or show no difference when compared with open surgery. Therefore, the only significant long-term advantage of laparoscopic surgery, as compared with open surgery, seems to be the higher satisfaction rate regarding the cosmesis of the surgical scar. There remains no clear explanation regarding the similarity of this comparative data between the two surgical techniques; feasible hypotheses are that indications for LC might be more easily proposed than those for open surgery. This could impact patient selection as well as patient expectation regarding laparoscopy[28]. Furthermore, patients selected for open surgery more frequently have a lower perception of QOL and more co-morbidities than matched laparoscopic patients prior to surgical intervention. These factors likely influenced outcomes and potentially introduced bias in the above-mentioned studies.

ADULT PATIENTS WITH CHOLELITHIASIS: IMPACT OF QOL FOLLOWING LC

The use of objective outcome measures after surgical procedures, even though non-disease specific, is helpful

for laparoscopic surgery such as cholecystectomy. Quintana *et al*[29] aimed to determine clinical variables that predicted changes in HRQOL using both instruments, GIQLI and SF-36. Patients were grouped according to diagnosis (complicated symptomatic cholelithiasis, including acute cholecystitis, choledocholithiasis, pancreatitis or cholangitis; uncomplicated symptomatic cholelithiasis; asymptomatic cholelithiasis) and surgical risk categories; patients were asked to complete a questionnaire before and 3 mo after cholecystectomy. The study concluded that cholecystectomy is the suitable treatment especially for patients with symptomatic cholelithiasis and low surgical risk since they experienced the highest QOL gains; whereas patients with asymptomatic cholelithiasis or high surgical risk experienced least improvement. Conversely, Mentes *et al*[30] observed significant GIQLI score improvements in both symptomatic and asymptomatic gallstone groups. However, the gallstone-related QOL improvements were particularly marked in symptomatic patients, indicating that gallstone patients with lower baseline GIQLI scores are more likely to benefit from LC. Thus, LC seems to be the appropriate intervention for patients with symptomatic gallstone and low surgical risk.

Alternatively, Vetrhus *et al*[31] evaluated gallstone-related acute cholecystitis *vs* symptomatic but non-complicated disease. They used QOL and pain surveys to compare chronic gallbladder disease outcomes between conservative observational treatment and cholecystectomy. The patients in this study answered standardized questions at baseline (before surgery), and at 6, 12 and 60 mo post-cholecystectomy. The observation group (no intervention) had a higher rate (36% *vs* 19%) of gallstone-related events, but the difference was not significant. When patients were grouped according to randomization or actual operative outcome (+/- cholecystectomy), the authors did not find any significant differences in pain or QOL measurements. The authors concluded that conservative treatment in acute cholecystitis did not significantly increase the risk of subsequent gallstone events, and importantly this did not influence the QOL outcome and pain measurements. Thus, conservative (non-operative) treatment and observation of acute cholecystitis would be an acceptable option and should at least be considered in high risk patients[27].

Another longitudinal QOL study from Taiwan provided data using the SF-36 questionnaire and GIQLI scores[32]. The preoperative SF-36 scores from gallstone patients were significantly inferior to an age- and sex-matched control population; LC effectively reduced gastrointestinal symptoms, confirmed by the improvement in GIQLI total, physical well-being, mental well-being, gastrointestinal digestion, and defecation subscale scores. Yet, certain authors' evidence indicates that some patients did not regain full GIQLI scores after surgery, deducing that some residual gastrointestinal discomfort remained. Indeed, some investigators described a persistent decrement in many of the SF-36 health dimensions at 12 mo following surgery; thus they identified different markers to evaluate QOL outcomes after surgery; they found that QOL improvements can be partially predicted by the preoperative direct bilirubin level and by the placement of a drainage tube intra-operatively. This aspect confirms data indicating that patients with worse preoperative health conditions may have greater gains in QOL improvement following LC surgery; moreover, QOL measures should consider potential variables that may act as confounding events. In fact, although there is no doubt that cholelithiasis may decrease the QOL during its acute symptomatic phase, the postoperative course after cholecystectomy, independent of the operative technique, might be potentially altered by other factors (bloating, slow digestion, *etc*.) that were not sufficiently controlled or distinguished by researchers, and could determine cholecystectomy as an overused procedure.

Finan *et al*[33] designed a study to determine gastrointestinal symptoms and QOL after cholecystectomy for better measurement of the change in QOL after surgery. In this study, SF-36 was employed along with a symptom survey that was designed to include both classic symptoms of biliary disease as well as other benign gastrointestinal (GI) diseases. Their results showed that LC significantly improved GI symptoms as well as QOL in subjects with symptomatic gallstone disease; the quantitative evaluation of GI symptoms allowed for analysis of symptom improvement by including patient perceived severity and distress. These results permitted the development of clear indications for operative management, supporting the effectiveness of cholecystectomy for elective biliary disease. In conclusion, in adult patients operated for cholelithiasis, QOL improved most in patients with symptomatic disease and average surgical risk; particular attention must be paid in regard to appropriate selection of patients, especially in terms of discrimination between biliary disease-related symptoms and other GI disorders.

IMPACT ON QOL OF LC FOR ACALCULOUS CHOLECYSTITIS

One of the most controversial and frequent dilemmas for surgeons in clinical practice is recurrent acalculous biliary pain. Surgical treatment of this disease represents a controversial issue, especially considering the similarities between its clinical presentation and that of other GI conditions. Therefore, clinical resolution cannot be guaranteed with surgical interventions and there is significant risk for decreased QOL following this procedure. Planells Roig *et al*[13] evaluated the QOL in patients with chronic acalculous cholecystitis in comparison to a control group of patients who underwent cholecystectomy for chronic calculous cholecystitis. They concluded that the prevalence of associated gastrointestinal symptoms

was similar for both groups, and QOL was similarly affected by both chronic diseases. The limitation of this work was primarily a disparity between the numbers of subjects (34 patients with chronic acalculous cholecystitis *vs* 297 with chronic calculous cholecystitis); moreover, the study population was a highly selected, though heterogeneous group of patients. A comprehensive and reproducible preoperative investigation for proper diagnosis of biliary disease has constituted an essential prerequisite for the appropriate selection of patients for surgery, and the appropriate exclusion for other GI disorders. Thus, the frustration due to the lack of understanding this disease consequently implies an impact in terms of post-surgical QOL for these patients. An accurate clinical selection seems to remain the most important criterion for surgical and healthcare expenditures in primary hepatobiliary centers.

CHANGES IN QOL FOLLOWING IATROGENIC INJURIES AFTER CHOLECYSTECTOMY

Unfortunately, with the introduction of LC, an increase in potentially dangerous injuries to the biliary tree has been observed, along with an increasing number of serious vascular lesions. Nowadays iatrogenic bile duct-related injuries (BDI) occur in less than 0.3% of all cholecystectomy procedures[34]. BDI are not always identified immediately during the surgical procedure and sometimes appear only in the postoperative course, mostly between days 1 and 5[35]. The clinical manifestations start with early biliary obstruction, biliary abdominal collection or biliary peritonitis, whereas late presentations include obstructive jaundice and ascending cholangitis. On this basis the optimal management of complications often advocates interventional procedures such as percutaneous drain placement or, sometimes, second-look surgery. The literature includes numerous studies confirming satisfactory technical and clinical approaches, demonstrating acceptable clinical outcomes, even in tertiary hepatobiliary centers. However, data is lacking regarding QOL. Only poor documentation of high quality care after bile-duct injuries exists. Results vary significantly between studies, and most recorded true BDI rather than simple cystic duct leaks.

Hogan *et al*[36] has recently published an interesting study, which compared an iatrogenic BDI study group with an age- and sex-matched control group, which underwent uncomplicated cholecystectomy. The SF-36 form was administered to the patients at a median postoperative time of 12 years (range, 2 mo to 20 years). The authors finally concluded that QOL of the surviving patients following BDI seems to be favorable to that after uncomplicated LC. Other studies showed different results; in particular, Sarmiento *et al*[37] and Melton *et al*[40] showed favorable comparisons between BDI and a control group whereas Boerma *et al*[39] and Moore *et al*[38] found that the BDI group had lower QOL scores. However, Boerma's work has been criticized, although they had the largest series[37,39]: for example, patient enrollment included those with cystic duct as well as peripheral hepatic injuries (e.g., leakage, 30%), which technically do not represent BDI. Furthermore, different QOL instruments were used for measuring health-related impact, invalidating any potential comparison between groups. Sarmiento *et al*[37] assessed QOL with the SF-36 questionnaire with a minimum follow-up of 5 years; the QOL after surgical biliary reconstruction compared favorably with that of patients undergoing uneventful LC. Melton *et al*[40] assessed QOL of patients after surgical reconstruction of major bile duct injury from LC with a median follow-up of 59 mo. Although using different survey instruments, the conclusions of the studies are quite similar, and all found that major BDI should be managed surgically, which constitutes a definitive therapy (although more invasive), and is not punctuated by repetitive interventions; in fact, patients with BDI managed endoscopically often require repeat intervention resulting in a worse QOL. In any case, an equivalence of QOL in BDI and uncomplicated LC is quite surprising and points to a possible bias. Patients with the most severe BDI may die, thus QOL cannot be assessed. Moreover, the numbers of patients included were small and in general, the instruments employed were nonspecific.

CONCLUSION

Many studies in the literature lack systematic data regarding QOL outcomes after cholecystectomy. Reported works have conflicting data and sometimes several limitations (i.e., small sample size, single-institution experience), and thus may not be generalizable. A general agreement is that postoperative QOL depends on preoperative clinical status; moreover the first essential criterion for an improvement in subjective change in QOL is accurate preoperative diagnosis. In fact, appropriate patient selection for surgery represents the most important criteria guiding the patients' subjective feeling after cholecystectomy, independent of the selected surgical technique. On the other hand, an effective way to investigate the factors that may influence subjective QOL outcomes would be to measure the satisfaction rate pre- and post-surgery, and repeatedly after surgical treatment; a QOL assessment is generally suggested at 1 and 6 mo postoperatively. On this basis, symptomatic patients usually gain more QOL from a surgical intervention (open or laparoscopic) in terms of long-term well-being. Even though LC improves QOL faster than open surgery, long-term results are only slightly better or show no difference compared with those of open surgery; at the same time, these data should be considered as a mean, and might be limited by study design (e.g., small sample size, biased and confounding variables). The only certain and significant long-term advantage of laparoscopic surgery might be the higher satisfaction rate in regard to

scar cosmesis, in the absence of complications.

In conclusion, although sensitive and responsive instruments for the measurement of post-cholecystectomy QOL exist, more research is needed to identify modifications that could lead to significant improvements.

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REVIEW

Review of dynamic contrast-enhanced ultrasound guidance in ablation therapy for hepatocellular carcinoma

Yasunori Minami, Masatoshi Kudo

Yasunori Minami, Masatoshi Kudo, Department of Gastroenterology and Hepatology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan
Author contributions: Minami Y drafted the manuscript and wrote the final version of the manuscript; Kudo M reviewed and approved the last version of the manuscript.
Correspondence to: Yasunori Minami, MD, PhD, Department of Gastroenterology and Hepatology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. m-kudo@med.kindai.ac.jp
Telephone: +81-72-3660221 Fax: +81-72-3672880
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Abstract

Local ablative techniques-percutaneous ethanol injection, microwave coagulation therapy and radiofrequency ablation (RFA)-have been developed to treat unresectable hepatocellular carcinoma (HCC). The success rate of percutaneous ablation therapy for HCC depends on correct targeting of the tumor *via* an imaging technique. However, probe insertion often is not completely accurate for small HCC nodules, which are poorly defined on conventional B-mode ultrasound (US) alone. Thus, multiple sessions of ablation therapy are frequently required in difficult cases. By means of two breakthroughs in US technology, harmonic imaging and the development of second-generation contrast agents, dynamic contrast-enhanced harmonic US imaging with an intravenous contrast agent can depict tumor vascularity sensitively and accurately, and is able to evaluate small hypervascular HCCs even when B-mode US cannot adequately characterize the tumors. Therefore, dynamic contrast-enhanced US can facilitate RFA electrode placement in hypervascular HCC, which is poorly depicted by B-mode US. The use of dynamic contrast-enhanced US guidance in ablation therapy for liver cancer is an efficient approach. Here, we present an overview of the current status of dynamic contrast-enhanced US-guided ablation therapy, and summarize the current indications and outcomes of reported clinical use in comparison with that of other modalities.

Key words: Dynamic contrast-enhanced ultrasound; Hepatocellular carcinoma; Percutaneous ethanol injection; Radiofrequency ablation

Peer reviewers: Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain; London Lucien Ooi, Professor, Chairman, Division of Surgery, Singapore General Hospital, 1 Hospital Drive, 169608, Singapore

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INTRODUCTION

Hepatic resection forms part of the conventional treatment for patients with primary liver cancers; however, the majority of hepatocellular carcinomas (HCCs) are not suitable for curative resection at the time of diagnosis. Difficulties of surgical resection may be related to size, site, and number of tumors, vascular and extrahepatic involvement as well as liver function of the patient[1-4]. There is a need to develop a simple and effective technique for treatment of unresectable HCCs; therefore, local ablative techniques [percutaneous ethanol injection (PEI), microwave coagulation therapy (MCT) and radiofrequency ablation (RFA)] have emerged in clinical practice to expand the pool of patients considered for

liver-directed therapies[5-8]. In particular, RFA is not associated with some of the side-effects of other ablative techniques[9]. Thus, RFA is currently more widely accepted due to the ease of use, safety, reasonable cost and applicability to minimally invasive techniques[10].

Percutaneous ablation therapy for HCC is widely performed under real-time sonographic guidance. The success rate of percutaneous RF ablation depends on correct targeting *via* an imaging technique. However, multiple sessions of ablation therapy are often required for small HCCs, which are poorly defined on conventional B-mode ultrasound (US) alone[11]. There are two particular situations in which B-mode US cannot adequately characterize the tumors[12]. The first is the presence of residual HCC nodules after ablation, because B-mode US findings cannot adequately differentiate between treated and viable residual tumor tissue. The second is the presence of naïve HCC nodules among many large regenerated nodules in cirrhotic liver. Color Doppler and power Doppler have increased the sensitivity of hepatic lesion detection compared to that using gray-scale US, but these modalities do not provide levels of sensitivity comparable to those of contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI)[13-16]. However, two breakthroughs in US technology, harmonic imaging and the development of second-generation contrast agents, have demonstrated the potential to dramatically broaden the scope of US diagnosis of hepatic tumors[14-16]. Dynamic contrast harmonic US can depict tumor vascularity sensitively and accurately, and is able to evaluate small hypervascular HCCs even when B-mode US cannot adequately characterize the tumors[17-21]. Therefore, contrast-enhanced harmonic US is expected to improve the detectability of HCC nodules, and decrease the number of sessions required for ablation of HCC in difficult cases[22,23].

This paper reviews the evidence for the use of dynamic contrast-enhanced US guidance in ablation of HCC, and illustrates the potential of the techniques for improving the targeting in percutaneous ablation therapy.

DIAGNOSIS AND TREATMENT OF HEPATOCELLULAR CARCINOMA

HCC can be diagnosed radiologically, without the need for biopsy if the typical imaging features are present[20,24,25]. This requires a contrast-enhanced study (dynamic CT or MRI). HCC enhances more intensely than the surrounding liver in the arterial phase, whereas the presence of 'washout' persists in the delayed phase. Tumor markers including alpha-fetoprotein and descarboxyprothrombin have been used for the diagnosis of HCC.

The management of HCC involves multiple disciplines including hepatology, surgery, diagnostic and interventional radiology, oncology, and pathology[20,25-27]. One has to consider several patient and tumor factors including the severity of underlying liver disease, tumor bulk, and associated comorbidities, as well as several practice-setting factors including availability and expertise in surgical resection, transplantation, and ablative therapies. RFA is basically recommended for HCC nodules with a maximum diameter of 3 cm in patients with not more than three tumors who are contraindicated for surgery.

DYNAMIC CONTRAST-ENHANCED ULTRASOUND

Contrast agents

Levovist (Schering, Berlin, Germany) is a first-generation US agent made of galactose[28]. A trace of palmitic acid is added as a surfactant to stabilize the resultant microbubbles. These bubbles have a weak encapsulating shell and are easily destroyed by US exposure. The contrast effect of Levovist is based on the destruction of microbubbles by high mechanical index (MI) pulses. In addition, Kupffer cells phagocytose Levovist microbubbles; therefore, liver parenchymal findings are obtained as Kupffer imaging in the postvascular phase at least 10 min after administration.

Sulfur hexafluoride microbubbles (SonoVue; Bracco SpA, Milan, Italy), perflutren lipid microbubbles (Definity; Bristol-Myers Squibb, North Billerica, MA), perflutren protein microbubbles (Optison; GE Healthcare, Buckinghamshire, United Kingdom), and perfluorocarbon microbubbles (Sonazoid; Daiichi-Sankyo, Tokyo, Japan) are second-generation contrast agents[29-33]. These microbubbles provide stable nonlinear oscillation in a low power acoustic field because of the hard shell of these bubbles, producing great detail in the harmonic signals in real-time. The only second-generation contrast agent that can be taken up by Kupffer cells in the liver is Sonazoid. Sonazoid microbubbles accumulate in the liver parenchyma over time[34,35].

Generally, few drug toxicities have been reported; these being pain at the point of injection, sense of heat and sense of cold. The incidence of complications was shown not to differ from historical controls (1.7%, P = 0.867 by Fisher's exact probability test)[36].

Imaging and procedure

In point of fact, there is a clinical need for high resolution and real-time imaging for dynamic contrast-enhanced US guidance in ablation therapy.

Using Levovist, real-time images of tumor enhancement by the simultaneous collapse of microbubbles caused by high mechanical index pulses can be obtained in the early vascular phase only[28]. The collapse of microbubbles in viable HCC lesions is seen as white flashes on the screen[12]. However, maintaining real-time US imaging for guidance reduces the enhancement period to approximately one minute after injection because Levovist microbubbles are easily disrupted (Figure 1). Therefore, great skill is required because the procedure time is too short to search for enhanced HCC nodules and insert the probe.

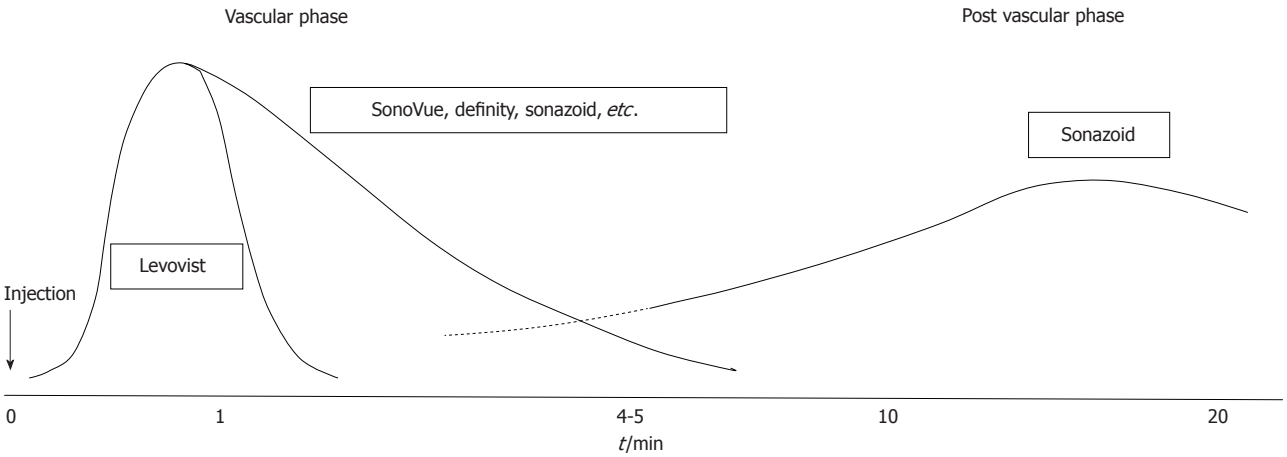

Figure 1 ***In vivo*** **kinetics of intravenous contrast ultrasound agents in the liver.**

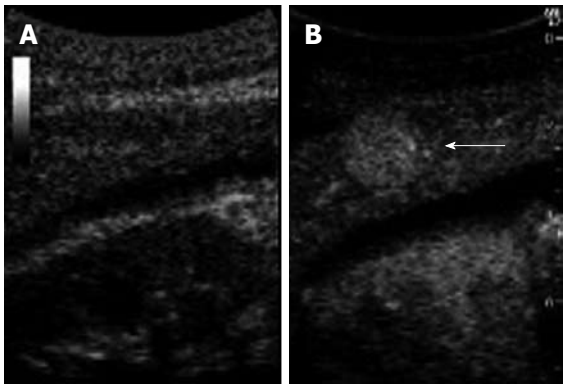

Figure 2 A 67-year-old man with a 1.5-cm hepatocellular carcinoma nodule located in segment 3 of the liver. A: B-mode ultrasound (US) cannot clearly depict the hepatocellular carcinoma (HCC) nodule; B: Contrast-enhanced US shows enhancement of HCC focus (arrow) in early vascular phase after administration of sonazoid.

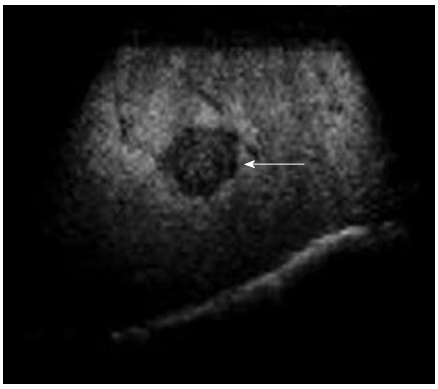

Figure 3 A 70-year-old man with a 2.0-cm hepatocellular carcinoma nodule located in segment 6 of the liver. Contrast-enhanced ultrasound using sonazoid shows the defect (arrow) imaging in post-vascular phase. The defect lesion can be targeted for insertion of a single needle by extending the time limitation.

Using second-generation contrast agents, real-time findings are better than those reported with Levovist[37] (Figure 1). Hypervascular HCC shows a short early arterial flushlike enhancement for less than 20 s, followed by homogeneous enhancement of the lesion in the late phase under low MI imaging (Figure 2). Needle insertion can be performed between the early arterial phase to late vascular phase in which maximum lesion conspicuity is observed[38]. In particular, HCCs have been visualized as defects in the liver parenchyma in the post-vascular phase only with Sonazoid use (Figure 1)[39-43]. Therefore, we can use these defect lesions as a target for insertion of a single needle (Figure 3). In patients who had previously undergone ablation for HCC, demonstration of viable nodules among all nodules detected in the post-vascular phase was achieved by injecting an additional new dose of Sonazoid in order to confirm tumor vascularity before needle insertion[44]. This defect-reperfusion US imaging is extremely useful in the depiction and confirmation of HCCs that are otherwise undetectable on US.

CLINICAL OUTCOMES

Treatment sessions and local tumor progression

Table 1 shows the treatment sessions of percutaneous ablation guided by contrast-enhanced US for HCC in published papers[12,36,45-49]. Numata *et al*[45] first reported that nine HCC nodules were successfully treated with percutaneous ablation therapy guided by intravenous contrast-enhanced US. These nine lesions were not detected on conventional US but were depicted on real-time contrast-enhanced harmonic gray-scale US with Levovist (incomplete local treatment, $n = 4$; small new lesion, $n = 5$). Since 2004, second-generation contrast agents of US have been used in percutaneous ablation guided by contrast-enhanced US. Particularly with Sonazoid use, complete tumor necrosis has been achieved in 94% with a single session of RF ablation[49]. In cases of HCC that are not clearly demarcated by B-mode US, dynamic contrast-enhanced sonography-guided RFA and are efficient approaches for guiding ablation.

Table 1 Treatment sessions of percutaneous ablation guided by contrast-enhanced ultrasound for hepatocellular carcinoma

Author[Ref.]	Year	Procedure	*n*	Contrast agent	Tumor size (mean, cm)	Treatment sessions (mean)
Numata *et al*[45]	2003	PEI, RFA	9	Levovist	1.4	ND
Minami *et al*[12]	2004	RFA	21	Levovist	1.7	1.05
Solbiati *et al*[46]	2004	RFA	51	SonoVue	ND	ND
Numata *et al*[47]	2008	RFA	15	Sonazoid	ND	1.04
Maruyama *et al*[48]	2009	PEI, RFA	42	Sonazoid	1.3	ND
Miyamoto *et al*[49]	2009	RFA	52	Sonazoid	ND	1.04
Minami *et al*[50]	2010	RFA	108	Sonazoid	1.7	1.1
Masuzaki *et al*[36]	2010	RFA	291	Sonazoid	1.6	1.33

HCC: Hepatocellular carcinoma; ND: Not described; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation.

Table 2 Local tumor progression rates of percutaneous ablation guided by contrast-enhanced ultrasound for hepatocellular carcinoma

Author[Ref.]	Year	Procedure	*n*	Tumor size (mean, cm)	Follow-up (mean, mo)	Local tumor progression (%)
Maruyama *et al*[48]	2009	PEI, RFA	42	1.3	8.6	0
Minami *et al*[50]	2010	RFA	108	1.7	4.3	0
Masuzaki *et al*[36]	2010	RFA	291	1.6	ND	2.1
Miyamoto *et al*[51]	2010	RFA	17	1.6	11	12

HCC: Hepatocellular carcinoma; ND: Not described; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation.

The local tumor progression rates after RFA have ranged from 0% to 12% during the follow-up period[47,36,49,50] (Table 2). The risk of local tumor progression increases with size, but the local tumor progression rates of small HCCs were markedly dependent on whether or not the center of the HCC nodule was penetrated by the RF needle.

Dynamic contrast-enhanced US guidance vs conventional B-mode US guidance

The effectiveness of contrast harmonic sonographic guidance for RFA of HCC was evaluated in comparison with conventional B-mode US guidance[12,36,51] (Table 3) (Level of evidence: grade B, level 2b). Dynamic contrast-enhanced US significantly helps in the placement of RFA electrodes in hypervascular HCCs that cannot be adequately depicted by B-mode sonography. In a randomized controlled study, the number of treatment sessions was significantly lower in the contrast harmonic US group (mean, 1.1 ± 0.2 *vs* 1.4 ± 0.6, $P = 0.037$)[51]. Treatment analysis showed that the complete ablation rate after a single treatment session was significantly higher in the contrast harmonic US group than in the B-mode US group (94.7% *vs* 65.0%, $P = 0.043$). Moreover, Masuzaki *et al*[36] reported in a large-scale study that the detectability of tumor nodules was 83.5% in conventional US and 93.2% in contrast-enhanced US ($P = 0.04$). The number of RFA sessions was 1.33 ± 0.45 with contrast-enhanced US as compared to 1.49 ± 0.76 in the historical controls ($P = 0.0019$). The number of RFA sessions required for complete ablation could be decreased in contrast-enhanced US-assisted RFA.

Few toxicities using US contrast agents have been reported, therefore the incidence of complications did not differ from that reported in patients treated by RFA alone[12,51].

Advances in techniques: Tumors abutting the diaphragm

HCC nodules abutting the diaphragm are difficult to depict because of ultrasound scatter due to pulmonary air. However, contrast-enhanced US through artificial pleural effusion can depict tumor vascularity in HCC. Thus, percutaneous RFA guided by contrast-enhanced US with artificial pleural effusion is an efficient approach[36,52]. Thirteen tumors were treated by contrast-enhanced US-guided RFA with artificial pleural effusion, and complete tumor necrosis was achieved in a single session in 12 lesions (92.3%)[52]. It took approximately 1 wk for pleural effusions to spontaneously resolve.

OTHER MODALITIES

Computed tomography guidance and computed tomography fluoroscopy

CT has high spatial resolution, good contrast, wide field of view, good reproducibility, and applicability to bony and air-filled structures. Potential advantages of CT guidance include confirmation of probe placement in relation to the tumor, improved visualization of the extent of ablation, and good correlation with actual lesion size[53-56] (grade C, level 3b). The use of a CT-guided method can be expected to reduce the rate of local tumor progression associated with percutaneous RFA. Laspas *et al*[53] reported that the ablation success rate was 87.3% (281/322 HCC nodules), and the survival rates at 1 year, 3 years and 5 years were 94.8%, 73.1% and 51.1%, respectively. Another merit is that the efficacy of treatment can be evaluated using contrast-enhanced CT immediately after treatment. Despite the advantages of CT, there are several limitations such as the increased time that is necessary for the procedure and exposure of the patient to ionizing radiation.

CT fluoroscopy guidance combines the high spatial resolution and good contrast resolution inherent in contrast-enhanced CT with the immediacy of fluoroscopic monitoring. Under CT fluoroscopy using either CT arteriography or iodized oil injection, we can target and puncture hepatic malignancies using a percutaneous ethanol injection needle. Real-time CT fluoroscopy is useful to guide the needle puncture and to monitor ethanol injection in small hepatic malignancies (grade C, level 3b). Takayasu *et al*[57] reported that the overall success rate in puncturing the lesions was 94.4% (17/18 sessions), the average number of punctures was 3.3, and this significantly decreased after the introduction of a puncture guide compared with freehand puncture ($P < 0.01$). However, the operator's hands are directly exposed to the beam of CT fluoroscopy, posing a potentially serious problem.

Magnetic resonance imaging guidance

MRI with its high soft tissue contrast can be advanta-

Table 3 Treatment sessions of radiofrequency ablation: Dynamic contrast-enhanced ultrasound guidance *vs* conventional B-mode ultrasound guidance

Author[Ref.]	Year	Study type	*n* (CEUS/B-mode)	Tumor size, (mean, cm) (CEUS/B-mode)	Mean treatment sessions (CEUS *vs* B-mode)	*P* value
Minami *et al*[12]	2004	Case control study	21/25	1.7/1.7	1.05 *vs* 2.0	0.002
Minami *et al*[53]	2007	RCT	19/20	1.2/1.3	1.1 *vs* 1.4	0.043
Masuzaki *et al*[36]	2010	Case control study	291/291	1.9/1.9	1.33 *vs* 1.49	0.0019

CEUS: Contrast-enhanced ultrasound; HCC: Hepatocellular carcinoma; RCT: Randomized controlled trial.

geous, and the capability of MRI for multiplanar imaging can be of value for needle placement and surveillance of the ablation procedure. Most of the current open MR scanners operate between 0.2 and 0.5 T, while clinical MR systems with a closed cylindrical design allow for significantly higher field strengths of up to 3.0 T or even more. While open MR systems allow for online monitoring of the puncture and easy replacement of the RF needle within a wide range, closed-bore MR systems improve lesion conspicuity and tumor delineation[58-63] (grade C, level 3b). Wu *et al*[59] reported that MRI and optical navigation system-guided ablation procedures were successfully performed on all 32 patients (36 tumor sites), and the 6- and 12-mo overall survival rates were 96.8% and 90.6%, respectively. Although MRI can be used to obtain reference images in ablation therapy, RF needle puncture is actually performed under sonographic guidance. Therefore, an MR-guided system can be used for ablation monitoring, but not for puncture guidance.

CO_2-enhanced ultrasound (ultrasound angiography)

CO_2-enhanced sonography is a sensitive means of detecting small HCC lesions. Kudo *et al*[64] reported that the detection rate of tumor hypervascularity on CO_2-enhanced sonography (86%) showed that it was more sensitive than digital subtraction arteriography (70%) and CT with iodized oil (82%). Imari *et al*[65] reported that CO_2-enhanced sonography is useful for the detection of hypervascular HCC and PEI treatment of lesions not detectable by conventional US. After direct intra-arterial injection of CO_2, enhancement of the tumor lasts approximately 10-60 min. This enhancement provides sufficient time to perform percutaneous ablation therapy (grade C, level 3b). Chen *et al*[66] reported that thirty-four (64.2%) of the 53 tumors showed complete necrosis after treatment, and the cumulative 1-, 2- and 3-year survival rates of patients who underwent CO_2-enhanced sonographically-guided percutaneous ethanol injection were 81%, 71% and 44%, respectively. However, nodules may become unclear because bubbles become trapped and accumulated in sinusoids with repeated injections of CO_2 microbubbles[67,68]. In addition, this method involves angiographic procedures that are invasive.

Virtual computed tomography sonography

Cross-sectional multiplanar reconstruction images from almost isovoxel volume data can be used for virtual sonographic visualization. This technique is available for patients with HCCs that became enhanced in the arterial phase of dynamic CT but were not well visualized with conventional B-mode US. Virtual CT sonography using magnetic navigation [real-time virtual sonography (RVS); HITACHI Medico, Tokyo, Japan] provides cross-sectional images of CT volume data corresponding to the angle of the transducer in the magnetic field in real-time[69-70]. RVS displays a real-time synchronized multiplanar CT image in precisely the same slice of the US plane. Thus, RVS can be used for real-time needle insertion guidance, especially for nodules demonstrated on CT, but not on US (grade C, level 3b). It has been reported that the technical success rate after a single treatment session was significantly higher in the virtual CT sonography group ($P = 0.017$)[71]. However, RVS has a weakness in that imaging gaps might be attributable to variations in the depth of breath-holding on CT and US examinations, as well as in the fact that the distance error between the magnetic sensor attached to the ultrasonic transducer and the magnetic generator becomes greater on intercostal US examination.

CONCLUSION

Percutaneous ablation therapy guided by dynamic contrast-enhanced US is an efficient approach for HCCs that are not clearly demarcated by B-mode US in both untreated and locally recurrent HCC cases. Moreover, second-generation microbubbles could facilitate dynamic contrast-enhanced US guidance of ablation therapy by extending the time limitation, simplifying the procedure, and improving detectability. RF ablation guided by second generation microbubble-enhanced US could become easier and be an efficient approach for hepatic malignancies that are not clearly depicted on B-mode US.

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ORIGINAL ARTICLE

Moxibustion down-regulates colonic epithelial cell apoptosis and repairs tight junctions in rats with Crohn's disease

Chun-Hui Bao, Lu-Yi Wu, Yin Shi, Huan-Gan Wu, Hui-Rong Liu, Rong Zhang, Li-Qing Yu, Jin-Hai Wang

Chun-Hui Bao, Lu-Yi Wu, Yin Shi, Huan-Gan Wu, Hui-Rong Liu, Rong Zhang, Li-Qing Yu, Jin-Hai Wang, Shanghai Institute of Acupuncture-Moxibustion and Meridian, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China
Author contributions: Shi Y and Bao CH designed the research; Bao CH, Wu LY and Zhang R performed the experiments; Liu HR and Wang JH analyzed the data; Bao CH and Yu LQ wrote and revised the manuscript; and Shi Y and Wu HG supervised the research and edited the manuscript.

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Correspondence to: Yin Shi, PhD, MD, Shanghai Institute of Acupuncture-Moxibustion and Meridian, 650 South Wanping Road, Xuhui District, Shanghai 200030, China. flysy0636@163.com
Telephone:+86-21-64383910 Fax: +86-21-64644238
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Abstract

AIM: To investigate the effects of moxibustion on down-regulation of the colonic epithelial cell apoptosis and repair of the tight junctions in rats with Crohn's disease (CD).

METHODS: Sixty male Sprague-Dawley rats were randomly divided into a normal control (NC) group, a model control (MC) group, an herbs-partitioned moxibustion (HPM) group, a mild-warm moxibustion (MWM) group and a salicylazosulphapyridine (SASP) group, with 12 rats in each group. The CD model rats were treated with trinitrobenzene sulphonic acid to induce intestinal inflammation. The rats in the HPM and MWM groups were treated at the Tianshu (ST25) and Qihai (CV6) acupoints once daily for 14 d, and the SASP group was fed SASP twice daily for 14 d. No additional treatment was given to the MC and NC groups. The microstructure of the colonic epithelium was observed under a transmission electron microscope, the transepithelial resistance was measured using a short-circuit current, colonic epithelial cell apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling assay, and the expression of occludin, claudin-1 and zonula occludens-l (ZO-1) in the colonic epithelial junction was determined by Western blotting and immunofluorescence staining.

RESULTS: Compared with the MC group, the microstructure of the colonic epithelial barrier was significantly improved in rats treated with HPM, MWM or SASP, meanwhile, the current flow was reduced significantly, with values of 168.20 ± 6.14 *vs* 99.70 ± 3.13, 99.10 ± 4.28 and 120.30 ± 3.65 mA, respectively (P = 0.001). However, the HPM and MWM groups had higher current flow rates than the SASP group (99.70 ± 3.13, 99.10 ± 4.28 *vs* 120.30 ± 3.65 mA, P = 0.001). The number of the apoptotic colonic epithelial cells in HPM, MWM and SASP groups was largely reduced (61.5 ± 16.91 *vs* 15.5 ± 8.89, 14.8 ± 6.27 and 24.7 ± 9.68, respectively (P = 0.001); and the expression of occludin, claudin-1 and ZO-1 in the MWM and HPM groups was significantly enhanced (0.48 ± 0.10, 0.64 ± 0.09 *vs* 0.18 ± 0.05 for occludin, 0.12 ± 0.02, 0.17 ± 0.03 *vs* 0.05 ± 0.01 for claudin-1, and 0.08 ± 0.01, 0.11 ± 0.01 *vs* 0.02 ± 0.01 for ZO-1). And in SASP group, the expression of occludin and ZO-1 was also significantly increased (0.27 ± 0.04 *vs* 0.18 ± 0.05 for occludin and 0.05 ± 0.01 *vs* 0.02 ± 0.01 for ZO-1), but there was no significant difference for claudin-1. The HPM and MWM groups had higher expression of occludin, claudin-1 and ZO-1 than the SASP group.

CONCLUSION: HPM and MWM treatment can down-regulate apoptosis of colonic epithelial cells, repair tight junctions and enhance colonic epithelial barrier function in rats with CD.

Key words: Moxibustion; Colonic epithelial cells apoptosis; Tight junctions; Colonic epithelial barrier; Crohn's disease; Rats

Peer reviewer: Dr. Marco Scarpa, PhD, Gastroenterology Section, Department of Surgical and Gastroenterological Sciences, University of Padova, via Giustiniani 2, Padova 35128, Italy

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INTRODUCTION

Crohn's disease (CD) is a chronic and non-specific inflammatory disease that may affect any part of the gastrointestinal tract from the mouth to the anus, thereby causing a wide variety of symptoms. CD has been mostly seen in Western industrialized countries. However, in recent years, the number of individuals affected by CD in China has significantly increased[1]. The exact cause of Crohn's disease is still unknown. In Western medical practices, treatment options include steroids, immunosuppressants and 5-aminosalicylic acid. However, these treatments are restricted to controlling symptoms, maintaining remission, and preventing relapse. Currently, there is no known pharmaceutical or surgical cure for Crohn's disease[2]. Although biologic therapies provide hope for CD patients, the financial cost and the significant side effects limit their application[2].

Moxibustion has been used in China for more than 4000 years to treat various diseases, including gastrointestinal diseases, such as Crohn's[3], ulcerative colitis[4] and irritable bowel syndrome[5]. Mild-warm moxibustion (MWM) and herbs-partitioned moxibustion (HPM) are critical components of moxibustion therapy. MWM is a type of moxa stick moxibustion that is performed by holding an ignited moxa stick a certain distance above the patient's skin, keeping the spot warm and making it reddened, but not burnt. HPM is performed by placing an herbal cake (traditional Chinese medicinal formula dispensing) on the patient's acupoints, followed by the placement and ignition of moxa cones, composed of refined mugwort floss, on the herbal cake to treat diseases. Previously, our group has used moxibustion in the clinic to treat mild or moderate CD, and the efficacy of this treatment can reach nearly 75%[6]. However, the underlying mechanisms of moxibustion treatment of CD remain elusive. Therefore, in this study, we used a rat model with trinitrobenzene sulfonic acid (TNBS)-induced CD and studied the role of moxibustion, as HPM and MWM, in repairing the colonic epithelial barrier using histomorphology, molecular biology, proteinology and electrophysiology.

Several factors have also been shown to contribute to the incidence of CD. For example, defective epithelial barrier function, which can be measured as increased intestinal permeability, has been implicated in CD[7] and can predict relapse during clinical remission[8,9]. However, increased permeability is also present in a subset of unaffected first-degree relatives of patients with CD[10-13] or other functional bowel diseases[14]. It has been demonstrated that damage of the colonic epithelial barrier, as in colonic epithelial death and the connective tissue damage, promotes CD in animal models[15] and humans[16-18]. The mechanisms underlying the loss of barrier function and permeability defects are thought to have a great potential in defining inflammation bowel disease (IBD) pathogenesis and guiding the development of novel treatment for IBD[19].

Therefore, the aim of this study was to investigate whether HPM and MWM can effectively treat CD and to clarify the underlying mechanisms regulating this process. To determine whether HPM and MWM are able to repair the colonic epithelial barrier, we examined the effects of moxibustion on colonic epithelial microstructure and transepithelial resistance, apoptosis of colonic epithelial cells and the expression of the proteins involved in colonic epithelial tight junctions, including occludin, claudin-1 and zonula occludens-l (ZO-1).

MATERIALS AND METHODS

Materials

Sprague-Dawley (SD) rats (male, Specific Pathogen Free, 150 ± 10 g) were purchased from Shanghai University of Traditional Chinese Medicine. All experimental protocols were approved by the Animal Research Ethics Committee of Shanghai University of Traditional Chinese Medicine, No. 09042.

Crohn's disease model establishment

Sixty male SD rats were randomly divided into a normal control (NC) group, a model control (MC) group, an HPM group, an MWM group and an SASP group, with 12 rats in each group. The CD models were established according to Morris' method[20]. Prior to model establishment, the rats were fasted and given water for 24 h. The rats were weighed, and 2% sodium pentobarbital (30 mg/kg) was administered through intraperitoneal injection (ip). The rats were anally injected with a TNBS solution mixed in 50% alcohol at a 1:2 ratio using a rubber tube. All groups of rats received the TNBS injection, with the exception of the normal control group, which received normal saline. The rubber tube was put into the anus 6-8 cm deep. Following the injection, the head of the rat was pushed down for about 1 min to prevent loss of the injected solution. The injection was repeated every 7 d for 4 wk. When the experimental CD rat models were completed, one rat was randomly selected from

each group to ascertain whether the CD models were successful by hematoxylin eosin staining.

Treatment

After the experimental CD rat models were confirmed to be successfully established, the rats were exposed to different treatments. In the HPM group, moxa cones (0.5 cm in diameter and 0.6 cm in length) made of refined mugwort floss were placed on an herbal cake [medicinal formula dispensing (radix) Aconiti praeparata, (cortex) Cinnamomi, *etc.*] at the Tianshu (ST25) and Qihai (CV6) acupoints and ignited. ST25 and CV6 were used to regulate intestinal functions. Two moxa cones were used for each treatment once daily for 14 d. In the MWM group, moxa sticks were ignited 1-2 cm above ST25 and CV6 for 10 min, with the temperature of the local area maintained at 43 ℃ ± 2 ℃. The rats in the SASP group were fed with SASP, which was prepared at the proportion of 1:0.018[21], twice a day for 14 d. The rats in the MC and NC groups did not receive any treatment. After the treatment, five groups of rats were sacrificed simultaneously. The distal colons were dissected at a length of 6 cm.

Transmission electron microscope

Samples (rats colonic mucosa) were cut into 1 mm^3 strips, fixed for 4 h at 4 ℃ in 5% glutaraldehyde, fully washed for 3 times in 0.1 mol/L phosphate buffered saline (PBS), postfixed for 2 h at 4 ℃ in 2% osmium tetroxide, dehydrated in a graded series of ethanols and embedded in Epon 812, cut into ultrathin sections (75 nm) and then stained with uranyl acetate and lead citrate. Sections were viewed in a HITACHI H-600 electron microscope at 80 kV (HITACHI, Tokyo, Japan).

Transepithelial resistance measurement

Colonic epithelial cells were isolated and immediately purified according to Evans *et al*[22]. Transepithelial resistance (Rt) experiments were performed[23] at least 1 wk after seeding the cells on filters, when the Rt of the monolayers reached stable values. The ussing chamber (A and B chamber) (Sigma, United States) used was modified to allow intact inserts to be placed into the chamber. The apical side of the colonic epithelium was directed to the A chamber, and the basolateral side of the colonic epithelium was directed to the B chamber. Both A and B chamber reservoirs contained Krebs-Henseleit solution with 5% oxygenation at 37 ℃. Short-circuit current, open-circuit transepithelial voltage, and transepithelial resistance were recorded by an Axon 2B clamp (Axon, United States). The data were analyzed by p Clamp 8.0 software (Axon, United States).

Western blotting

Western blotting analysis was conducted, as described by Zeissig *et al*[24] We randomly selected six rat colon samples from each group for detection. Rat colon samples were homogenized with a dounce homogeniser in lysis buffer containing 20 mmol/L Tris, pH 7.4, 5 mmol/L magnesium chloride, 1 mmol/L EDTA, 0.3 mmol/L ethyleneglycol tetra-acetic acid, 1 μL/mL aprotinin, 16 μg/mL benzamidine/hydrochloric acid, 10 μg/mL phenanthroline, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 2 mmol/L phenylmethyl-sulphonyl fluoride, 210 μg/mL sodium fluoride, 2.16 mg/mL β-glycerophosphate, 18.4 μg/mL sodium vanadate and 1 μL/mL trypsin inhibitor. The lysates were passed through a needle, and the insoluble material was removed by centrifugation (350 × *g* for 5 min at 4 ℃). The supernatant was centrifuged at 43 000 × *g* for 30 min at 4 ℃, and the pellets were resuspended in lysis buffer. The protein concentrations were determined using a Pierce bicinchoninic acid assay. Aliquots of 5 μg of each sample were separated by polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane. The blots were blocked for 2 h in 5% non-fat milk in phosphate-buffered saline and overnight in 5% bovine serum albumin in phosphate-buffered saline (at 4 ℃) before incubation with primary antibodies for 90 min at room temperature. Primary rabbit monoclonal immunoglobulin (Ig)G antibodies directed against occludin (1:200) (BD, United States), claudin-1 (1:200) (Invitrogen, United States) and ZO-1 (1:200) (Santa Cruz, United States) were used. Peroxidase-conjugated goat anti-rabbit IgG (1:1000) (Bioss, Beijing, China) and chemiluminescence fluid A and B (1:1) (Boster, Wuhan, China) were mixed for 1 h and 1 min, respectively. The reactive bands were detected using chemiluminescent reagents (Pierce Company, Minneapolis, United States).

Immunofluorescence staining

Rat colon samples were washed with 4 ℃ normal saline, fixed in 10% formalin for 24 h, embedded in paraffin, cut into sections and heated at 60 ℃ for 20 min. The sections were soaked in dimethyl benzene twice for 10 min, soaked in 100%, 95% and 70% alcohol for 5 min, and washed with water. A total of 2000 mL of 0.01 mol/L natrium citricum buffer solution (pH 6.0) was added to the pressure kettle, and the colon sections were placed on a staining rack in the pressure kettle. The samples were processed for 1-2 min at 0.142 MPa, removed, and quickly washed with water, followed by an additional wash with PBS for 5 min. Goat serum was added to the samples for 20 min at room temperature, and the superfluous fluid was removed. The samples were incubated with primary monoclonal antibody diluted 1:1000 (occludin Ab, claudin-1 Ab, ZO-1 Ab) in blocking buffer overnight at 4 ℃. The samples were incubated at 37 ℃ for 45 min and were washed 3 times for 2 min with PBS. The samples were incubated with secondary antibody diluted 1:10 000 (FITC-conjugated anti-rabbit) (Abcam, United States) in blocking buffer for 20 min at room temperature. Next, the samples were washed 3 times for 2 min with PBS. The samples were incubated with 5-10 mL DAPI staining solution for 10 min and were washed 4 times for 5 min with PBS. One drop of 50% glycerine was added to the sections, and laser confocal microscopy (Nikon, Japan)

was used to detect the expression of occludin, claudin-1 and ZO-1. The protein expression was evaluated in the 5 fields of vision.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

Transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) staining was performed using the Dead-End™ Fluorometric TUNEL System (Promega, Madison, WI). A total of 5×10^7/mL cells were fixed with a 4% formaldehyde solution for 10 min and washed twice for 5 min with PBS. The slides were equilibrated with equilibration buffer and were incubated for 30 min at 37 ℃ with recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer. The negative control sections were incubated with control incubation buffer without the rTdT enzyme. The slides were analyzed using a Leica TCS SP5 confocal microscope (Germany).

Statistical analysis

Results were expressed as mean ± SD. Statistical analyses were performed using SPSS 13.0 (SPSS Inc. Chicago, Illinois). Differences in mean were compared by one way analysis of variance. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Microstructure of rat colonic epithelial tissue in different groups

In the NC group (Figure 1A and B), the morphology of the colonic epithelia, the cell membrane and the nuclear membrane were integral. The nucleolus could be seen clearly. In the cytoplasm, both the endoplasmic reticulum and mitochondria could be seen clearly. The junction of the epithelial cells was tight. The junctions among the cells were not broadened. We also observed abundant secretion by the cells, and there were villi present on the cell surfaces.

In the MC group (Figure 1C and D), the colonic epithelial cells were reduced in size, the integrity of the epithelial membrane and the nuclear membrane was compromised, the nuclei were absent, and most of the organelles inside the cytoplasm were absent. The MC group colonic epithelial cells contained bubbles inside the cytoplasm, and the number of mitochondria was significantly reduced. The junctions between the epithelial cells were loose, and a significant broadening of the colonic cells was observed. No villi could be detected on the cell surface.

In the colonic epithelial cells of the SASP group (Figure 1E and F), the integrity of the cell and nuclear membranes were disrupted, the nuclei of the cells were absent, the structure of the organelles inside the cytoplasm could not be seen clearly, and the number of mitochondria was relatively normal. In addition, the size of the colonic epithelial cells was significantly reduced, and bubbles of different sizes were present in the cytoplasm. The junctions between the epithelial cells were not tight, and the intracellular space was enlarged. No villi could be seen on the cell surfaces.

In the colonic epithelial cells of the HPM group (Figure 1G and H), the integrity of the nuclear membranes were almost normal. The nuclei of the cells were clear, the number of the organelles was reduced and the cell membranes were injured. Many mitochondria could be observed, the junctions between the epithelial cells were relatively tight, the intracellular spaces were increased, and some villi could be seen on the cell surfaces.

In the colonic epithelial cells of the MWM group (Figure 1I and J), the integrity of the nuclear membrane was relatively normal. The nuclei of the cells were present, a few bubbles appeared in the cytoplasm, and many mitochondria could be detected. The junctions between the epithelial cells were tight, while some intracellular spaces were tight and some intracellular spaces were broadened. Particle secretion was detected in the intracellular space. There were a few villi on the cell surfaces.

Table 1 Comparison of current value and current flow of rat colonic epithelium in different groups (n = 10, mean ± SD)

Group	Ω/cm^2	mA
NC	0.8603 ± 0.08396	58.60 ± 5.5217
MC	0.2976 ± 0.01099[a]	168.20 ± 6.1427[a]
SASP	0.4160 ± 0.01275[a,c]	120.30 ± 3.6530[a,c]
MWM	0.5054 ± 0.0223[a,c,e]	99.10 ± 4.2804[a,c,e]
HPM	0.5020 ± 0.01581[a,c,e]	99.70 ± 3.1287[a,c,e]

[a]$P < 0.01$ *vs* normal group; [c]$P < 0.01$ *vs* model group; [e]$P < 0.01$ *vs* SASP group. NC: Normal control; MC: Model control; SASP: Salicylazosulphapyridine group; MWM: Mild-warm moxibustion group; HPM: Herbs-partitioned moxibustion group.

Function of rat colonic epithelial barrier in response to moxibustion treatment

Compared with normal rats, the current flow of the colonic epithelial cells was significantly reduced in the MC group (168.20 ± 6.14 *vs* 58.60 ± 5.52 mA, respectively, $P = 0.001$), but the transepithelial resistance was increased (0.30 ± 0.01 *vs* 0.86 ± 0.08, respectively, $P = 0.001$) (Table 1). Following the HPM, MWM or SASP treatment, the transepithelial resistance was increased, and the current flow was decreased compared with normal rats. Compared with the MC group, the transepithelial resistance was significantly increased in rats treated with HPM, MWM or SASP, with values of 0.30 ± 0.01 *vs* 0.50 ± 0.02, 0.51 ± 0.02, 0.42 ± 0.01, respectively ($P = 0.001$). Meanwhile, the current flow was reduced, with values of 168.20 ± 6.14 *vs* 99.70 ± 3.13, 99.10 ± 4.28, 120.30 ± 3.65 mA, respectively ($P = 0.001$). However, we found that the current flow in the HPM and SASP groups was enhanced compared with the SASP group, with values of 0.50 ± 0.02, 0.51 ± 0.02 *vs* 0.42 ± 0.01 and 99.70 ± 3.13, 99.10 ± 4.28 *vs* 120.30 ± 3.65 mA, respectively ($P = 0.001$), but was lower than the flow rate of the normal group. These data suggest

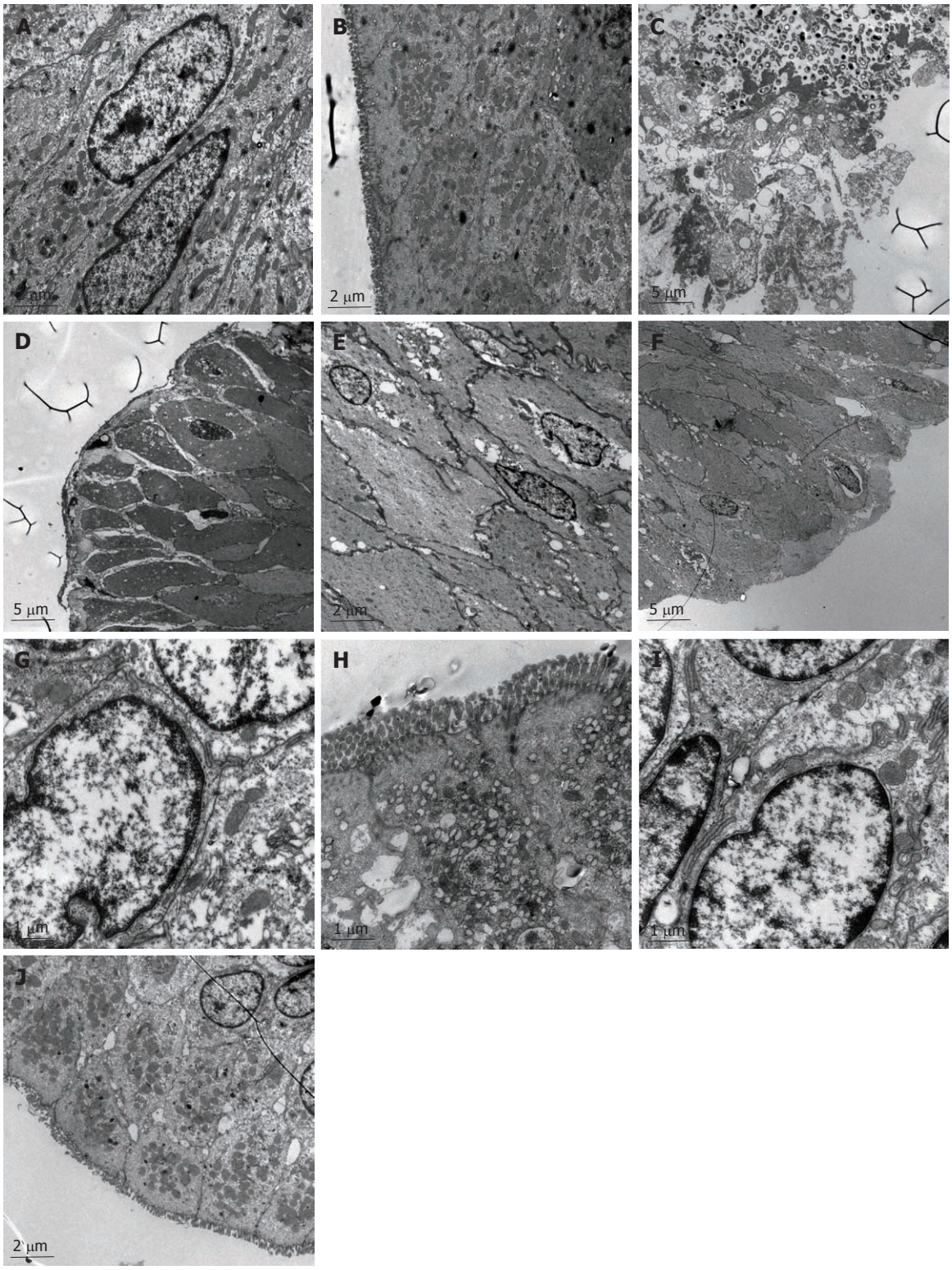

Figure 1 Microstructure of rat colonic epithelial tissues in different groups. A, B: Normal group (6000 ×, 6000 ×); C, D: Model group (2500 ×, 2500 ×); E, F: Salicylazosulphapyridine group (6000 ×, 2500 ×); G, H: Mild-warm moxibustion group (12 000 ×, 12 000 ×); I, J: Herbs-partitioned moxibustion group (12 000 ×, 4000 ×).

that the CD rats treated with HPW, MWM or SASP had colonic epithelial cells with significantly increased transepithelial resistance, which may further cause enhanced repair and/or protection of the colonic epithelial barrier (Figure 2A and B).

Reduced apoptosis of rat colonic epithelial cells in response to moxibustion treatment

Compared with the NC group, the number of apoptotic colonic epithelial cells was significantly increased in the MC group (61.5 ± 16.91 *vs* 11.5 ± 4.48, respectively, *P*

Table 2 Comparison of number and rate of apoptosis of rat colonic epithelial cells in different groups (n = 10, mean ± SD)

Group	Number	Rate (%)
NC	11.5 ± 4.48	0.0220 ± 0.0085
MC	61.5 ± 16.91[a]	0.0996 ± 0.0257[a]
SASP	24.7 ± 9.68[a,c]	0.0454 ± 0.0210[a,c]
MWM	14.8 ± 6.27[c,f]	0.0271 ± 0.0132[f]
HPM	15.5 ± 8.89[c,f]	0.0269 ± 0.0139[f]

[a]P < 0.01 *vs* normal group; [c]P < 0.01 *vs* model group; [f]P < 0.05 *vs* SASP group. NC: Normal control; MC: Model control; SASP: Salicylazosulphapyridine group; MWM: Mild-warm moxibustion group; HPM: Herbs-partitioned moxibustion group.

Table 3 Expressions of occludin, claudin-1 and zonula occludens-1 in rat colonic epithelium in different groups (n = 10, mean ± SD)

Group	Occludin	Claudin-1	ZO-1
NC	1.26 ± 0.12	0.25 ± 0.04	0.28 ± 0.02
MC	0.18 ± 0.05[b]	0.05 ± 0.01[b]	0.02 ± 0.01[b]
SASP	0.27 ± 0.04[b,c]	0.08 ± 0.02[b]	0.05 ± 0.01[b,d]
MWM	0.48 ± 0.10[b,d,e]	0.12 ± 0.02[b,d,e]	0.08 ± 0.01[b,d,f]
HPM	0.64 ± 0.09[b,d,f]	0.17 ± 0.03[a,d,f]	0.11 ± 0.01[b,d,f,g]

[a]P < 0.05, [b]P < 0.01 *vs* normal group; [c]P < 0.05, [d]P < 0.01 *vs* model group; [e]P < 0.05, [f]P < 0.01 *vs* SASP group; [g]P < 0.01 *vs* MWM group. ZO-1: Zonula occludens-l; NC: Normal control; MC: Model control; SASP: Salicylazosulphapyridine group; MWM: Mild-warm moxibustion group; HPM: Herbs-partitioned moxibustion group.

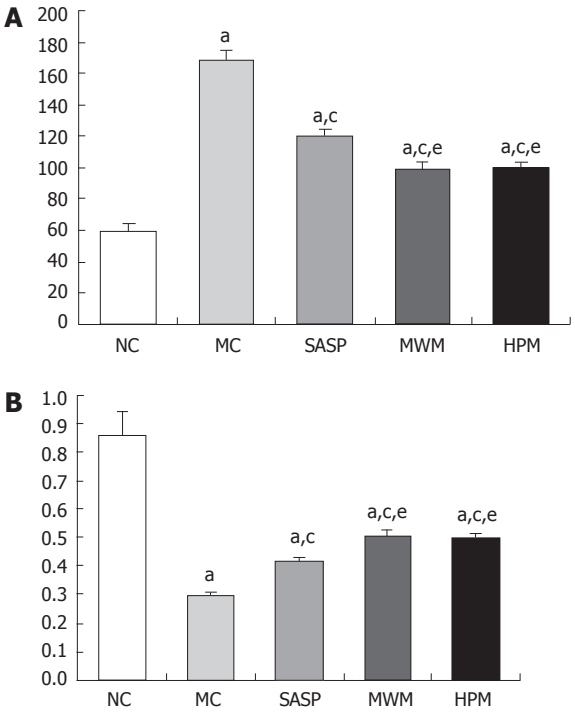

Figure 2 Current flow of rat colonic epithelial cells in different groups. A: The current flow of the rat colonic epithelium; B: The electric resistance of the rat colonic epithelium. NC: Normal control; MC: Model control; SASP: Salicylazosulphapyridine group; MWM: Mild-warm moxibustion group; HPM: Herbs-partitioned moxibustion group. [a]P < 0.01 *vs* normal group; [c]P < 0.01 *vs* model group; [e]P < 0.01 *vs* SASP group.

= 0.001) (Table 2). However, the number of colonic epithelial cells undergoing apoptosis decreased in the MWM, HPM and SASP groups (61.5 ± 16.91 *vs* 15.5 ± 8.89, 14.8 ± 6.27, 24.7 ± 9.68, respectively, P = 0.001). However, no significant differences in colonic epithelial cell apoptosis were found among the HPM, MWM and SASP groups. In addition, compared with the NC group, the differences in the number of apoptotic colonic epithelial cells were not statistically significant between the MWM and HPM groups; however, there was a difference in apoptosis between the SASP and NC groups (24.7 ± 9.68 *vs* 11.5 ± 4.48, respectively, P = 0.017). Moreover, the number of apoptotic colonic epithelial cells in the MC group was significantly increased compared with the NC group (0.10 ± 0.03 *vs* 0.02 ± 0.09, respectively, P = 0.001), and compared with the MC group, the number of apoptotic colonic epithelial cells in the HPM, MWM and SASP groups was also largely reduced (0.03 ± 0.01, 0.03 ± 0.01, 0.05 ± 0.02 *vs* 0.10 ± 0.03, respectively, P = 0.001). There was a larger number of apoptotic cells in the colonic epithelium in the SASP group than in the MWM or HPM groups (0.05 ± 0.02 *vs* 0.03 ± 0.01, 0.03 ± 0.01, respectively, P = 0.024 and P = 0.025). These data suggested that the MWM and HPM were able to down-regulate the apoptosis of colonic epithelial cells in rats with CD (Figure 3).

Expression of occludin, claudin-1 and zonula occludens-l in rat colonic epithelium by Western blotting

Compared with the NC group, the expressions of occludin, claudin-1 and ZO-1 were significantly reduced in the MC, SASP, MWM and HPM groups (Table 3). Compared with the MC group, the expressions of occludin, claudin-1 and ZO-1 in the MWM and HPM groups were significantly increased (0.48 ± 0.10, 0.64 ± 0.09 *vs* 0.18 ± 0.05, P = 0.002 and P = 0.001, respectively, for occluding; 0.12 ± 0.02, 0.17 ± 0.03 *vs* 0.05 ± 0.01, P = 0.001 for claudin-1; and 0.08 ± 0.01, 0.11 ± 0.01 *vs* 0.02 ± 0.01, P = 0.001 for ZO-1). And in SASP group, the expressions of occludin and ZO-1 were significantly increased (0.27 ± 0.04 *vs* 0.18 ± 0.05, P = 0.043 for occludin and 0.05 ± 0.01 *vs* 0.02 ± 0.01, P = 0.001 for ZO-1), but there was no significant difference for claudin-1. Moreover, the HPM and MWM groups had higher expression of occludin, claudin-1 and ZO-1 than the SASP group (0.48 ± 0.10, 0.64 ± 0.09 *vs* 0.27 ± 0.04, respectively, P = 0.015 and P = 0.001 for occluding; 0.12 ± 0.02, 0.17 ± 0.03 *vs* 0.08 ± 0.02, P = 0.021 and P = 0.001 for claudin-1; and 0.08 ± 0.01, 0.11 ± 0.01 *vs* 0.05 ± 0.01, P = 0.002 and P = 0.001 for ZO-1). The expression of ZO-1 between MWM and HPM groups was significantly different (0.08 ± 0.01 *vs* 0.11 ± 0.01, P = 0.001), but there was no significant difference for occludin and claudin-1 (Figure 4).

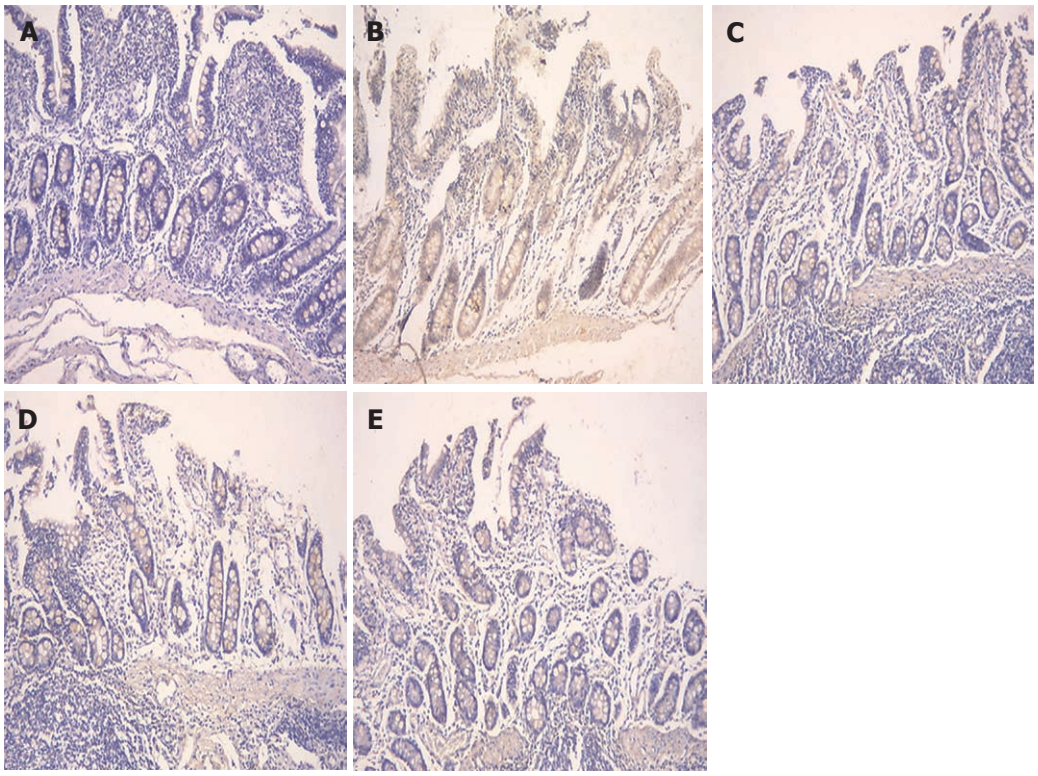

Figure 3 Apoptosis of colonic epithelial cells. A: Normal group (100 ×); B: Model group (100 ×); C: Salicylazosulphapyridine group (100 ×); D: Mild-warm moxibustion group (100 ×); E: Herbs-partitioned moxibustion group (100 ×).

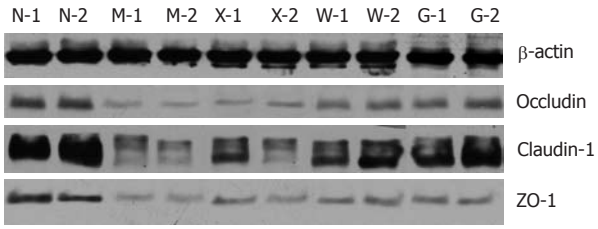

Figure 4 Expression of occludin, claudin-1 and zonula occludens-l in rat colonic epithelium. N: Normal group; M: Model group; X: Salicylazosulphapyridine group; W: Mild-warm moxibustion group; G: Herbs-partitioned moxibustion group.

Expressions of occludin, claudin-1 and zonula occludens-l in rat colonic epithelium by immunofluorescence staining

The expression of occludin (Figure 5), claudin-1 (Figure 6) and ZO-1 (Figure 7) was detected in the tight junctions and subjunctional lateral membranes of surface and crypt enterocytes under confocal immunofluorescence microscope, and the strength of the immunofluorescence signals was significantly reduced in these sites in the MC group. However, in the HPM, MWM or SASP groups, the signal significantly increased as HPM > MWM > SASP, which was consistent with the results obtained using Western blotting.

DISCUSSION

CD is a chronic, recurrent and refractory disease. It primarily causes abdominal pain, diarrhoea with abdominal mass, fistulisation and intestinal obstruction. Patients with CD usually have symptoms, including loss of appetite, sallow complexion, spontaneous perspiration, night sweat, insomnia and loss of energy and weight. The cause of this disease still remains unclear. Although the current pharmaceutical treatments are helpful in temporarily controlling the primary symptoms, they are unable to cure the disease.

Moxibustion has been used to treat various diseases in China and in other countries. Joos *et al*[3] found that Crohn's Disease Activity Index can be decreased from 250 ± 51 to 163 ± 56 (P = 0.003) in patients treated with acupuncture and moxibustion. Furthermore, the researchers observed that the general conditions of the patients that received acupuncture and moxibustion were also improved compared with the controls. We have used HPM and MWM to treat patients with mild and moderate CD. We observed that HPM and MWM alleviates abdominal pain and diarrhoea and improves sallow complexion, loss of energy and insomnia in CD patients[6]. So far, no side effects have been observed. However, the mechanisms underlying moxibustion-induced CD alleviation are undefined.

In this study, we observed under transmission electron microscope that there were reduced apoptosis, clearer cell structures and greater numbers of mitochondria in the colonic epithelial cells of rats treated with HPM or MWM compared with the rats in the MC or SASP groups. In addition, compared with the MC group, we also found that apoptosis of rat colonic epi-

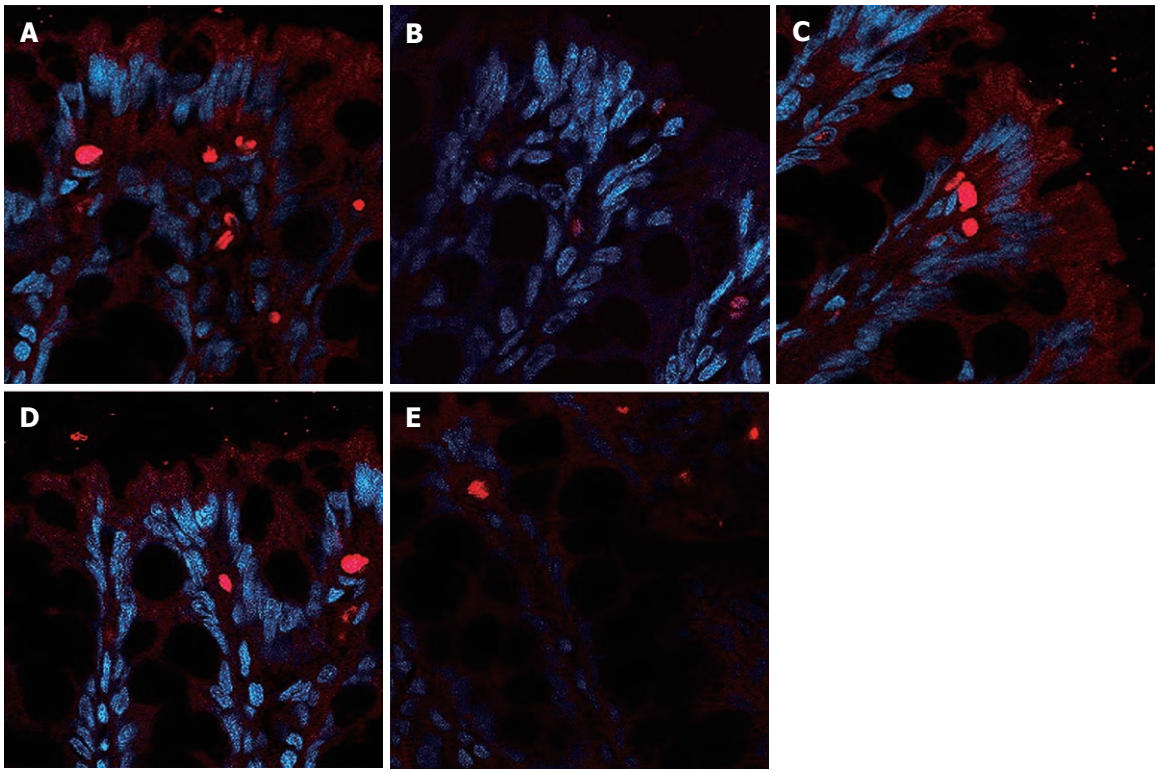

Figure 5 Expression of occludin in rat colonic epithelium. A: Normal group; B: Model group; C: Salicylazosulphapyridine group; D: Mild-warm moxibustion group; E: Herbs-partitioned moxibustion group.

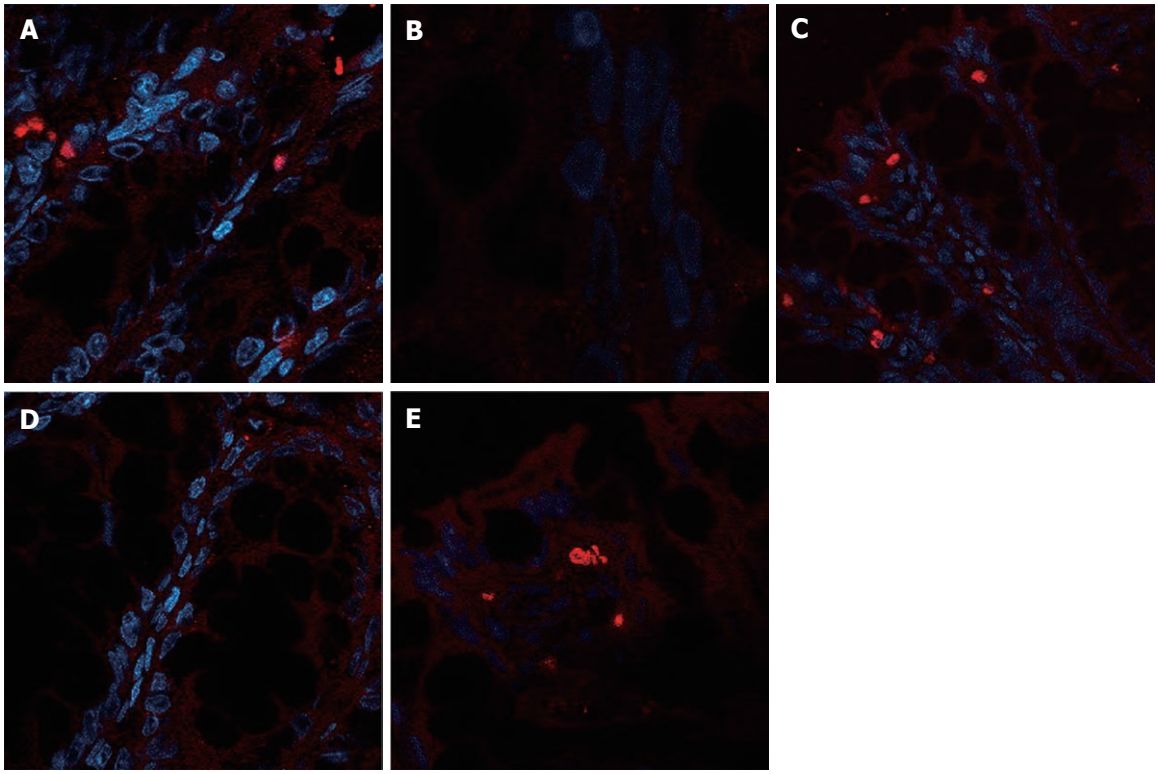

Figure 6 Expression of claudin-1 in rat colonic epithelium. A: Normal group; B: Model group; C: Salicylazosulphapyridine group; D: Mild-warm moxibustion group; E: Herbs-partitioned moxibustion group.

thelial cells was significantly reduced in the HPM, MWM and SASP groups. Previously, we found that HPM and MWM could reduce the apoptosis of the colonic epithelial cells in rats with ulcerative colitis, which was medi-

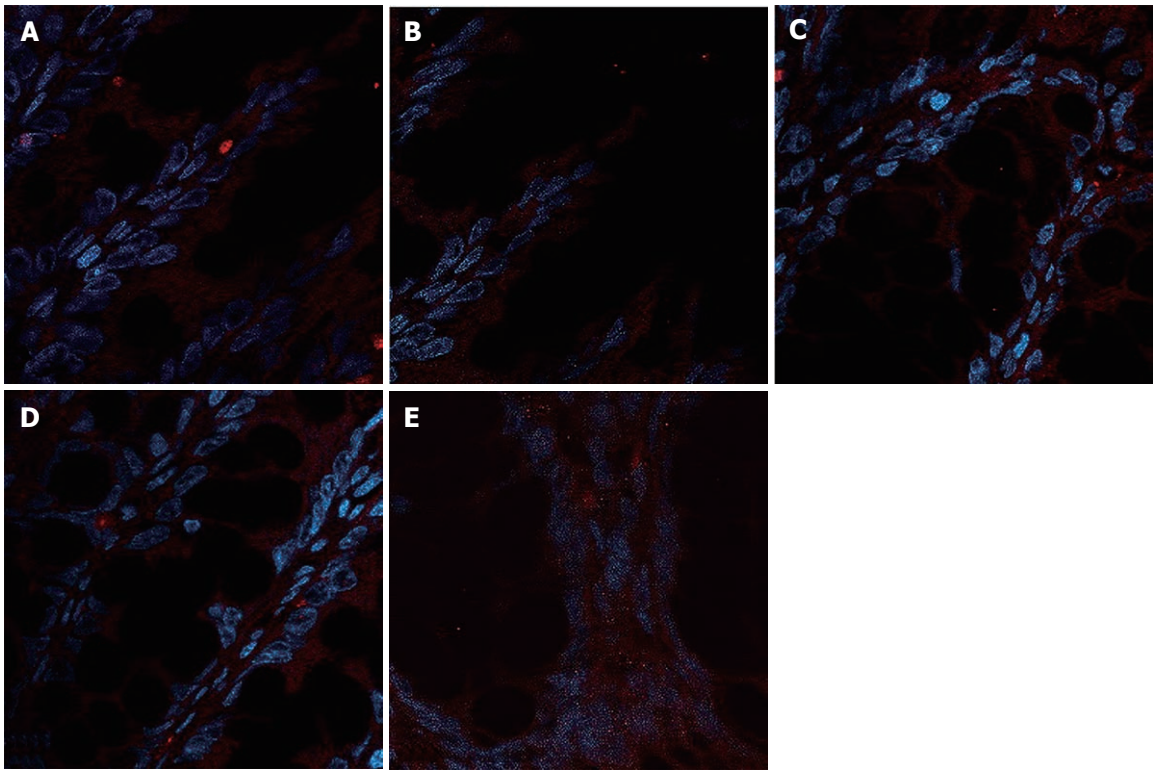

Figure 7 Expression of zonula occludens-I in rat colonic epithelium. A: Normal group; B: Model group; C: Salicylazosulphapyridine group; D: Mild-warm moxibustion group; E: Herbs-partitioned moxibustion group.

ated by the Bcl-2/Bax and Fas/FasL pathways[25].

The balance between epithelial cell apoptosis and proliferation is pivotal for the maintenance of mucosal integrity in the intestine[26]; Di Sabatino *et al*[27] found that dysregulation of this balance is caused by an increase in apoptosis, which leads to cellular loss and tissue atrophy and is likely to be involved in the pathogenesis of CD. This increase was not mediated by a Fas-Fas ligand or by abnormal E-cadherin distribution. Increased matrix metalloproteinase-1 release from lamina propria mononuclear cells may be one of the possible mechanisms responsible for the increased apoptosis of enterocytes in CD. Another study showed that an increase of tumor necrosis factor-alpha mucosal release may promote enterocyte apoptosis in CD[28]. Whether similar mechanisms are used by moxibustion to reduce apoptosis in colonic epithelia needs further investigation.

In addition, we investigated the effect of HPM or MWM on the tight junctions of colonic epithelial cells. We observed that the epithelial cell junction was tighter, the intracellular space was relatively broadened and the expression of occludin, claudin-1 and ZO-1 involved in the tight junctions was significantly enhanced in the HPM and MWM groups as compared with the MC and the SASP groups. In addition, we found that the electric current of the cells in the MC group was significantly higher than in the other groups, and the electric current of the epithelial cells in the HPM or MWM groups was lower than in the SASP group.

The tight junction seals the space between adjacent epithelial cells, and in intact gastrointestinal epithelia, tight junction permeability is the rate-limiting step that defines the overall epithelial permeability[14]. Increased permeability of the tight junctions is thought to contribute to CD[24,29]. Occludin, the claudins and ZO-1 are key proteins in the tight junctions[30]. Patients with active or inactive CD have increased intestinal permeability and differential expression of these tight junction proteins[31,32], which may be associated with abnormal expression of the proinflammatory cytokines TNF-α, IFN-γ and IL-13[33-36]; whether moxibustion increases the expression of the proteins involved in tight junctions through similar mechanisms or *via* other mechanisms requires further investigation.

Overall, we found that HPM or MWM administration of the Tianshu (ST25) and Qihai (CV6) acupoints can reduce colonic epithelial cell apoptosis and repair the intracellular tight junctions in rats with CD, which results in a significant improvement of their barrier function and a decrease in epithelial permeability. Our findings provide the novel mechanisms by which moxibustion can significantly improve CD symptoms. These findings suggest that the moxibustion therapy can be used as an effective treatment for Crohn's disease.

ACKNOWLEDGMENTS

We want to thank Dr. Xiao-Ping Zhang for his help and technical support in this study.

COMMENTS

Background

Crohn's disease (CD) is a chronic, recurrent and refractory disease. The pathophysiology of CD has not been completely elucidated, making its treatment difficult. Previously, the authors used moxibustion in the clinic to treat mild and moderate CD, and the efficacy of moxibustion has reached nearly 75%. However, the regulatory effect of moxibustion on CD is still unknown.

Research frontiers

Defective epithelial barrier function is considered to be a critical factor that promotes CD. In this study, the authors investigated the effect of moxibustion on the colonic epithelial barrier.

Innovations and breakthroughs

Moxibustion at ST25 and CV6 has been found to be effective against CD. Both HPM and MWM are able to prevent the apoptosis of colonic epithelial cells, repair tight junctions, and enhance the function and integrity of the colonic epithelial barrier in rats with CD.

Applications

The experimental data can be used in further studies on moxibustion therapy in treatment of CD.

Peer review

This is an interesting study reporting how moxibustion can repair the colonic epithelial barrier in rats with Crohn's disease.

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BRIEF ARTICLE

Burden of celiac disease in the Mediterranean area

Luigi Greco, Laura Timpone, Abdelhak Abkari, Mona Abu-Zekry, Thomas Attard, Faouzi Bouguerrà, Paskal Cullufi, Aydan Kansu, Dusanka Micetic-Turk, Zrinjka Mišak, Eleftheria Roma, Raanan Shamir, Selma Terzic

Luigi Greco, Laura Timpone, European Laboratory for Food Induced Diseases, Federico II University of Naples, 80131 Naples, Italy
Abdelhak Abkari, Hopital des Enfant Ibn Rochd de Casablanca, 20100 Casablanca, Morocco
Mona Abu-Zekry, Children's Hospital, Gastrointestinal Unit, Cairo University, 12611 Giza, Egypt
Thomas Attard, University of Malta, Medical School, Guardamangia MSD 06, Malta
Faouzi Bouguerrà, Hopital des Enfants de Tunis, 1006 Tunis, Tunisia
Paskal Cullufi, Department of Gastroenterology and Hepatology, University Hospital Center "Mother Teresa", nr 371 Tirana, Albania
Aydan Kansu, Department of Pediatric Gastroenterology, Ankara University, Dikimevi/Mamak, Ankara 06100, Turkey
Dusanka Micetic-Turk, University Medical Centre, Paediatric Department, Ljubljanska, 2000 Maribor, Slovenia
Zrinjka Mišak, Children's Hospital Zagreb, 10000 Zagreb, Croatia
Eleftheria Roma, Athens University, Aghia Sophia Children's Hospital, Goudi, 11527 Athene, Greece
Raanan Shamir, Schneider Children's Medical Center of Israel, Tel-Aviv University, Petach-Tikva 49202, Israel
Selma Terzic, University Clinical Center Tuzla, Children's Hospital, 75000 Tuzla, Bosnia Herzegovina
Author contributions: Greco L, Timpone L, Abkari A, Abu-Zekry M, Attard T, Bouguerrà F, Cullufi P, Kansu A, Micetic-Turk D, Mišak Z, Roma E, Shamir R and Terzic S directly participated in the study, including substantial contributions to conception and design of the study and acquisition of data; Greco L, Timpone L and Shamir R analyzed the data, wrote the manuscript and provided statistical analysis of data.
Supported by European Laboratory for Food Induced Diseases, Federico II University of Naples
Correspondence to: Luigi Greco, Professor of Medicine, Chief, European Laboratory for Food Induced Diseases, Federico II University of Naples, 80131 Naples, Italy. ydongre@unina.it
Telephone: +39-081-7463275 Fax: +39-081-7462375
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Abstract

AIM: To estimate the burden of undiagnosed celiac disease (CD) in the Mediterranean area in terms of morbidity, mortality and health cost.

METHODS: For statistics regarding the population of each country in the Mediterranean area, we accessed authoritative international sources (World Bank, World Health Organization and United Nations). The prevalence of CD was obtained for most countries from published reports. An overall prevalence rate of 1% cases/total population was finally estimated to represent the frequency of the disease in the area, since none of the available confidence intervals of the reported rates significantly excluded this rate. The distribution of symptoms and complications was obtained from reliable reports in the same cohort. A standardized mortality rate of 1.8 was obtained from recent reports. Crude health cost was estimated for the years between symptoms and diagnosis for adults and children, and was standardized for purchasing power parity to account for the different economic profiles amongst Mediterranean countries.

RESULTS: In the next 10 years, the Mediterranean area will have about half a billion inhabitants, of which 120 million will be children. The projected number of CD diagnoses in 2020 is 5 million cases (1 million celiac children), with a relative increase of 11% compared to 2010. Based on the 2010 rate, there will be about 550 000 symptomatic adults and about 240 000 sick children: 85% of the symptomatic patients will suffer from gastrointestinal complaints, 40% are likely to have anemia, 30% will likely have osteopenia, 20% of children will have short stature, and 10% will have abnormal liver enzymes. The estimated standardized medical costs for symptomatic celiac patients during the delay between symptom onset and diagnosis (mean 6 years for adults, 2 years for children) will be about €4 billion (€387 million for children) over the next 10 years. A delay in diagnosis is expected to increase mortal-

ity: about 600 000 celiac patients will die in the next 10 years, with an excess of 44.4% *vs* age- and sex-matched controls.

CONCLUSION: In the near future, the burden of CD will increase tremendously. Few Mediterranean countries are able to face this expanding epidemic alone.

Key words: Pediatric; Celiac disease; Short stature; Anemia; Osteopenia; Purchasing power parity; Standardized mortality rate; Mediterranean area

Peer reviewer: Ron Shaoul, MD, Director, Pediatric Gastroenterology and Nutrition Unit, Meyer Children's Hospital, Rambam Medical Center, PO Box 9602, Haifa 31096, Israel

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INTRODUCTION

Recent epidemiological studies show that the prevalence of celiac disease (CD) is underestimated not only in Europe, but also among the populations of Mediterranean regions such as the Middle East and North Africa[1-3], where its prevalence is similar to that recently observed in Western countries[4]. Indeed, in these two regions, a very high prevalence of CD has recently been reported both in the general population and in at-risk groups[2]. These high frequencies are associated with the widespread consumption of wheat and barley[1,5] and the high frequency of the DR3-DQ2 CD-predisposing haplotypes in these populations[6,7]. But these factors alone do not satisfactorily account for the spread of the CD epidemic in recent years[8,9]. The prevalence of CD among the general population varies from 0.14% to 1.17%[10-20]: 1%-1.3% in Turkey[10-12], 0.6%-0.96% in Iran[13,14], 0.5% in Egypt[15], 0.6% in Tunisia and Israel[16-19], and < 0.5% in Jordan, Lebanon and Kuwait[5,20]. Among high-risk groups [including patients with a positive family history, insulin-dependent diabetes mellitus (IDDM), thyroiditis] the prevalence of CD ranges from 2.4% to 44%, assessed by serological markers and biopsy[21-24].

Egypt, and indeed all North African countries, were significant producers of wheat, and largely used barley for beer brewing; they were considered the "granary" of Romans for over 4 centuries. Bread, mostly made of wheat flour and called "the survival" in some local languages[1], has been a staple food for thousands of years. Similarly, the widespread use of couscous [from grossly milled durum wheat (*Triticum durum*)] dates back over 2000 years. But the use of wheat and other gluten-containing cereals is also increasing in the countries where it has been a staple for centuries[25,26].

The diffusion of pasta across all the Mediterranean countries is relatively recent and stems from the industrial development of grain processing. Unfortunately, a side effect of this positive dispersal may be the enormous increase in gluten intolerance, which is at a truly epidemic level. CD is now a widespread public health problem that also involves the populations of developing countries, as well as China and India[27,28]. However, this epidemic is not fully recognized since a sizeable number of cases are neither diagnosed nor cared for. In many Mediterranean countries, few cases are diagnosed because of the low level of awareness, knowledge and skill to deal with the problem, the lack of diagnostic resources and the attribution of CD symptoms to other, similar, illnesses[5,20]. The low awareness of CD often leads to a delay in diagnosis, which contributes to an excess of medical costs (CD includes growth failure, infant malnutrition, gastrointestinal diseases, anemia and more than 20 associated symptoms and conditions) and mortality.

All partners taking part in this study agreed that, to date, the best available estimation of CD-associated medical cost was that reported by Long *et al*[29], and supported by Hershcovici *et al*[31]. The annual medical cost in the year preceding the diagnosis of CD, excluding diagnostic costs, was estimated to be $5023/patient, $1764 more than the cost of the same patients in the year after diagnosis[29]. In the four years preceding the diagnosis of symptomatic CD, the direct medical cost was estimated to be $11 037/patient. For a symptom- and age-matched control individual, not affected by CD, the cost after 4 years was estimated at $7073, with a difference of $3964 (about $1000/patient per year). This difference is due to increased in-patients admissions, out-patient cost, laboratory tests, radiology, and office visits[29]. The diagnosis of CD resulted in a 30% reduction in direct medical expenditure. A similar 30% reduction in direct medical costs after diagnosis of CD was reported by Green *et al*[30]; the mean medical expenditure decreased from $8502 per capita to $7133 for the 2 years after diagnosis of CD.

The CD epidemic is the largest epidemic of food-induced permanent disease in the Euro-Mediterranean region. Very few countries of this region are able to face this expanding problem. The aim of this study was to estimate what the burden of CD will be in the near future, and how the CD epidemic will affect morbidity, mortality and health costs. We aim to provide stakeholders with a reliable prediction of the incoming picture of CD in the Mediterranean area, and so enable them to take action to face this epidemic.

MATERIALS AND METHODS

Population statistics

For statistics regarding each country in the Mediterra-

nean area, we accessed authoritative international sources (World Bank, World Health Organization and United Nations). Population size, median age, number of children (0-14 years), population growth rate, birth rate, death rate, infant mortality rate and literacy were retrieved and validated across multiple sources. The projected population from 2010 to 2020 was computed by adopting the 2008 growth rate as a constant over the following decade because the predicted rate of change of the growth rate would have not significantly affected our estimate. The number of children was incremented yearly by the birth rate and corrected for the infant mortality rate although mortality from 1 to 14 years is minimal in all the countries included in this evaluation.

Celiac disease

The prevalence of CD among the populations of Mediterranean countries, such as the Middle East and North Africa[1-3], is similar to that recently observed in Western countries[4]. The prevalence of CD among the general population varies from 0.14% to 1.17%[10-20]: 1%-1.3% in Turkey[10-12], 0.6%-0.96% in Iran[13,14], 0.5% in Egypt[15], 0.6% in Tunisia and Israel[16-19], and < 0.5% in Jordan, Lebanon and Kuwait[5,20]. An overall prevalence rate of 1% cases/total population was finally estimated to better represent the frequency of the disease in the area, since none of the available confidence intervals of the reported rates significantly excluded this 1% rate. The rate of symptomatic *vs* asymptomatic patients was obtained from several reliable reports from the area[3,9,10,17]. In summary, 85% of symptomatic patients are likely to suffer from gastrointestinal symptoms, which include diarrhea, abdominal pain, vomiting, irritable bowel, and gastritis[5,13,20,32-37]. Among the non-gastrointestinal complaints, the available estimates suggest 20% of children are affected by short stature[5,20,33-35,37], 40% of all cases are affected by anemia[5,20,32,36,37], 30% are afflicted by osteopenia[32,33,35,37], and 10% by abnormal liver enzymes[37,38].

Mortality has been reported in excess of 1.8 compared to age- and sex-matched controls[31,39,40]. The risk of cancer in undiagnosed adults is significantly increased and the mortality is almost doubled in the total cohort of affected persons compared with the general population[41,44].

Crude medical costs

Crude health costs were estimated for the years between symptoms and diagnosis only for symptomatic adults and children, and were standardized for purchasing power parity (PPP) to account for the different economic profile among Mediterranean countries. Since gross national product is different across countries, the PPP is based on the law of one price; in the absence of transaction costs, identical goods will have the same price in different markets. The PPP equalizes the purchasing power of different currencies for a given basket of goods, thereby providing a standardized estimate of cost across countries.

We assume that the cohort of CD without symptoms does not increase the average medical cost compared to non CD individuals (but this should also be revised, since a significant number of patients identified by screening had a posteriori clinical symptoms). Therefore, medical costs are estimated only for 1:7 adults and 1:5 children with CD symptoms.

Table 1 Excess need of health resources before the diagnosis of celiac disease

	n	Adult cost (€)	*n*	Child cost (€)
In-patient admission	2	9818	1	2254
Out-patient admission	1	879	1	586
Medical consultations	3	100	3	150
Specialist consultations	2	150	1	50
Lab test	4	446	2	297
Total per patient		11 393		3337

For each individual adult we assigned (on the basis of the reports cited and the clinical experience of the study partners), a minimal period of 6 years of delay between symptom onset and diagnosis of the disease[45,46], while this delay was two years for each assigned child with CD[9,20]. During that period an adult with CD required, in excess of age- and sex-matched controls, at least: 2 in-patient admissions, 1 out-patient admission, 3 primary medical consultations, 2 specialized consultations, and 4 laboratory tests. Similarly, children needed at least: 1 in-patient admission and 1 out-patient admission, 3 medical consultations, 1 specialized consultation and 2 laboratory tests (Table 1).

Estimated medical costs

The costs of health services were estimated based on the 2007 costs of the Italian National Health Service (NHS) which is similar to that of several European countries. We summed the total costs of the medical services required for each child or adult patient to obtain a standardized cost/per patient before the diagnosis of CD was made (Table 1). In this way, we obtained an estimation of the financial load (only for medical expenses) of symptomatic patients. The estimated cost according to the Italian NHS was then standardized for each country according to its PPP index. The total load of medical expenses for each country was calculated by multiplying the individual cost by the number of symptomatic patients estimated (adults and children).

Summary of reference data

(1) CD prevalence = 1%; incidence: new cases/year estimated at 1% of the live births, corrected for infant mortality rate; (2) symptomatic adults: 1 of every 7 cases, children 1:5 cases; (3) mortality of the total CD cohort: standardized mortality rate 1.8 compared to age- and sex-matched population; (4) delay between symptoms and diagnosis: adults 6 years, children 2 years; (5) associated conditions: 10%-15% of the total cohort - autoimmune disorders 30% (Turkey 1.9%, Iran 33%) and IDDM 10% (6.7%-18.5%); (6) complications: 16% of symptomatic CD patients; and (7) non gastrointestinal

Table 2 Populations now and after 10 years

	Population	Children 0-14	Median age (yr)	Population growth rate (%)	Children 0-14 in 10 yr	Total population in 10 yr
Albania	3 619 778	853 883	29.9	0.5	901 667	3 822 345
Algeria	33 769 669	8 878 665	26.6	1.2	9 999 566	38 032 972
Bosnia	4 590 310	673 770	39.8	0.3	696 962	4 748 317
Cyprus	792 604	154 445	35.5	1.7	182 623	937 214
Croatia	4 491 543	708 683	41	-0.1	705 006	4 468 242
Egypt	81 713 517	25 983 672	24.8	2	31 776 575	99 931 053
France	64 057 790	11 894 698	39.4	0.5	12 564 088	67 662 729
Greece	10 722 816	1 531 606	41.8	0.1	1 551 169	10 859 777
Israel	7 112 359	1 989 312	29.1	1.7	2 347 869	8 394 303
Italy	58 126 212	7 870 226	43.3	0	7 833 314	57 853 596
Lebanon	3 971 941	1 032 888	29.3	1.1	1 153 096	4 434 197
Libya	6 173 579	2 048 548	23.9	2.2	2 539 599	7 653 427
Malta	403 532	66 112	39.5	0.4	68 805	419 967
Morocco	34 343 219	10 473 478	25	1.1	11 683 138	8 309 775
Syria	19 747 586	7 146 569	21.7	2	8 716 754	24 086 361
Slovenia	2 007 711	273 464	41.5	0	273 655	2 009 117
Spain	40 525 002	5 864 419	41.1	0.1	5 906 780	40 817 729
Tunisia	10 383 577	2 413 484	29.2	1	2 660 713	11 447 236
Turkey	71 892 807	17 545 890	27.7	1.3	19 965 025	81 805 009
Mediter	458 445 552	107 403 812	33.2	0.9	121 526 405	507 693 365

Table 3 Prevalence of celiac disease in the next 10 years[1]

	Estimated celiacs today	Estimated celiac children today at 1%	Projected prevalence of CD in next 10 yr	Projected celiac children in next 10 yr
Albania	36 198	8539	38 223	9017
Algeria	337 697	88 787	380 330	99 996
Bosnia	45 903	6738	47 483	6970
Cyprus	7926	1544	9372	1826
Croatia	44 915	7087	44 682	7050
Egypt	817 135	259 837	999 311	317 766
France	640 578	118 947	676 627	125 641
Greece	107 228	15 316	108 598	15 512
Israel	71 124	19 893	83 943	23 479
Italy	581 262	78 702	578 536	78 333
Lebanon	39 719	10 329	44 342	11 531
Libya	61 736	20 485	76 534	25 396
Malta	4035	661	4200	688
Morocco	343 432	104 735	383 098	116 831
Syria	197 476	71 466	240 864	87 168
Slovenia	20 077	2735	20 091	2737
Spain	405 250	58 644	408 177	59 068
Tunisia	103 836	24 135	114 472	26 607
Turkey	718 928	175 459	818 050	199 650
Mediter	4 584 456	1 074 038	5 076 934	1 215 264

[1]Population prevalence estimated at minimum rate of 1%. CD: Celiac disease.

symptoms: short stature 20% (only children), anemia 40% (20%-80%), osteopenia 30% (30%-50%), abnormal liver function 10% (Turkey 38%, Iran 25%)

RESULTS

Table 2 shows the population growth, number of children aged 0-14 years and the predicted figures for the year 2020, calculated based on a constant growth rate. The Mediterranean area will have about half a billion individuals by the year 2020, more than 100 million of which will be children aged 0-14 years. This estimate is likely to be in the low range, since some countries with a large population are likely to grow at a higher rate than this estimate before the year 2010.

Table 3 shows the prevalence of CD in each country in 2010 and the predicted prevalence in 2020. Within 10 years, the Mediterranean area will have to face more than 5 million cases of CD, one million of which will be in children. The large majority will not have clear symptoms and their diagnosis and care will be significantly delayed. Among the adult CD population, about 550 000 will present symptoms, while only 240 000 out of the 1 million estimated celiac children will be symptomatic. Table 4 shows the estimated number of clinical complaints associated with the CD epidemic. It is likely that more than 48 000 children will be affected by growth failure, there will be 317 000 cases of anemia and 238 000 individuals will be afflicted with osteopenia. Table 5 shows the estimated financial burden of the CD epidemic. There is no scope for a detailed calculation of costs, which will be related more to the availability of and access to medical services than to the actual cost of the service, but these figures help to understand the financial burden of the undiagnosed disease. European countries may not be impressed by these estimates but, for several other Mediterranean countries, these predicted costs might be a consistent load to the gross national product. More than €4 billion is a prudent estimate; only crude medical costs are included, not individual or social cost.

Table 6 shows the estimated number of deaths in the celiac disease cohort and the excess of deaths compared to age- and sex-matched controls. At the present rate, there will be more than 250 000 CD-related deaths in the Mediterranean area in 2020.

DISCUSSION

Celiac disease is a very common chronic disease that

Table 4 Symptoms and diseases associated with symptomatic cases

	Symptomatic adults next 10 yr 1:7	Symptomatic children next 10 yr 1:5	Gastrointestinal symptoms	Anaemia	Osteopenia	Abnormal liver	Children with short stature
Albania	4172	1803	5079	2390	1793	598	361
Algeria	40 048	19 999	51 040	24 019	18 014	6005	4000
Bosnia	5788	1394	6104	2873	2154	718	279
Cyprus	1078	365	1227	577	433	144	73
Croatia	5376	1410	5768	2714	2036	679	282
Egypt	97 364	63 553	136 779	64 367	48 275	16 092	12 711
France	78 712	25 128	88 264	41 536	31 152	10 384	5026
Greece	13 298	3102	13 940	6560	4920	1640	620
Israel	8638	4696	11 333	5333	4000	1333	939
Italy	71 458	15 667	74 056	34 850	26 137	8712	3133
Lebanon	4687	2306	5944	2797	2098	699	461
Libya	7305	5079	10 527	4954	3715	1238	1016
Malta	502	138	543	256	192	64	28
Morocco	38 038	23 366	52 194	24 562	18 421	6140	4673
Syria	21 957	17 434	33 482	15 756	11 817	3939	3487
Slovenia	2479	547	2573	1211	908	303	109
Spain	49 873	11 814	52 433	24 675	18 506	6169	2363
Tunisia	12 552	5321	15 193	7149	5362	1787	1064
Turkey	88 343	39 930	109 032	51 309	38 482	12 827	7986
Mediter	551 667	243 053	675 512	317 888	238 416	79 472	48 611

Table 5 Excess cost of undiagnosed symptomatic celiac patients

	Purchasing power parity	Standardized cost for an adult in 6 yr of delay, €	Standardized cost for a child in 2 yr of delay, €	Total cost for adults in the next 10 yr, €	Total cost for children in the next 10 yr, €	Total cost of symptomatic in the next 10 yr, €
Albania	7.164	2804	821	11 698 575	1 481 020	13 179 595
Algeria	6.869	2688	787	107 662 164	15 748 296	123 410 460
Bosnia	7.361	2881	844	16 673 654	1 176 265	17 849 919
Cyprus	17.7	6928	2029	7 468 819	741 246	8 210 065
Croatia	28.54	11 171	3272	60 057 866	4 613 886	64 671 751
Egypt	6.123	2396	702	233 320 214	44 609 799	277 930 013
France	33.68	13 181	3861	1 037 513 563	97 017 277	1 134 530 840
Greece	29.88	11 695	3426	155 520 664	10 627 416	166 148 080
Israel	28.39	11 112	3255	95 985 202	15 284 247	111 269 450
Italy	29.11	11 393	3337	814 080 086	52 279 538	866 359 625
Lebanon	14.23	5568	1631	26 097 336	3 761 033	29 858 369
Libya	14.33	5608	1643	40 966 178	8 342 757	49 308 935
Malta	23.58	9230	2704	4 630 407	372 044	5 002 450
Morocco	4.604	1802	528	68 540 194	12 332 575	80 872 769
Syria	4.7	1839	539	40 388 186	9 393 156	49 781 342
Slovenia	29.69	11 619	3403	28 807 411	1 862 767	30 670 178
Spain	33.7	13 189	3863	657 786 976	45 639 366	703 426 342
Tunisia	8.254	3230	946	40 548 541	5 035 255	45 583 796
Turkey	12.48	4883	1430	431 358 582	57 108 945	488 467 527
Mediter	17.92	7012	2054	3 879 104 619	387 426 887	4 266 531 506

affects adults and children in all wheat-consuming countries. It has also recently been reported in countries where its prevalence was previously unknown, such as China[27]. For more than two decades, we have been discussing the difference in the prevalence of CD among countries in Europe, North America and South America, and the conclusion is that there is no country where CD prevalence is significantly different from the overall prevalence of about 1%. Interestingly, the prevalence, at a global level, is not related either to the amount of wheat consumed by each country or to the prevalence of the human leukocyte antigen (HLA) DR3-DQ2 and DR4-DQ8 haplotype worldwide[47].

An excess prevalence of CD has been reported in an isolated population in North Africa and in a large population in Sweden, but again it is plausible that this excess prevalence reflects a bias related to the cohort rather than a true excess. The prevalence of CD is increasing worldwide, including in Europe[4], China[27] and India[28]. The only region where it has not yet been described is Central Africa, and this may be explained by the absence in this region of HLA predisposing haplotypes, and of polymorphisms of the major non-HLA genes, namely *SH2B3, IL12A, SCHIP, IL18RAP,* and *IL1RL1*, among others[47,48]. Recently, Barada *et al*[2] from Lebanon produced a comprehensive report of the situation in the

Table 6 Excess mortality in undiagnosed cases[2]

	Projected prevalence of CD in the next 10 yr	Death rate, deaths/1000 individuals	Population expected deaths (next 10 yr)	Celiac deaths in next 10 yr	Excess celiac deaths in next 10 yr
Albania	38 223	5.1	193 793	3488	1550
Algeria	380 330	4.6	1 764 730	31 765	14 118
Bosnia	47 483	8.6	409 780	7376	3278
Cyprus	9372	6.4	59 982	1080	480
Croatia	44 682	11.8	525 018	9450	4200
Egypt	999 311	4.9	4 876 635	87 779	39 013
France	676 627	8.6	5 791 930	104 255	46 335
Greece	108 598	10.5	1 141 363	20 545	9131
Israel	83 943	5.4	455 811	8205	3646
Italy	578 536	10.7	6 201 905	111 634	49 615
Lebanon	44 342	6	267 382	4813	2139
Libya	76 534	3.4	260 982	4698	2088
Malta	4200	8.4	35 193	633	282
Morocco	383 098	4.7	1 815 883	32 686	14 527
Syria	240 864	3.7	896 013	16 128	7168
Slovenia	20 091	9.2	184 839	3327	1479
Spain	408 177	10	4 077 691	73 398	32 622
Tunisia	114 472	5.2	595 256	10 715	4762
Turkey	818 050	6	4 908 301	88 349	39 266
Mediter	5 076 934	7	34 462 486	620 325	275 700

[2]Undiagnosed celiac patients have 1.8 standard mortality rate[39]. CD: Celiac disease.

countries that face the Mediterranean Sea, thereby increasing the awareness of CD in the area.

The EUROMED program supports several health-promoting activities across the Mediterranean, such as the surveillance of infectious diseases program and the Program for Transplants and Oncology EuroMed (Cancer Registries Network, Cancer screening and early diagnosis program, Mediterranean Transplant Network). Italy has requested that the CD epidemic be included in these programs (www.eeas.europa.eu/euromed/index_en.htm). The first step in facing this epidemic is to estimate the burden of CD in the area. Here we provide a reliable and simple picture of the present situation and a prediction of the development of the CD epidemic in the next 10 years, up to 2021.

The prediction obtained by simple straightforward calculations is impressive. Mediterranean countries will have to be prepared to deal with a considerable number of CD patients in the near future. There will be more than 5 million cases, one million of which will be children. But, more than the overall figures, each country will be especially concerned about the national figures. Our estimates are conservative figures, since we estimated a constant population growth over the next ten years, whereas the faster growing countries may have a more rapid growth rate than slower growing countries. Data on symptoms and common clinical problems are available only for symptomatic individuals, while a considerable percentage of so-called "asymptomatic" subjects notoriously report significant complaints a posteriori[49]. A limitation of this study is related to the uncertainties inherent in any prediction given the wide confidence intervals of rates. However, the starting 1% prevalence rate is not only very robust, because of innumerable replications, but it also probably underestimates rather than overestimates the problem[4,28,50]. The rate of symptomatic versus asymptomatic individuals is also fairly conservative.

The financial burden estimate is not aimed to acquire more precision; we provide a gross figure for the spectrum of resources needed in each country for the services required by symptomatic patients. The priority issue is the availability of services; in many African countries, services are mostly only available in large cities and specialized health institutions. In the rural areas, the availability of services can be far less than that required. Hence, the cost of these services should, sadly, be subtracted from the total financial burden. This impending cohort of CD patients does require, and moreover will require, access to health services as inpatients or outpatients, for medical consultations, laboratory tests and, after diagnosis, financial support for a lifelong gluten-free diet. There is universal concern and many countries demand the expertise and support for dissemination of know how and capacity building for the management of CD.

The EuroMed - MEDICEL project (www.medicel.unina.it) offers a platform to analyze the problem and develop strategies, but active national plans are required to face the burgeoning epidemic, and the heavy burden that it will place on the health and the finances of the population.

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COMMENTS

Background

The incidence of celiac disease (CD) (i.e., permanent gluten intolerance), is

increasing in all countries in which there is awareness of this intolerance. In all Western countries, including the United States and South America, the observed prevalence of the disease went from 1:1000 individuals to more than 1:100 individuals in two decades. However, large series of cases have recently been reported from "new" countries like India, China, North Africa and the Middle East. Celiac disease is expanding over and above any predicted trend, and has taken on the semblance of a real epidemic.

Research frontiers

This expanding "epidemic" raises a series of unanswered research questions related to the following hot topics: **(1) the weight of environmental factors in the** increase of CD; **(2) the genetic profile associated with predisposition to CD; (3)** population differences in terms of genetic and environmental factors; **and (4)** the development of "sensitivity" to gluten.

Innovations and breakthroughs

In next 10 years, the Mediterranean area will have about half a billion inhabitants, 120 million of whom will be children. The projected number of CD cases in 2020 will be 5 million cases (1 million celiac children), with a relative increase of 11% compared to 2010. At a 2010 constant rate, there will be about 550 000 symptomatic adults and 240 000 sick children: 85% of patients will suffer from gastrointestinal complaints, 40% are likely to have anemia, 30% will be afflicted with osteopenia, 20% of children will have short stature and 10% will have abnormal liver enzymes. The estimated standardized medical costs for symptomatic celiac disease during the years of delay between onset of symptoms and diagnosis (mean: 6 years for adults, 2 years for children) will be about €4 billion (€387 million for the children) over the next 10 years. A delay in diagnosis is expected to increase mortality; about 600 000 deaths will occur among individuals affected by CD in the next 10 years, with an excess of 44.4% compared to age- and sex-matched controls.

Applications

The data produced in this study provide a picture of the cohort of patients affected by CD that will develop over the next 10 years in each country of the Mediterranean Basin. Stakeholders and health professionals in each country now have the figures with which it is possible to base adequate plans to face this epidemic. The diagnostic protocol must be simplified and made available not only in specialized centers, usually in large cities, but it should be especially important in rural districts.

Terminology

CD: Celiac disease is a permanent intolerance to gluten based on a genetic predisposition; **Projected prevalence: The number of celiac cases that are** expected to be present over the next 10 years; **Excess mortality: Undiagnosed** celiac cases have twice the risk of death compared to age- and sex-matched controls. If the expected cases are not diagnosed, there will be more than 200 000 excess deaths in the Mediterranean area; **Growth failure: 20% of children** (about 50 000) with undiagnosed CD are affected by weight loss and short stature, due to a growth failure.

Peer review

The paper is well written and deals with an important problem people are continuously facing.

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BRIEF ARTICLE

^{13}C-methacetin breath test reproducibility study reveals persistent CYP1A2 stimulation on repeat examinations

Anna Kasicka-Jonderko, Anna Nita, Krzysztof Jonderko, Magdalena Kamińska, Barbara Błońska-Fajfrowska

Anna Kasicka-Jonderko, Anna Nita, Krzysztof Jonderko, Magdalena Kamińska, Barbara Błońska-Fajfrowska, Department of Basic Biomedical Science, School of Pharmacy, Medical University of Silesia, PL-41-205 Sosnowiec, Poland
Author contributions: Kasicka-Jonderko A and Jonderko K designed the research, analysed the data and wrote the paper; Nita A and Kamińska M performed the research, collected and analysed the data; Błońska-Fajfrowska B was involved in providing financial support for this work and approved the final version.
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Correspondence to: Dr. Anna Kasicka-Jonderko, Department of Basic Biomedical Science, School of Pharmacy, Medical University of Silesia, Kasztanowa street 3, PL-41-205 Sosnowiec, Poland. akj@sum.edu.pl
Telephone: +48-32-2699830 Fax: +48-32-2699833
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Abstract

AIM: To find the most reproducible quantitative parameter of a standard ^{13}C-methacetin breath test (^{13}C-MBT).

METHODS: Twenty healthy volunteers (10 female, 10 male) underwent the ^{13}C-MBT after intake of 75 mg ^{13}C-methacetin p.o. on three occasions. Short- and medium-term reproducibility was assessed with paired examinations taken at an interval of 2 and 18 d (medians), respectively.

RESULTS: The reproducibility of the 1-h cumulative ^{13}C recovery (AUC_{0-60}), characterized by a coefficient of variation of 10%, appeared to be considerably better than the reproducibility of the maximum momentary ^{13}C recovery or the time of reaching it. Remarkably, as opposed to the short gap between consecutive examinations, the capacity of the liver to handle ^{13}C-methacetin increased slightly but statistically significantly when a repeat dose was administered after two to three weeks. Regarding the AUC_{0-60}, the magnitude of this fixed bias amounted to 7.5%. Neither the time gap between the repeat examinations nor the gender of the subjects affected the ^{13}C-MBT reproducibility.

CONCLUSION: ^{13}C-MBT is most reproducibly quantified by the cumulative ^{13}C recovery, but the exactitude thereof may be modestly affected by persistent stimulation of CYP1A2 on repeat examinations.

Key words: ^{13}C-Methacetin; Breath test; Isotope application in medicine; Liver; Reproducibility

Peer reviewer: George Papatheodoridis, MD, Assistant Professor in Medicine and Gastroenterology, 2nd Department of Internal Medicine, Athens University Medical School, Hippokration General Hospital of Athens, 114 Vas Sophias Ave, 115 27 Athens, Greece

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INTRODUCTION

There is no surprise that non-invasive diagnostic procedures in medicine attract much attention from either side participating in the search of any cause of malfunctioning of the organism-patients would definitely prefer methods sparing them the necessity of encroaching the body integrity, like for example a liver biopsy, whereas physicians too would prefer to avoid inflicting pain on their patients. A brilliant idea of per oral administration of ^{13}C-enriched substrates in order to get information on the metabolic efficiency or functional mass of the liver is a

practical answer to the requirement outlined above. Continuous research work has brought about considerable progress and nowadays it is possible to assess by means of ^{13}C breath tests the microsomal, the cytosolic, and the mitochondrial function of the liver[1-4]. A look at pertinent literature shows that during the past decade, from among the compounds applied for microsomal ^{13}C breath tests, ^{13}C-methacetin has steadily been making its way to be recognized as the most frequently used substrate. A number of features support its usefulness as a functional liver probe: a fast metabolism to acetaminophen and $^{13}CO_2$ by cytochrome P450 1A2 (CYP 1A2), safety at low doses applied for a breath test, and a low cost[1-4]. Accordingly very promising results on its diagnostic usefulness were obtained in patients with chronic hepatitis C virus infection[5,6], primary biliary cirrhosis[7,8], non-alcoholic steatohepatitis[9], and various stages of liver cirrhosis[10-14], including those awaiting a liver transplantation[15].

A vital asset of any measurement or diagnostic method used in medical practice is an ability to provide reproducible results. Quite surprisingly a search of data on the reproducibility of the ^{13}C-methacetin breath test (^{13}C-MBT) revealed this item as being almost a completely blank research area. We decided therefore to search in this prospective study for a quantitative parameter which would offer the best reproducibility for a standard ^{13}C-MBT.

MATERIALS AND METHODS

Subjects

Twenty healthy non-obese subjects, 10 female and 10 male were invited to enter the study; their mean age was 25 years (range 21-31 years), and their average body mass index was 22.38 ± 0.64 kg/m^2. During a screening interview the participants declared themselves as being in full health according to the World Health Organisation criteria[16]. Among the participants three (1 male and 2 female) were habitual cigarette smokers. All the subjects categorically denied a systematic use of significant amounts of alcoholic beverages. None of the volunteers did take any medication except for oral hormonal contraception which was used by 4 female. Exclusion criteria to attend the study comprised a history of surgery affecting the digestive tract anatomy, with the exception of appendectomy, current use of any drugs which might affect gastrointestinal motility, and pregnancy. In every subject the normal structure and size of the liver was confirmed by means of an ultrasonographic examination performed by a qualified investigator.

The study was conducted in accordance with the Helsinki Declaration, and every volunteer gave written consent to participate after getting information as to the aim, protocol and methodology of the project. During the introductory interview the subjects were instructed not to eat any food with a naturally increased ^{13}C content, such as products made of maize, cane sugar, pineapple, or kiwi fruit for 48 h preceding the examination[17-19].

Experimental protocol

Every subject underwent three examination sessions, held on separate days. In half of the volunteers the two first sessions were taken 2-4 d apart, and the third one was pursued 2-3 wk later. In the other half of the subjects the schedule was inversed, i.e. the first two examination measurements were taken 2-3 wk apart, followed by a third one 2-4 d after the second one. The assignment of the order of intervals separating the sessions (short-long or long-short) was randomized. Accordingly, the assessment of the short-term reproducibility involved 20 pairs of examinations separated by a median 2 d break (range 2-4 d), whereas the medium-term reproducibility was evaluated on 20 pairs of the most distant examinations, i.e. taken at a median interval of 18 d (range 17-23 d).

The volunteers came to the laboratory in the morning, after a 12-h overnight fast and abstaining from cigarette smoking (if applicable). In the female volunteers the examinations were taken always within the same phase of their menstrual cycle. After a 15-min rest in a sitting position, necessary for stabilization of the metabolism, a basal sample of the exhaled air was collected. At the time point designated "0" the subjects took 75 mg ^{13}C-methacetin (code INC590P, Euriso-Top SA, Saint-Aubin, France; according to the certificate of analysis, the manufacturer guarantees ⩾ 99% isotopic ^{13}C atom enrichment determined by proton NMR) orally dissolved in 200 mL unsweetened black tea. Samples of expiratory air for $^{13}CO_2$ measurement were collected at 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 40, 50, 60, 75, 90, 105, 120, 150 and 180 min[20]. The procedure of collecting the breath air was standardized: the subjects took in breath and held it for 10 s, then steadily blew the air into a special aluminium covered plastic bag of 1.1 L capacity (Fischer Analysen Instrumente GmbH, Leipzig, Germany) equipped with a mouthpiece and an unidirectional valve[21,22]. The bag was closed with a plastic cork immediately at the end of the exhalation and stored for analysis. The subjects remained seated quietly and fasted throughout the whole period of the collection of the samples.

Measurement of $^{13}CO_2$ and derivation of the breath test parameters

Concentrations of $^{13}CO_2$ in the probes of the exhaled air were measured with the use of a non-dispersive isotope-selective infrared spectrometry apparatus (IRIS, manufactured by Wagner Analysen Technik Vertriebs GmbH, Hamburg, Germany; a model equipped with 16 ports for simultaneous mounting of bags with air samples was used). Following the procedures described previously[20,23,24], curves of the momentary and cumulative recovery of ^{13}C in the exhaled air relative to the administered oral dose of ^{13}C-methacetin were constructed within the time domain of 0 to 180 min. Subsequently the following parameters were derived: D_{max} - the maximum momentary ^{13}C recovery in expiratory air, T_{max} - the time elapsing from the intake of the substrate until the occurrence of the D_{max}, and AUC_i - the cumulative ^{13}C recovery within

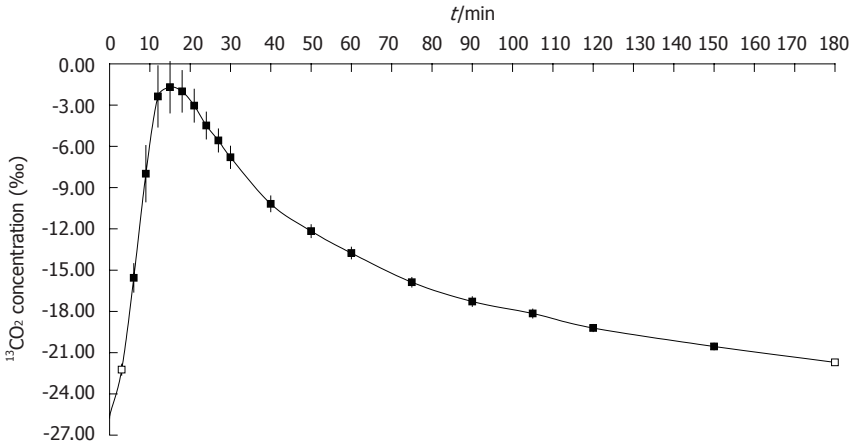

Figure 1 Time course of the $^{13}CO_2$ content within the total pool of exhaled CO_2 after peroral administration of 75 mg ^{13}C-methacetin in 20 healthy volunteers who each underwent three breath tests on separate days. Filled rectangles represent the time points at which the rise in $^{13}CO_2$ concentration was statistically significantly different from the baseline measurement, open rectangles stand for values not statistically different from the baseline. The values shown are mean ± SE.

Table 1 Reproducibility of the ^{13}C-methacetin breath test

	D_{max}		T_{max}		$AUC_{0\text{-}60}$	
	S_term	M_term	S_term	M_term	S_term	M_term
CVp	16.23%	16.46%	30.57%	32.49%	10.00%	10.00%
RC	15.55% dose/h	16.80% dose/h	16.86 min	16.73 min	5.84% dose	5.86% dose
Bias proportional	N	N	N	Y	N	N
Bias fixed	N	Y	N	N	N	Y
$Delta_{0.05}$	3.80% dose/h	3.51% dose/h	4.0 min	3.8 min	1.43% dose	1.26% dose

T_{max}: Time to reach the maximum momentary ^{13}C elimination; D_{max}: Maximum momentary ^{13}C elimination; $AUC_{0\text{-}60}$: 60-min cumulative ^{13}C elimination in expiratory air, respectively; S_term: Short term reproducibility assessment on the basis of 20 pairs of ^{13}C-methacetin breath tests separated by a median 2-d break; M_term: Medium-term reproducibility evaluation involved 20 pairs of examinations accomplished at a median interval of 18 d. CV_P: Coefficient of variation for paired examinations; RC: Repeatability coefficient. $Delta_{0.05}$: The least difference detectable at $P = 0.05$ level, two-tailed in the case of 20 paired examinations.

a time span i, which was calculated by integration of the respective part of the momentary ^{13}C recovery curve.

Statistical analysis

The data were subjected to the Bland and Altman statistic for calculation of repeatability coefficients and check for a proportional or fixed bias[25,26]. In addition the reproducibility of breath test parameters was expressed in terms of the coefficient of variation for paired examinations, CV_p[27,28]. Sets of absolute values of individual differences in paired measurements were compared with the use of the paired or unpaired *t* test (where appropriate) in order to check if factors such as the time gap between the examinations, or the gender of the subjects affected the reproducibility. Other statistical methods comprised the use of a repeated measures analysis of variance (R_ANOVA) followed by a *post hoc* check on the significance of differences between means, which was accomplished with the Tukey's honest significant difference test[29]. Statistical significance was set at the $P < 0.05$ level, two-tailed. The results are presented as mean ± SE. All statistical analyses were performed with the use of Statistical 6.0 software[30].

RESULTS

Similar basal concentrations of $^{13}CO_2$ in the expiratory air were observed on the three study days, amounting to -25.79‰ ± 0.25‰, -25.44‰ ± 0.21‰ and -25.59‰ ± 0.22‰. After oral intake of the ^{13}C-methacetin solution an expected biphasic course of $^{13}CO_2$ content within the exhaled air was observed, characterized by a rapid rise followed by a less steep decline. R_ANOVA indicated that when referred to the basal situation, the rise in breath $^{13}CO_2$ was statistically significant between the 6th and the 150th minute (Figure 1).

Basic reproducibility assessment

As shown in Table 1, the T_{max} exhibited rather an unsatisfactory reproducibility, whereas the D_{max} displayed a fair reproducibility, supposedly sufficient from the point of view of diagnostic applications of the breath test. The reproducibility of the cumulative ^{13}C recovery relative to the administered substrate dose was strongly dependent on the time span included for the calculation of this parameter, and improved rapidly with inclusion of the subsequent measurement points (Figure 2). The $AUC_{0\text{-}60}$ attained a CV_p of 10%-identical in both the case of the

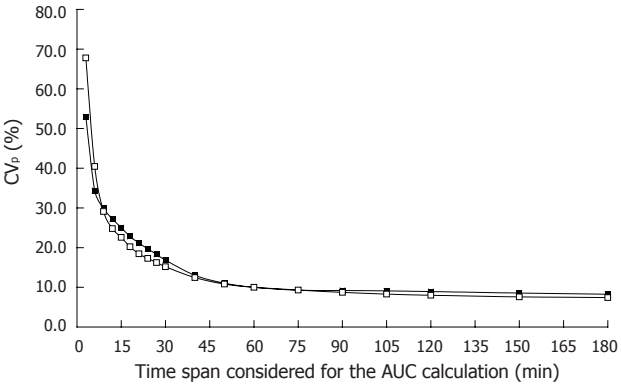

Figure 2 Relationship of the reproducibility of the cumulative ^{13}C recovery, expressed in terms of the coefficient of variation for paired examinations (CV_P), on the time span taken for the calculation of the area under the curve. The short-term reproducibility (open rectangles) assessment involved performance of 20 pairs of ^{13}C-methacetin breath tests separated by a median 2-d break, whereas 20 pairs of examinations accomplished at a median interval of 18 d were considered for the evaluation of the medium-term reproducibility (filled rectangles). AUC: Area under the curve.

short- or the medium-term reproducibility. The inclusion of data from beyond the 60 min did not bring about much refinement of the reproducibility of the AUC.

No statistically significant difference between the short- and medium-term reproducibility of the breath test parameters was found. Moreover, none of the parameters considered had a different short- or medium-term reproducibility in men and women.

Bland and Altman statistics of reproducibility

Bland and Altman plots pertaining to representative parameters of the ^{13}C-MBT are provided in Figure 3. Bland and Altman statistics revealed no proportional bias in either D_{max} or $AUC_{0\text{-}60}$ (Table 1). The same applied to the short-term reproducibility of T_{max}, whereas analysis of its medium-term reproducibility showed that the slope of the linear regression of the between-day differences on the corresponding means of the paired measurements was statistically significantly different from zero (Table 1).

The Bland and Altman statistic disclosed a fixed bias in the case of the medium-term reproducibility of either the D_{max} or the $AUC_{0\text{-}60}$. It means that the mean differences between the paired measurements taken a median of 18 d apart differed statistically significantly from zero. A closer look at the pertinent differences indicated that the ability of the liver to handle ^{13}C-methacetin increased when a repeat dose was administered after two to three weeks (D_{max}: 34.60% ± 2.75% dose/h on the first administration *vs* 39.08% ± 2.77% dose/h on the second administration, $P = 0.015$; $AUC_{0\text{-}60}$: 20.80% ± 0.99% dose on the first administration *vs* 22.27% ± 0.98% dose on the second administration, $P = 0.017$). No such effect was observed if two doses of ^{13}C-methacetin were administered in close sequence [D_{max}: 34.26% ± 2.41% dose/h on the first administration *vs* 34.91% ± 2.68% dose/h on the second administration, not significant (NS); $AUC_{0\text{-}60}$: 21.16% ± 0.96% dose on the first administration *vs* 21.03% ± 1.07% dose on the second administration, NS]. A graphical representation of the phenomenon observed is provided in Figure 4.

DISCUSSION

Without any doubt provision of reproducible measurement results is a feature of paramount importance, expected to be assured by methods designed for research and/or clinical applications in medicine[28,31-33]. Breath tests performed with the use of stable isotopes, such as ^{13}C, cannot be considered an exception in this respect, especially because during the past decade their clinical use has been constantly growing[24,34,35]. An increase in the number of relevant scientific papers indicates that, from among the tests dedicated to assess the activity of cytochrome P450, known as the microsomal breath tests, the ^{13}C-MBT has lately taken the lead. Accordingly, Yaron Ilan in his very recent review[36] provides evidence-based arguments that the ^{13}C-MBT is a powerful tool to aid hepatologists in bedside decision making.

While searching the literature we came across only one study which was aimed at the evaluation of the reproducibility of the ^{13}C-MBT. Petrolati *et al*[15] performed repeat measurements separated by an interval of 12 wk in 10 healthy volunteers. This scarcity of data encouraged us to undertake a systematic, prospective study on the reproducibility of the quantitative measures of a standard ^{13}C-MBT. Much effort was given in order to assure comparable conditions while performing the ^{13}C-MBT. Accordingly, all the examinations were started at the same time in the morning, a 15-min rest always preceded the collection of the basal samples of expiratory air, the procedure of taking the breath samples was standardized, and comfortable surroundings were provided to the volunteers so that they could stay relaxed while maintaining a sitting position throughout the examination. Moreover, the female participants were always examined in the same phase of the menstrual cycle. While designing the study protocol we decided to adopt the currently accepted mode of administration of ^{13}C-methacetin, namely a fixed oral dose of 75 mg[5,6,8,13,15,20,37,38]. It should be noted, however, that formerly other body mass adjusted dosage regimens were applied, encompassing 5 mg/kg in the pioneer work by Krumbiegel *et al*[39], then 2 mg/kg[7,11,12,40], as well as 1 mg/kg[10,41], and finally Iikura *et al*[42] applied in infants 0.5 mg/kg ^{13}C-methacetin.

Taking into account the investigative nature of our research, we generously took as many as 19 samples of breath air throughout 3 h. This approach appears to be precedential in nature, because for routine clinical use just a few measurement points are usually considered[15,42]. Nine samples were collected in the study by Zipprich *et al*[13] (at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after application of the substrate), and twelve samples of the expiratory air-every 15 min for 3 h of observation – were taken by Ciccocioppo *et al*[41]. A team of German researchers from Bochum collected thirteen samples-at 5, 10, 15, 20,

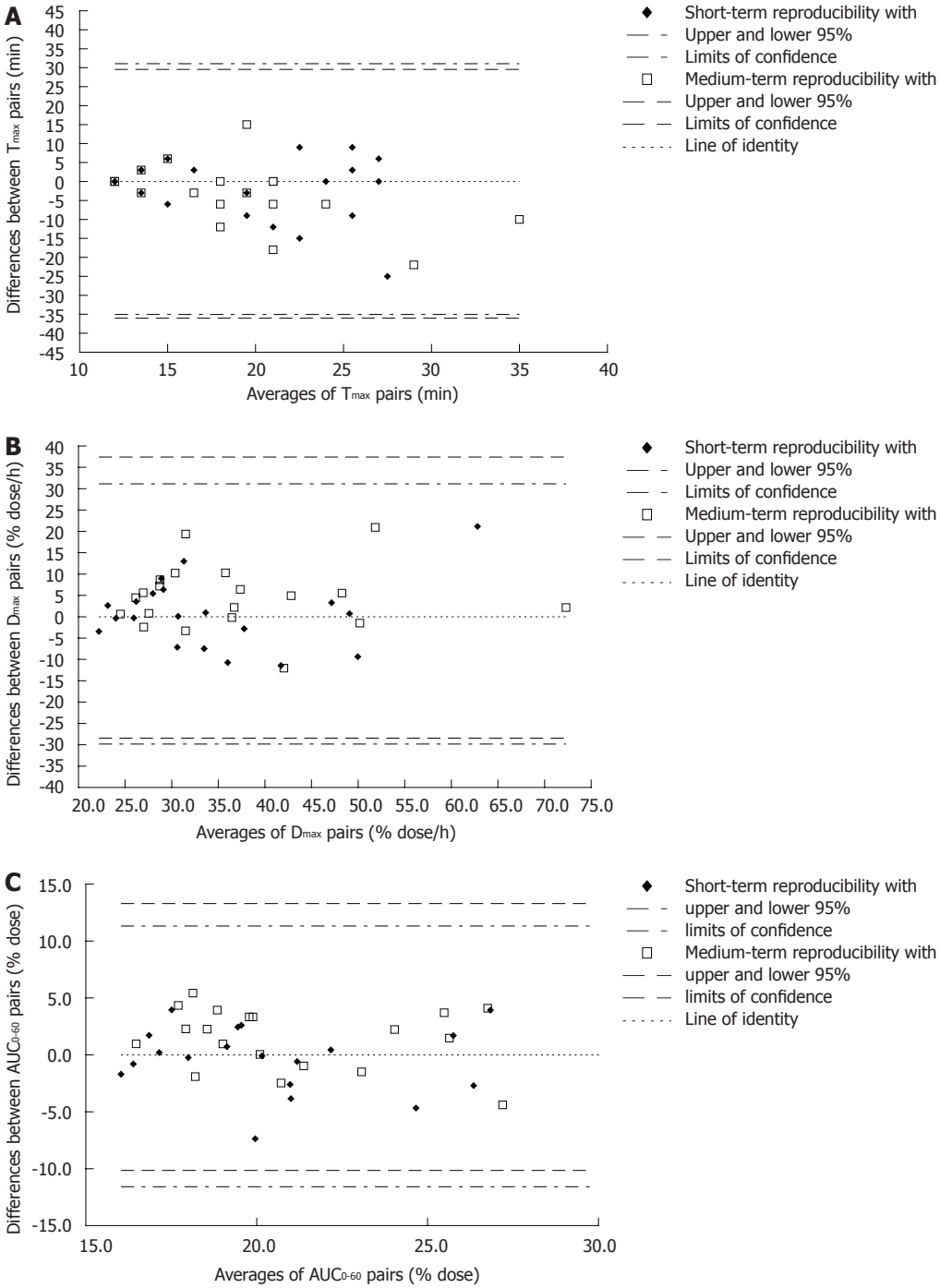

Figure 3 Bland and Altman statistics (plot of differences between pairs *vs* their means) of the short- (filled diamonds) and medium-term (open rectangles) reproducibility of the ^{13}C-methacetin breath test. T_{max}: Time to reach the maximum momentary ^{13}C elimination (panel A); D_{max}: Maximum momentary ^{13}C elimination (panel B); $AUC_{0\text{-}60}$: 60-min cumulative ^{13}C elimination in expiratory air (panel C). On each panel the respective borders of the 95% confidence intervals are plotted.

30, 40, 50, 60, 80, 100, 120, 150 and 180 min after administration of ^{13}C-methacetin[12]. One should mention that our frequent sampling of breath air (an aliquot was taken every 3 min during the first half hour) is similar to the new approach recently proposed by Lalazar *et al*[6,38] who introduced a "continuous" ^{13}C-MBT wherein samples of breath air are taken every 2-3 min and analysed with a laser-based device.

Our intention was to determine the length of the T_{max} with the greatest exactitude possible. Nevertheless, with the CV_p between 30.6% and 32.5%, the reproducibility of this parameter appeared to be a bit disappointing. Apparently the T_{max} may therefore not be the most useful parameter for the purposes of clinical decision making. On the other hand, we established on the basis of the within-subject study protocol that the repeatability of the T_{max} was sufficient to discern a difference in its length as small as 4 min ($P = 0.05$ level, two-tailed, $n = 20$).

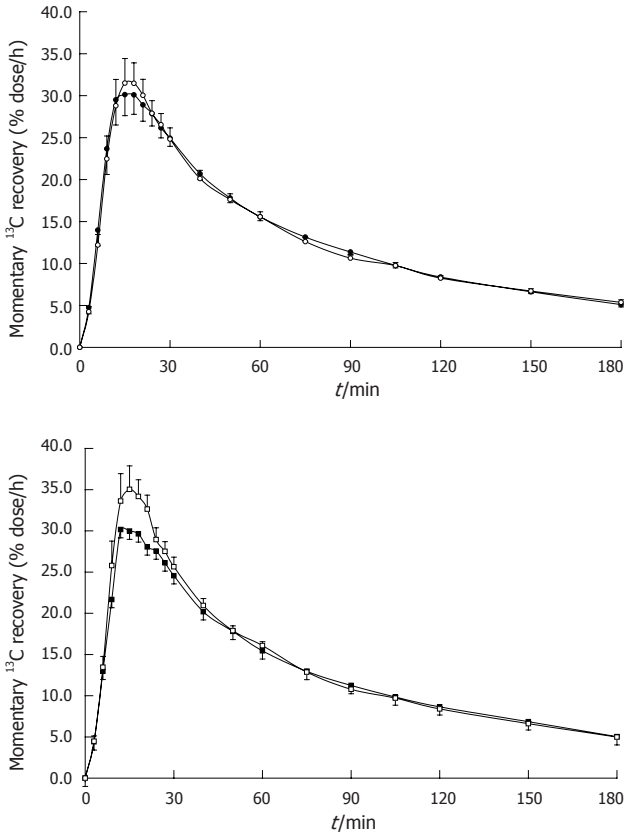

Figure 4 Corresponding curves of the momentary ^{13}C recovery in breath air after peroral administration of 75 mg ^{13}C-methacetin in 20 healthy volunteers if two examinations were taken at a median of 2 d apart (panel A) or if the examinations were separated by a median 18 d gap (panel B). Open symbols, the first administration, filled symbols, the second administration, the values shown are mean ± SE. See the text for the results of the statistical comparison of the matching D_{max} and $AUC_{0\text{-}60}$ values.

D_{max} (CV_p of 16%) showed better reproducibility, whereas the most reliable with regard to its repeatability appeared to be cumulative ^{13}C recovery in expiratory air. At this point it is important to emphasize that the reproducibility of the cumulative ^{13}C recovery was dependent on the time span considered for its calculation. Hence the CV_p improved rapidly with inclusion of the subsequent measurement points, achieving a reasonable value of 10% for the period comprising the first 60 min following the administration of the substrate. Consideration of later measurement points offered only a small further improvement in the reproducibility of the AUC. Our finding provides an argument in support of limiting the time for sample collection to 1 h when performing the ^{13}C-MBT-which is a standpoint coherent with the procedure proposed lately by the research group headed by Yaron Ilan from Israel[6,38].

Of great importance is the finding that in the case of each of the considered parameters, characterizing quantitatively the result of the ^{13}C-MBT, the reproducibility depended neither on the time gap between the repeat examinations nor on the gender of the subjects. Thus a convincing proof of reliability of this test has been obtained. Quite unexpectedly a thorough analysis of reproducibility, involving the application of the Bland and Altman statistic, disclosed the existence of a fixed bias in the case of the medium-term reproducibility of the D_{max} and the AUC_{60}. It was found that those two parameters increased statistically significantly if the repeat examinations were separated by a two- to three-week gap, which was not the case when the results of examinations taken at a close sequence were compared. A similar trend can, however, be discerned when looking at the results obtained by Petrolati *et al*[15] in their attempt to evaluate the reproducibility of the ^{13}C-MBT. They found that the 45-min cumulative ^{13}C recovery ($AUC_{0\text{-}45}$) in breath air after peroral intake of 75 mg ^{13}C-methacetin amounted to 17.5% ± 2.8% dose and to 18.8% ± 4.3% dose on the first and the second occasion, respectively. Although the two means did not differ statistically significantly (P = 0.30), it is evident that the $AUC_{0\text{-}45}$ determined on the second occasion increased by 7.4%. Quite a similar magnitude of increment was determined in our study-the $AUC_{0\text{-}60}$ rose by 7.5% on the repeat measurement taken after two to three weeks (P = 0.017), whereas it remained unchanged with an average difference of -0.6% (NS) between measurements performed a median of 2 d apart. A delayed and persisting stimulation of a minor, but statistically significant, magnitude of the CYP1A2 capacity to metabolize methacetin would therefore be inferred. It remains unclear if the phenomenon disclosed would have a clinically relevant impact. It is rather obvious that not all patients with liver disease require performance of a series of ^{13}C-MBTs, which most likely may pertain to those awaiting liver transplantations[15]. At present one cannot predict whether a stimulation of CYP1A2 observed on repeated ^{13}C-MBT in healthy volunteers will occur also in patients with major impairment of the liver functional reserve. Nevertheless the error in estimating the cumulative ^{13}C recovery in breath air caused by a stimulation of CYP1A2 by ^{13}C-methacetin seems to be quite small, not exceeding 8% according to our results, as well as those obtained by Petrolati *et al*[15].

Summing up, the study renders evidence that the ^{13}C-MBT provides satisfactorily reproducible results. Remarkably, the cumulative ^{13}C recovery appear to be the most reproducible quantitative index of the test, the computation of which over the first hour following administration of the substrate offers a reasonable 10% coefficient of variation, regardless of the time span separating the repeat measurements. One should be aware that the exactitude of this parameter may be modestly affected by a persistent stimulation of CYP1A2 on repeat examinations.

COMMENTS

Background

Great progress has been made during the past two decades with respect to the diagnostic use of stable isotopes in hepatology - it is now possible to assess by means of ^{13}C breath tests the microsomal, the cytosolic, or the mitochondrial function of the liver. From the point of view of a patient those tests are particularly attractive as in many instances they may result in avoidance of the inconvenience of a repeat liver biopsy.

Research frontiers

A crucial asset of any measurement or diagnostic method used in medical practice is provision of reproducible results. Stable isotope breath tests cannot be exempted from this scrutiny. Quite surprisingly, the reproducibility of one of the most popular among the ^{13}C breath tests applied in hepatology, the ^{13}C-methacetin breath test (^{13}C-MBT) has not been sufficiently examined before.

Innovations and breakthroughs

This prospective study is the first one dedicated to thorough evaluation of the repeatability of the ^{13}C-MBT. According to the study results, cumulative ^{13}C recovery is the most reproducible quantitative index of the ^{13}C-MBT, the computation of which over the first hour following administration of the substrate offers a 10% coefficient of variation, regardless of the time span separating the repeat measurements. An additional finding is that in the case of repeat examinations the exactitude of this parameter may be modestly affected by a persistent stimulation of CYP1A2 responsible for a fixed bias which amounted to 7.5%.

Applications

Promising results on the usefulness of the ^{13}C-MBT to evaluate reliably and accurately the metabolic liver functional reserve were documented in patients with various stages of liver cirrhosis, including those awaiting a liver transplantation, as well as those with chronic hepatitis C virus infection, primary biliary cirrhosis, or non-alcoholic steatohepatitis.

Terminology

The breath tests dedicated to get information on the metabolic efficiency of the liver are based on an elegant but simple idea of monitoring the elimination of $^{13}CO_2$ in expiratory air after per oral administration of ^{13}C-enriched substrates. Depending on the chemical structure of such substrates, the microsomal, the cytosolic, or the mitochondrial function of the liver may be evaluated noninvasively.

Peer review

The study was conducted carefully and the paper is well written.

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BRIEF ARTICLE

Lamivudine resistance mutations in patients infected with hepatitis B virus genotype D

Orhan Yıldız, Bilgehan Aygen, Nese Demirtürk, Tuna Demirdal, Dilara Inan, Taner Yıldırmak, Arzu Kantürk, Ediz Tütüncü, Hepatitis B Study Group

Orhan Yıldız, Bilgehan Aygen, Department of Infectious Diseases and Clinical Microbiology, Medical School of Erciyes University, 38039 Kayseri, Turkey
Nese Demirtürk, Tuna Demirdal, Department of Infectious Diseases and Clinical Microbiology, Medical School of Kocatepe University, 03030 Afyon, Turkey
Dilara Inan, Department of Infectious Diseases and Clinical Microbiology, Medical School of Akdeniz University, 07058 Antalya, Turkey
Taner Yıldırmak, Arzu Kantürk, Okmeydani Training and Research Hospital of the Ministry of Health, 34384 Istanbul, Turkey
Ediz Tütüncü, SB Diskapi Yildirim Beyazit Training and Research Hospital of the Ministry of Health, 06110 Ankara, Turkey
Hepatitis B Study Group, Sıla Akhan, Celal Ayaz, Mustafa Çelen, Nurcan Baykam, Kenan Hızel, Uner Kayabas, O Faruk Kokoglu, Hasan Ucmak, Sukran Kose, Resit Mıstık, Oral Oncul, Nese Saltoglu, Lutfu Savas, Yusuf Onlen, Necla Tulek
Author contributions: Yıldız O and Aygen B designed the study, directed its implementation, supervised all the field activities, contributed to the analysis and interpretation of the data, and to the redaction of the manuscript; Demirtürk N, Demirdal T, Inan D, Yıldırmak T, Kantürk A and Tütüncü E contributed to the analysis and interpretation of the data; all authors read and approved the final manuscript.
Correspondence to: Orhan Yıldız, MD, Associate Professor, Erciyes Universitesi, Tıp Fakültesi, Enfeksiyon Hastalıkları Kliniği, 38039 Kayseri, Turkey. oyildiz@erciyes.edu.tr
Telephone: +90-352-4374937 Fax: +90-352-4374948
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Abstract

AIM: To determine the distribution of viral genotypes for primary or acquired lamivudine resistance.

METHODS: A total of 283 patients with chronic hepatitis B virus (HBV) infection (245 patients with chronic hepatitis B and 38 inactive hepatitis B surface antigen carriers) were included in the study. The HBV genotype was determined by using quantitative real-time polymerase chain reaction and sequence analysis, and tyrosine-methionine-aspartate-aspartate (YMDD) motif mutations were determined using the reverse transcriptase hybridization method.

RESULTS: Lamivudine resistance was determined in a total of 25 (10.7%) chronic hepatitis B patients. Eight subjects (4%) had primary resistance to lamivudine, and 17 (53.1%) had secondary resistance to lamivudine. Genotype D, which was isolated from 267 of the patients with chronic HBV infection, was the dominant genotype in Turkey.

CONCLUSION: Identification of YMDD motif mutations should have a positive impact on the selection of proper antiviral medication for patients, even for those who are nucleoside naïve.

Key words: Hepatitis B virus; Genotype; Resistance; Lamivudine; Tyrosine-methionine-aspartate-aspartate mutation

Peer reviewer: Eric WC Tse, MB, PhD, Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China

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INTRODUCTION

Chronic infection with hepatitis B virus (HBV) is an

important cause of morbidity and mortality worldwide[1]. The objective in the treatment of this disease is to cease viral replication and provide improvement in liver histopathology; thereby preventing complications such as cirrhosis and hepatocellular carcinoma and the associated mortality[2].

Lamivudine is a synthetic nucleoside analogue that suppresses viral replication by inhibiting viral polymerase activity[3]. Although lamivudine is an effective and well-tolerated medication, duration of treatment is quite long, and resistance is an important issue[4]. Resistance to lamivudine is associated with mutations in the HBV polymerase gene. The most frequent mutation that develops with lamivudine is the tyrosine-methionine-aspartate-aspartate (YMDD) mutation of the HBV polymerase gene. It has been shown that all lamivudine resistant patients had a HBV YMDD mutation[5]. Among them, 41.3% of the patients had the rtL180M/M204V mutation, 25.7% had the rtL180M/M204I mutation and 33% had the rtM204I mutation. The frequency of mutation increases proportionally with the duration of lamivudine treatment, and the five-year post-treatment frequency is about 65%-70%[6,7]. On the other hand, resistance to lamivudine might also be seen in nucleoside naïve chronic hepatitis B patients or inactive HBsAg carriers. This resistance is associated with structural changes in the DNA polymerase enzyme gene[8,9]. The recommended methods of treatment aimed at preventing resistance include the use of more potent agents, changing the treatment in patients in whom the disease progressed during treatment, and the use of combined antiviral agents.

There are eight HBV genotypes (A-H), and the distribution of these genotypes varies by geographic region. Genotype A and C are prevalent in the American continent, whereas the prevalent genotypes are B and C in Asian countries, D in Europe, and E in Africa[10-13].

The aims of the present study were to determine the dominant viral genotype in chronic HBV infection in Turkey, and determine the prevalence of primary or acquired lamivudine resistance by genotype.

MATERIALS AND METHODS

This is a multi-center study including 17 centers from Turkey between January and September 2007. Patients between the ages of 18 and 65 years who were inactive HBsAg carriers or who had chronic hepatitis B (regardless of antiviral treatment status) were included in this study. Patients with co-infections with hepatitis C virus, hepatitis D virus or human immunodeficiency virus, along with patients with primary or secondary causes of liver disease other than hepatitis B (e.g., autoimmune hepatitis, steatohepatitis, hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficiency, and severe cardiopulmonary disease) were excluded from the study. This study was approved by the Local Ethics Committee of the Department of Medicine at the Erciyes University in Turkey. A written informed consent was obtained from all participants before laboratory tests were performed.

Laboratory tests

The complete blood count was determined using an automated cell counter (Coulter LH750). Liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in serum using standard commercial kits (Boehringer, Mannheim, Germany). All samples were screened to detect HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc (total), and anti-HBc IgM, by using third-generation microparticle enzyme immunoassays (Abbott Laboratories, Chicago, IL). HBV DNA was investigated by real-time polymerase chain reaction (PCR) (COBAS TaqMan 48 analyzer, Roche Diagnostics) according to manufacturer's instructions.

HBV genotypes were determined by DNA sequencing. HBV DNA was extracted from serum samples. HBV DNA polymerase gene region was amplified by nested-PCR using specific primers. PCR products were analyzed on UV transilluminator and purified from agarose gel. The purified products were sequenced by the Visible Genetics OpenGene system (Visible Genetics, Toronto, Ontario, Canada) and the Cy5.5 dye terminator cycle sequencing kit (Amersham, Pharmacia Biotech, Piscataway, NJ, United States). YMDD mutation in the HBV DNA polymerase gene was determined with RT hybridization (INNO-LiPA HBV DR-Innogenetics, Ghent-Belgium), according to manufacturer's instructions. A part of HBV domains B and C of the pol gene was amplified. The biotinylated PCR fragments were reverse hybridized using typing-membrane-based INNO-LIPA HBV DR strips. After hybridization, streptavidin labeled with alkaline phosphatase were added and bound to the previously formed biotinylated hybrids. Incubation with the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitro blue tetrazolium chromogen resulted in a purple- brown color development.

A percutaneous liver biopsy was performed at each center under local anesthesia (optional). A biopsy specimen of more than 1 cm in length with five to six portal tracts was collected. Chronic hepatic disease activity was classified according to the Knodell histological activity index (HAI)[14].

Diagnostic criteria

Chronic hepatitis B: (1) HBsAg positive for at least 6 mo; (2) serum HBV DNA > 20.000 IU/mL (> 10^5 copies/mL), often ranges between 2.000-20.000 IU/mL in HBeAg-negative patients; (3) persistent or intermittent elevation of ALT/AST levels; and (4) moderate or severe necro-inflammation.

Inactive HBsAg carriers: (1) HBsAg positive for at least 6 mo; (2) HBeAg-negative, anti-HBe-positive; (3) serum HBV DNA < 2.000 IU/mL (< 10^4 copies/mL); (4) persistently normal ALT/AST; and (5) no signs of hepatitis in liver biopsy.

Demographic features of the patients, possible routes of transmission of HBV, and the antiviral treatments

Table 1 Demographic features of study patients

	Chronic hepatitis B	Inactive HBsAg carriers	*P* value
No. of patients, *n* (%)	245 (86.6)	38 (13.4)	
Sex (male/female)	166/79	11/27	0.852
BMI (mean ± SD, kg/m^2)	25.9 ± 4.3	25.7 ± 3.6	0.914
Hemoglobin (mean ± SD, gr/dL)	14.9 ± 1.5	14.9 ± 1.6	0.949
White cell count (mean ± SD, /mm^3)	6577 ± 1783	6615 ± 1599	0.959
Thrombocyte count (mean ± SD, 1000/mm^3)	200 482 ± 57 762	219 727 ± 63 743	0.088
AST (mean ± SD, IU/L)	56.2 ± 51.3	25.2 ± 11.1	0.000
ALT (mean ± SD, IU/L)	91.6 ± 92.6	25.3 ± 13.2	0.000
HBeAg positivity, *n* (%)	57 (23.2)	0	
HBV DNA (mean ± SD copies/mL)	$4.9 \times 10^8 \pm 5.9 \times 10^9$	$2.0 \times 10^3 \pm 2.6 \times 10^3$	0.000
Histopathological findings, *n*[1]:143			
Total HAI score, median (range)	7 (1-18)		
PN and BN, median (range)	2 (0-7)		
ID and FN, median (range)	1 (0-4)		
PI, median (range)	2 (0-4)		
Fibrosis, median (range)	2 (0-4)		

[1]Number of patients who underwent liver biopsy. BMI: Body mass index; HAI: Histological activity index; PN: Piecemeal necrosis; BN: Bridging necrosis; ID: Intralobular degeneration; FN: Focal necrosis; PI: Portal inflammation; HBV: Hepatitis B virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 2 Levels of hepatitis B virus DNA and alanine aminotransferase in chronic hepatitis B by HBeAg positivity

	HBeAg-positive (*n* = 57)			HBeAg-negative (*n* = 210)			*P* value
	Min	Max	mean ± SD	Min	Max	mean ± SD	
ALT (IU/L)	17	537	121.6 ± 120.3	7	423	81.9 ± 77.4	0.012
HBV DNA (copies/mL)	1×10^4	9.3×10^{10}	$1.7 \times 10^9 \pm 1.2 \times 10^{10}$	1×10^4	1.4×10^9	$1 \times 10^8 \pm 2.3 \times 10^8$	0.15

ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

administered and their durations were all recorded. The decision to start antiviral therapy was based on the serum HBV DNA and aminotransferase levels, histological grade and stage at each center[15,16]. Lamivudine (100 mg/d) was administered orally in all eligible chronic hepatitis B patients. Resistance status was determined for patients receiving and not receiving lamivudine. The relationship between resistance and sex, body mass index, genotype, HBV DNA levels, ALT/AST levels, HBeAg positivity or negativity, and necro-inflammation and fibrosis in the liver were investigated in subjects with primary resistance.

Statistical analysis

"SPSS 12.0 for Windows" was used for statistical analyses. For continuous variables, descriptive statistics (mean, median, standard deviation, minimum and maximum) were calculated. For comparisons of categorized variables, the Chi-square test was used, and for continuous variables, the Student's *t* test and Mann-Whitney *U* test were used. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 283 patients with chronic HBV infection composed of 245 patients with chronic hepatitis B and 38 inactive HBsAg carriers were included in the study; 193 were males, and 90 were females. No differences were observed between the two groups except for the levels of HBV DNA and ALT/AST (Table 1). The histopathological findings of chronic hepatitis B patients who underwent optional liver biopsy are presented in Table 1. The median Knodell fibrosis score was 2 (0-4), and the median HAI was 7 (1-18).

Genotype determination with sequence analysis revealed that the HBV isolated from 267 patients were all of genotype D. Comparison of subgroups of chronic hepatitis B patients in terms of the "e" antigen revealed that the levels of ALT were higher in patients who are positive for HBeAg compared to those who were negative (121.6 U/L and 81.9 U/L, respectively, $P = 0.012$). However, there was no statistically significant difference between the levels of HBV DNA (1.7×10^9 copies/mL and 1×10^8 copies/mL, respectively, $P = 0.150$, Table 2).

HBV drug resistance

HBV was isolated from a total of 267 patients with chronic HBV infection and analyzed for YMDD resistance using INNO-LiPA HBV DR. Of these patients, 104 (39%) had a history of previous treatment with an antiviral, and 32 (30.8%) of these patients had received lamivudine. The mean duration of lamivudine treatment was 18.5 ± 14.5 mo in these subjects, and 17 of these (53.1%) developed secondary resistance. Primary resistance to lamivudine was determined in 8 (4%) out of 202 lamivudine naïve patients. The frequency of YMDD

Table 3 Rates of tyrosine-methionine-aspartate-aspartate mutation in patients with chronic hepatitis B virus infection *n* (%)

	Chronic hepatitis B			Inactive HBsAg carriers	Total
	Previous lamivudine treatment		Total		
	Yes[1]	No			
YVDD + YIDD positive	17 (53.1)	8 (4)	25 (10.7)	1 (3)	26 (9.7)
YMDD negative	15 (46.9)	194 (96)	209 (89.3)	32 (97)	241 (90.3)
Total	32	202	234	33	267

[1]Patients who are receiving or received lamivudine previously. YMDD: Tyrosine-methionine-aspartate-aspartate; YIDD: Tyrosine-isoleucine-aspartate-aspartate; YVDD: Tyrosine-valine-aspartate-aspartate.

mutation in patients with chronic hepatitis B is shown in Table 3. Wild-type HBV was determined in 241 (90.3%) patients. YMDD mutation was determined in 26 (9.7%) patients, and only one of these subjects (1/33, 3%) was an inactive HBsAg carrier.

No relationship was established between YMDD motif mutation and viral load, body mass index, liver function tests, HBeAg positivity and liver histopathology. Similarly, there was no difference in those same parameters between chronic hepatitis B patients with primary and secondary resistance to lamivudine.

DISCUSSION

Lamivudine is the first nucleoside analogue approved for the treatment of chronic hepatitis B infection. It has been used successfully for years in the treatment of chronic hepatitis B patients due to its rapid suppression of HBV DNA levels and perfect side effects profile. It is often received as a single daily 100 mg dosage and is generally well-tolerated[17]. Although lamivudine is a potent medication against HBV, the duration of the treatment is quite long, and resistance is an important issue [4]. It has been shown that YMDD mutations increase with the duration of lamivudine treatment in chronic hepatitis B patients treated with lamivudine for long periods of time[5-7].

In the study by Alvarado-Esquivel *et al*[18], the prevalence of wild-type HBV was found to be 94.9%, and the prevalence of genotypic resistance to lamivudine was reported as 2.6%. Sun *et al*[19], on the other hand, observed the YMDD mutation in 17% (42/247) of patients treated with lamivudine. These patients had used lamivudine for a mean duration of 533 d, and the cumulative incidence of YMDD mutation was 41.3%. The YMDD motif mutation has been investigated in the polymerase gene in 267 of 283 chronic hepatitis B patients. Among the subjects, 90.3% were infected with wild-type HBV. The frequency of the YMDD mutation was 10.7%, and the majority of these were chronic hepatitis B patients. However, the prevalence of YMDD mutation was lower in inactive HBsAg carriers (3%). This patient group was infected with viruses that replicate less compared to wild-type viruses. The cause of YMDD mutations and the effects of these mutant serotypes on hepatic activation are not known. The findings of the present study are in line with the literature, as mutations were observed in 53.1% of the total 32 patients whose lamivudine therapy duration was 18.5 mo (secondary resistance).

Sensitive diagnostic tests allow for the identification of rtM552I/V mutant HBV in patients receiving lamivudine at the third month of treatment. The wild-type virus re-emerges upon termination of treatment with lamivudine, and resistant serotypes emerge immediately upon re-initiation of treatment. However, lamivudine resistance has also been reported in chronic hepatitis B patients with no history of lamivudine use, and inactive HBsAg carriers (primary resistance). The frequency of primary resistance, which is associated with structural changes in the DNA polymerase enzyme gene, ranges between 0% and 27.7%[9,20-23]. Five (27.7%) out of 18 nucleoside naïve patients, and 4 out of 36 (11.1%) patients were found to have the YMDD mutation in studies by Kobayashi *et al*[20] and Kirishima *et al*[21], respectively. Both studies revealed that all patients who were positive for mutation were also anti-HBe positive. Heo *et al*[22] observed mutations in 3 (7.5%) out of 40 lamivudine naïve patients and reported HBeAg positivity in one of these subjects. However, no YMDD mutation was observed in the first study by Matsuda *et al*[9,23] performed on nucleoside naïve chronic hepatitis B patients, whereas 2 (2.8%) out of 71 subjects were found to have the mutation in their second study.

Akarsu *et al*[24] reported from Turkey that 13 (18.3%) out of 71 asymptomatic carriers of hepatitis B with no history of antiviral treatment were infected with lamivudine-resistant HBV. In another study from Turkey, 6 (7.8%) of 77 nucleoside naïve patients were reported to have the YMDD motif mutation[25]. In the present study, YMDD motif mutations were found in 8 (4%) out of 202 patients with no history of antiviral treatment. The reason for the marked difference between these studies might be due to differences in geographical location, mean age, age at infection, HBeAg positivity, HBV genotype and levels of viral load.

Some studies have shown that YMDD mutation is a feature of patients with low viral load[20,21]. However, no relationship was demonstrated in other studies between viral load and mutation[22-24]. In this study, no relationship was found between YMDD motif mutation and viral load, body mass index, liver function tests, HBeAg positivity and liver histopathology.

Studies from Turkey performed by Tuncbilek *et al*[25], Senturker *et al*[26] and Bozdayi *et al*[27] have reported that all examined patients (77, 23 and 41 patients, respectively) were infected with HBV genotype D. However, the results from the previous studies may not be generalizable to the larger population of Turkey due to the inadequate number of patients and limited geographic locations from which the data in these studies were obtained.

In the present study, all 267 Turkish chronic hepatitis B patients were infected with HBV genotype D according to the genotype analysis performed with the sequence analysis method. This multi-center and comprehensive study was the first to show the dominance of the genotype D in Turkey.

In conclusion, lamivudine therapy has been associated consistently with secondary resistance in chronic hepatitis B patients. The assessment of genotypic resistance prior to re-treatment of chronic hepatitis B patients with lamivudine or a different antiviral agent might increase the efficacy of treatment and prevent complications. In this study, the frequency of primary resistance to lamivudine was found to be 4% in nucleoside naïve patients with chronic hepatitis B. In addition, the identification of YMDD motif mutations prior to nucleoside treatment should have a positive impact on the selection of proper antiviral medication for patients, even for those who are nucleoside naïve.

COMMENTS

Background

Hepatitis B virus (HBV) infection is a worldwide problem, with potentially 400 million individuals with active HBV infection. HBV is a significant cause of morbidity and mortality from cirrhosis, liver failure, and hepatocellular carcinoma in these populations. Lamivudine is still used widely to treat HBV infection in Turkey. However, lamivudine resistance is a major concern.

Research frontiers

Eight genotypes of HBV (A-H) have been identified, and increasing evidence suggests that certain genotypes may have a significant impact on both the choice of treatment and clinical outcome, and is also affected by the patterns of resistance manifested by local strains. The authors studied the genotype profiles and lamivudine resistance patterns of HBV isolates among Turkish patients.

Innovations and breakthroughs

The tyrosine-methionine-aspartate-aspartate (YMDD) motif mutation has been investigated in the polymerase gene in chronic hepatitis B patients. The findings of the present study are in line with the literature. A significant proportion of patients with lamivudine resistance would not be able to receive proper antiviral medication and may run the risk of disease progression in Asia. Although it is expected that HBV genotype D to be dominant in Turkey, it was surprising that no other genotypes were detected.

Applications

Identification of YMDD motif mutations prior to nucleoside treatment might help in decisions regarding selection of antiviral therapy, and expected responses to treatment.

Peer review

In this manuscript, the authors have reported that the HBV genotype is prevalent in Turkey. The frequency of YMDD mutants was also determined. The methodology employed in this study was very much standard. The results are of significant local importance.

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BRIEF ARTICLE

Failed biliary cannulation: Clinical and technical outcomes after tertiary referral endoscopic retrograde cholangiopancreatography

Michael P Swan, Michael J Bourke, Stephen J Williams, Sina Alexander, Alan Moss, Rick Hope, David Ruppin

Michael P Swan, Michael J Bourke, Stephen J Williams, Sina Alexander, Alan Moss, Rick Hope, David Ruppin, Department of Gastroenterology and Hepatology, Westmead Hospital, Sydney 2145, Australia

Author contributions: Swan MP designed the study, wrote the manuscript and performed some of the procedures; Bourke MJ and Williams SJ supervised all procedures and performed some of the procedures; Bourke MJ designed the study and assisted in the writing of the manuscript; Alexander S and Moss A collected data and assisted in the care of patients; Ruppin D and Hope R performed some of the procedures.

Correspondence to: Dr. Michael J Bourke, Director of Gastrointestinal Endoscopy, Department of Gastroenterology and Hepatology, Westmead Hospital, Hawkesbury Road, Sydney 2145, Australia. michael@citywestgastro.com.au

Telephone: +61-2-96335055 Fax: +61-2-96333958

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Abstract

AIM: Prospective evaluation of repeat endoscopic retrograde cholangiopancreatography (ERCP) for failed Schutz grade 1 biliary cannulation in a high-volume center.

METHODS: Prospective intention-to-treat analysis of patients referred for biliary cannulation following recent unsuccessful ERCP.

RESULTS: Fifty-one patients (35 female; mean age: 62.5 years; age range: 40-87 years) with previous failed biliary cannulation were referred for repeat ERCP. The indication for ERCP was primarily choledocholithiasis (45%) or pancreatic malignancy (18%). Successful biliary cannulation was 100%. The precut needle knife sphincterotomy (NKS) rate was 27.4%. Complications occurred in 3.9% (post-ERCP pancreatitis). An identifiable reason for initial unsuccessful biliary cannulation was present in 55% of cases. Compared to a cohort of 940 naïve papilla patients (female 61%; mean age: 59.9 years; age range: 18-94 years) who required sphincterotomy over the same time period, there was no statistical difference in the cannulation success rate (100% *vs* 98%) or post-ERCP pancreatitis (3.1% *vs* 3.9%). Precut NKS use was more frequent (27.4% *vs* 12.7%) (P = 0.017).

CONCLUSION: Referral to a high-volume center following unsuccessful ERCP is associated with high technical success, with a favorable complication rate, compared to routine ERCP procedures.

Key words: Failed endoscopic retrograde cholangiopancreatography; Failed biliary cannulation; Unsuccessful biliary cannulation; Tertiary referral endoscopic retrograde cholangiopancreatography; Needle knife sphincterotomy; Biliary cannulation; Precut sphincterotomy; Post endoscopic retrograde cholangiopancreatography pancreatitis

Peer reviewer: Kyu Taek Lee, MD, PhD, Professor, Department of Medicine Samsung Medical Center, Sungkyunkwan, University School of Medicine, No. 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

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INTRODUCTION

Despite technical innovations, structured training programs and improved endoscopic imaging, failed biliary

cannulation during endoscopic retrograde cholangiopancreatography (ERCP) occurs in 5%-20% of all cases[1]. Achieving biliary cannulation during ERCP remains an important and transparent determinant of a successful procedure for biliary endoscopists and patients alike. The American Society for Gastrointestinal Endoscopy recommendations suggest a biliary cannulation rate of > 85% should be the goal for all endoscopists engaged in ERCP[2]. Differing rates of successful biliary cannulations have been reported from community and tertiary institutions[1,3,4], with higher rates (up to 98%) achieved in specialized tertiary institutions[5,6,7], which reflects higher workload and experience, greater expertise, and more frequent use of more advanced cannulation techniques.

The therapeutic options following failed biliary cannulation may include: (1) repeat endoscopic attempt; (2) percutaneous cholangiography; (3) endoscopic ultrasound (EUS)-guided bile duct puncture and drainage; and (4) surgical management. This study was undertaken to assess the clinical impact of a tertiary referral ERCP service for prior unsuccessful biliary cannulation.

MATERIALS AND METHODS

Over a 34-mo period to September 2009, patients referred for a repeat ERCP following a previous failed biliary cannulation were enrolled prospectively on an intention-to-treat basis. A number of different hospitals and endoscopists referred patients to our center following an unsuccessful unsuccessful biliary cannulation at ERCP. All the referring endoscopists were accredited specialist biliary endoscopists[8]. The indication for ERCP was evaluated and deemed appropriate to require a further attempt at biliary cannulation by both the referring specialist and our center. The referring specialist was asked to provide a reason (if any) as to the possible cause for the failed cannulation.

Procedures were performed under propofol-based sedation provided by an anesthetist. An Olympus Exera TJF-160R duodenoscope (Olympus Optical, Japan, Tokyo) was used.

The cannulation technique and algorithm has been previously described in detail elsewhere[7]. A wire-guided technique utilizing a triple-lumen sphincterotome (CleverCut3™; Olympus Medical) and a 400-mm long hydrophilic wire (Jagwire™, Boston Scientific, Spencer, IN, United States) was used initially in all cases. In the event that a precut needle knife sphincterotomy was needed, all procedures used the conventional (freehand papillotomy/deroofing) needle knife sphincterotomy (NKS) method (Figure 1).

As is standard practice at our center[7,9], all cannulation parameters were recorded at the time of the procedure, including time to cannulation, number of attempts on papilla, number of pancreatic cannulations, and cannulation techniques performed. All procedures were performed by or under the direct supervision of two experienced high-volume biliary endoscopists (MJB, SJW). Clinical follow-up was obtained at 24 h and 30 d post-procedure.

Table 1 Schutz grade for endoscopic retrograde cholangiopancreatography degree of difficulty for biliary procedures[11]

	Biliary procedures
Grade 1	Diagnostic cholangiogram
	Biliary cytology
	Standard sphincterotomy ± removal of stones < 10 mm
	Stricture dilatation/stent for extra-hepatic stricture or bile leak
Grade 2	Diagnostic cholangiogram with Billroth Ⅱ anatomy
	Removal of CBD stones > 10 mm
	Stricture dilatation/stent for hilar tumors or benign intrahepatic strictures
Grade 3	SOD manometry
	Cholangioscopy
	Any therapy with Billroth Ⅱ anatomy
	Removal of intrahepatic stones or any stones with lithotripsy

CBD: Common bile duct; SOD: Sphincter of Oddi dysfunction.

Patients referred after prior failed cannulation were compared with a cohort of naïve papilla patients undergoing ERCP for a biliary indication performed at our center during the same time period.

Failed biliary cannulation was defined as the inability to gain deep and free access to the bile duct. Cholangiography alone without deep instrumentation of the bile duct was not recorded as being successful. Complications were defined and recorded according to established consensus criteria[10]. The indication and complexity of the ERCP procedure was graded according to the modified Schutz criteria (Table 1)[11]. Only Schutz grade 1 cases were included in this study. Exclusion criteria were patient age < 18 years old, surgically altered anatomy (Roux-en-Y, Billroth Ⅱ partial gastrectomy), and duodenal obstruction. As a referral center, Schutz grade 2 and 3 cases were received and managed by our group but were not included in this study.

Primary endpoints were success of biliary cannulation and post-ERCP complications.

Statistical analysis was performed using StatPlus:mac LE, (AnalystSoft, Vancouver, BC, Canada). Student's *t* test was used to detect differences between the two groups. Statistical significance was accepted on the basis of a *P* value < 0.05.

RESULTS

Fifty-one patients [35 female (69%); mean age: 62.5 years; age range: 40-87 years] with previously unsuccessful biliary cannulation at previous ERCP were referred and prospectively enrolled. The main indications for biliary cannulation and therapeutic intervention (Table 2) in the tertiary referral group were choledocholithiasis (45.1%), pancreatic malignancy (17.7%) and benign biliary disease [i.e., primary sclerosing cholangitis (PSC), chronic pancreatitis related biliary stricture] (12%).

All referred ERCP procedures were assessed as grade 1 difficulty, indicating that they were applicable to be

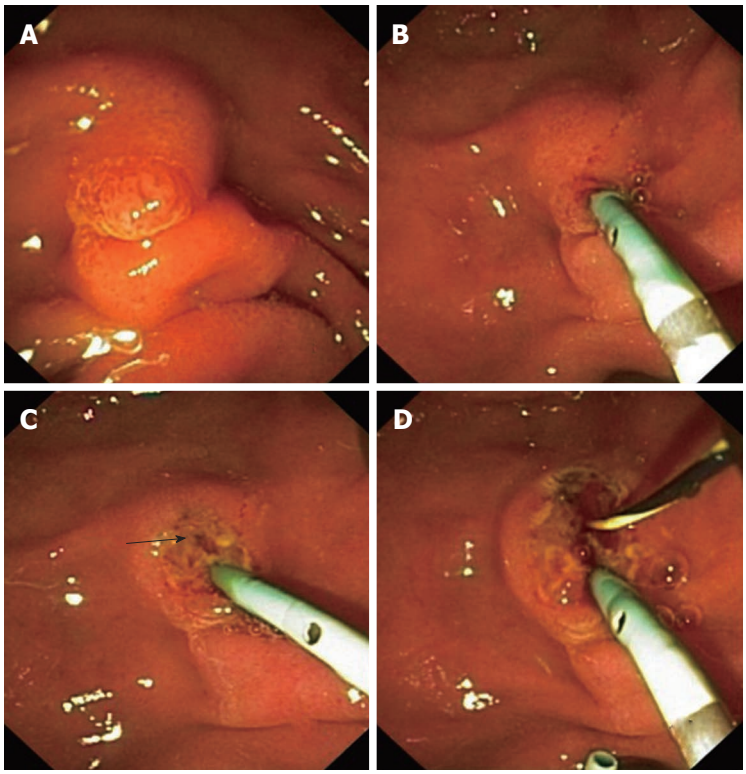

Figure 1 Technique of conventional/freehand/deroofing precut needle knife sphincterotomy. Case of previously failed biliary cannulation without an identifiable cause. A: Normal papillary appearance; B: After we were unable to cannulate selectively the biliary orifice after several pancreatic duct wire cannulations, a pancreatic stent was inserted; C: Precut needle knife sphincterotomy was performed with a cut directed from the ampullary orifice towards the 12 o'clock position. Biliary epithelium and bile noted (arrowpoint) at apex of the cut; D: Bile duct selectively cannulated with wire and sphincterotomy performed.

Table 2 Clinical indication for procedures *n* (%)

Indication	Tertiary referral cohort (*n* = 51)	Naïve papilla cohort (*n* = 940)	*P* value
Choledocholithiasis	23 (45.1)	446 (47.4)	0.78
Pancreatic malignancy	9 (17.7)	194 (20.6)	0.72
Benign biliary stricture/PSC	6 (11.8)	41 (4.4)	0.03
Bile leak	5 (9.8)	59 (6.3)	0.37
Cholangitis	4 (7.8)	57 (6.1)	0.48
Other (acute pancreatitis, suspected sphincter dysfunction)	4 (7.8)	143 (15.2)	0.22

PSC: Primary sclerosing cholangitis.

Table 3 Comparison of referred failed biliary cannulation ERCP and naïve papilla patients

	Non-referred naive papilla	Previous failed biliary cannulation	*P* value
No.	940	51	
Mean age (yr)	62.5 (40-87)	59.9 (18-96)	NS
Sex (female:male)	69:31	61:39	NS
Cannulation attempts	3.2 (1-14)	2.96 (1-8)	NS
Time to cannulation	4.2 min (0.08-41)	4.4 min (0.8-13.5)	NS
Cannulation success	98%	100%	1.00
NKS rate	12.7%	27.4%	0.017
PEP	3.1%	3.9%	1.00
Other ERCP complication	Bleeding 0.5% Perforation 0.2%	Nil	

ERCP: Endoscopic retrograde cholangiopancreatography; NKS: Needle knife sphincterotomy; PEP: Post-ERCP pancreatitis; NS: Not significant.

performed by all competent biliary endoscopists. Eleven (21.6%) patients had had a previously attempted precut NKS. Two patients (5%) had post-ERCP pancreatitis (PEP) following their initial unsuccessful biliary cannulation.

Biliary cannulation was successful in all 51 referred patients in a single procedure. A mean of 2.96 attempts (range: 1-8) was required to gain biliary access, with a mean time to successful cannulation of 4.4 min (range: 0.8-13.5 min). Fourteen patients (27.4%) required precut NKS. Eleven patients (21.6%) had had a previous precut NKS attempt before referral, of which 7 seven (64%) required a further NKS to gain biliary access. Two patients (3.9%, both choledocholithiasis indication) developed moderate severity PEP (requiring uncomplicated 5 d and 4 d inpatient stays, respectively). Definitive organic findings (choledocholithiasis, biliary sludge, stricture, malignancy) were identified in 42/51 patients (82%), with the remainder having non-specific features such as biliary duct dilatation only.

The naïve ERCP cohort comprised 940 patients (female 61%; male 39%; mean age: 59.9 years; age range: 18-96 years) and was not significantly different from the referred failed ERCP cohort (Table 3). Indications were similar to those in the referral cohort (Table 2). The majority of procedures were performed for either choledocholithiasis (47.4%) or pancreatic malignancy (20.6%). Cannulation success was 98% (920/940), and 12.7% required NKS to gain access to the bile duct. A mean 3.2

cannulation attempts (range: 1-14) was required to gain biliary access, with a mean time to successful cannulation of 4.2 min (range: 8 s-41 min). The complication rate was 3.8% overall: 3.1% PEP, 0.5% bleeding, and 0.2% perforation. Two patients had perforation following sphincterotomy and both were managed conservatively in hospital for 9 and 11 d, respectively.

No reason for the initial failed biliary cannulation was identified in 23 (45%) patients. The 28 patients (55%) with an identifiable reason had a wide range of possible explanations for initial failure, including long and mobile (floppy) papilla and intraduodenal segment of the common bile duct (n = 8, 29%), unstable position (n = 9, 32%), small papilla (n = 4, 14%) or periampullary diverticulum (n = 7, 25%). In two of the periampullary diverticular cases, the papilla could not be identified at the initial procedure.

Technical rates, rates of successful biliary cannulation and complications were comparable in both groups (Table 3). Only the use of precut NKS in the tertiary referral cohort was significantly different at 27.4% *vs* 12.7% (P = 0.017). Half of the NKS procedures were performed in patients with a previous NKS attempt. Correcting for this variable, the NKS rate in the referred group was still statistically significantly greater (20%) than in the comparison group ($P < 0.05$).

DISCUSSION

Following a failed cannulation attempt, the endoscopist, in consultation with the patient and family, should carefully consider the next management step. The procedural indication and the definitive need for therapeutic intervention should be re-evaluated. Non-invasive imaging including magnetic resonance cholangiography or EUS should be utilized if they are likely to add value before proceeding. This study demonstrates that following an unsuccessful attempt at biliary cannulation, a further attempt in a tertiary referral center achieves a high success rate with minimal complications. We have specifically examined Schutz grade 1 patients; a cohort typical of cases managed by appropriately trained and competent biliary endoscopists in routine practice. The current study's incidence of complications of 3.9% (which was limited to PEP) is not different from the large and carefully studied comparison group or from other large recently published studies[12,13].

Outcomes for a repeat endoscopic attempt following unsuccessful ERCP in heterogeneous groups of patients have been reported > 10 years previously[14-16]. The past decade has seen many changes in ERCP practice, coupled with enhanced awareness of the risk factors for complications, particularly PEP, and greater utilization of non-invasive imaging. The importance of dedicated training and continuous quality audit has also been more widely recognized. The endoscopes, accessories utilized and cannulation techniques used have also evolved. This contemporary study carefully examined a well-characterized group of Schutz grade 1 cases and confirmed that prior failure by a competent and accredited biliary endoscopist is not an absolute indication to seek an alternative strategy to access the biliary tree, nor is a repeat attempt associated with higher complications. In 1999, Ramirez *et al*[14] reported 47 patients with failed cannulation, of which 24 (51%) underwent repeat ERCP by the same endoscopist. Successful cannulation was achieved in 87.5%, without any complications being noted. Although a significant proportion of cases were Schutz 1 complexity, Billroth Ⅱ anatomy was present in 17% of cases and minor papillary cannulation was required in 2%. Kumar *et al*[16] in 1995 and Choudari *et al*[15] in 2000 documented referral to a tertiary center for a second ERCP attempt in 113 and 562 patients, respectively. Over 50% of cases were Schutz grade 2-3 and represented a high degree of difficulty, including > 20% of cases requiring manometry. Both studies achieved 96% successful cannulation, although complications (mostly pancreatitis) occurred in 10%-11% of patients. Unlike the current study, the higher proportion of more complex cases in these last two retrospective studies reduces the ability to extrapolate the results to more general endoscopic practice. It is quite reasonably argued that Schutz grade 2 and 3 cases should not be attempted outside of high volume, expert tertiary centers. The rate of NKS in the cohort of referred patients (27.4%) was significantly higher than in the naïve papilla group, as well as appreciably higher than the aforementioned studies of repeat ERCP following previously failed cannulation[13-15] and other recent studies[17]. Our unit has recently shown that, in experienced hands, NKS is not a risk for PEP[17]. The use of NKS in a referred group of previous unsuccessful cannulations should be considered a necessary and vital part of the armamentarium of an experienced ERCP endoscopist.

Increasingly compelling data are emerging to confirm that, when the clinical entity or its treatment is complex or attended by high morbidity, then referral to experienced high-volume centers results in superior procedural and patient outcomes and reduced costs[18,19]. Similarly, ERCP procedures in high-volume centers have confirmed benefits including improved cannulation rates, lower complication rates and better patient outcomes[20,21]. Patients who have failed one attempt at cannulation in competent hands self select themselves as a group of "difficult cannulation" patients and are therefore best managed in an experienced tertiary center.

In the event of failed ERCP cannulation, studies indicate that 50%-60% of patients have further therapeutic procedures-radiological, surgical or repeat endoscopic management[22,23]. Percutaneous trans-hepatic cholangiography drainage and stenting is a common interventional radiological procedure utilized in the management of malignant obstructive jaundice, as well as in the treatment of operative bile leaks and benign biliary strictures[24,25].The technical and clinical success of a radiological approach has been reported to be > 90%, however, complications

occur in 20%-30%, including cholangitis, bile leak, hemorrhage, post-procedural pain and stent/tube malfunction[26]. Protracted inpatient management is also usually necessary. The use of surgical management is of limited utility in non-malignant indications due to high morbidity (17%-37%) and high mortality (2%-5%)[27-29]. Recently, EUS-guided biliary drainage, either via rendezvous approach or direct puncture and stenting, has emerged as a viable therapeutic alternative[30,31]. This technique is used mainly in malignant obstructions, with technical and clinical success rates above 90% and 5%-15% complication rates. However, experience is currently limited to enthusiastic proponents of this technique and its durability is not well established.

The technical reasons for unsuccessful Schutz grade 1 biliary cannulation have not been previously documented. Although this study does not have an explanation for unsuccessful biliary cannulation in all cases (only 55% had an identifiable etiology), it does detail situations that intuitively may lead to more difficult cannulation, such as a long and mobile intraduodenal portion of the bile duct, periampullary diverticulum, an unstable position or a small papilla. Peripapillary diverticulum was cited as the cause of 14% of the unsuccessful biliary cannulations, despite two recent studies indicating no effect on cannulation rates[32,33].

The current study is limited by lack of randomization to an alternative therapeutic modality or a comparator group. Selection bias may also have been present because the referred patients were likely to have been considered more appropriate for a repeat ERCP attempt by the referrers than those not referred.

In conclusion, referral to an experienced high-volume center following an initial unsuccessful Schutz grade 1 biliary cannulation attempt is associated with high technical success and a favorable complication profile, compared to other routine naïve papilla ERCP procedures. We believe that a second ERCP procedure in a tertiary referral center should be the next management step following an unsuccessful biliary cannulation in this patient group. This type of clinical pathway should be part of the range of management options available to biliary endoscopists working outside high-volume tertiary centers.

COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is an invaluable endoscopic technique in the management of gallstones and bile duct pathology. The technique is not always successful and it is unclear what the next management step should be following a failed attempt.

Research frontiers

Knowledge in endoscopy continues to evolve on many fronts with an ever-expanding repertoire of indications and techniques. Evidence based justification is addressed with each new advance however more common dilemmas (such as what to do following a failed ERCP procedure) lack clear evidence. Repeat ERCP attempt has been evaluated in three previous studies, but all have involved a higher complexity than that what is seen in routine, everyday practice. Recent publications have addressed alternative therapeutic modalities but either lack widespread availability or inferior safety profiles.

Innovations and breakthroughs

This study demonstrated that in the setting of a low risk, uncomplicated patient a failed ERCP procedure can be successfully and safely managed by a repeat ERCP procedure *via* referral to a specialized, high volume endoscopy unit.

Applications

This type of clinical pathway following a failed ERCP attempt should be part of the range of management options available to biliary endoscopists working outside of high volume tertiary centres.

Terminology

ERCP is an advanced endoscopic technique that is used to access and visualize the bile ducts and pancreas, as well as to perform therapeutic management in the setting of gallstones, malignancy and post surgical complications.

Peer review

This paper deals with a clinical pathway in failed biliary cannulation. Results showed clear data about the high success rate of biliary cannulation in tertiary referral centre. Overall, the study clearly demonstrated a clinical pathway in failed biliary cannulation.

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BRIEF ARTICLE

Diagnostic utility of narrow-band imaging endoscopy for pharyngeal superficial carcinoma

Noboru Yoshimura, Kenichi Goda, Hisao Tajiri, Yukinaga Yoshida, Takakuni Kato, Yoichi Seino, Masahiro Ikegami, Mitsuyoshi Urashima

Noboru Yoshimura, Kenichi Goda, Yukinaga Yoshida, Department of Endoscopy, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Hisao Tajiri, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Takakuni Kato, Yoichi Seino, Department of Otorhinolaryngology, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Masahiro Ikegami, Department of Pathology, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Mitsuyoshi Urashima, Clinical Research and Development, The Jikei University School of Medicine, Tokyo 105-8461, Japan
Author contributions: Yoshimura N, Goda K and Yoshida Y performed the endoscopies and evaluated the endoscopic findings; Kato T and Seino Y ordered narrow-band imaging magnified endoscopy to inspect oropharyngeal and hypopharyngeal mucosal sites; Tajiri H assisted with the study design; Ikegami M established the histological diagnoses; Urashima M was responsible for the statistical analysis of the data; and Yoshimura N and Goda K wrote the paper.
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Correspondence to: Kenichi Goda, MD, PhD, Department of Endoscopy, The Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan. kengoendoscopy@hotmail.co.jp
Telephone:+81-3-34331111 Fax: +81-3-34594524
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Abstract

AIM: To investigate the endoscopic features of pharyngeal superficial carcinoma and evaluate the utility of narrow-band imaging (NBI) for this disease.

METHODS: In the present prospective study, 335 patients underwent conventional white light (CWL) endoscopy and non-magnified/magnified NBI endoscopy, followed by an endoscopic biopsy, for 445 superficial lesions in the oropharynx and hypopharynx. The macroscopic appearance of superficial lesions was categorized as either elevated (< 5 mm in height), flat, or depressed (not ulcerous). Superficial carcinoma (SC) was defined as a superficial lesion showing high-grade dysplasia or squamous cell carcinoma on histology. The color, delineation, and macroscopic appearances of the lesions were evaluated by CWL endoscopy. The ratio of the brownish area/intervascular brownish epithelium (IBE), as well as microvascular proliferation, dilation, and irregularities, was determined by non-magnified/magnified NBI endoscopy. An experienced pathologist who was unaware of the endoscopic findings made the histological diagnoses. By comparing endoscopic findings with histology, we determined the endoscopic features of SC and evaluated the diagnostic utility of NBI.

RESULTS: The 445 lesions were divided histologically into two groups: a non-SC group, including non-neoplasia and low-grade dysplasia cases, and an SC group. Of the 445 lesions examined, 333 were classified as non-SC and 112 were classified as SC. There were no significant differences in age, gender, or the location of the lesions between the patients in the two groups. The mean diameter of the SC lesions was significantly greater than that of non-SC lesions (11.0 ± 7.6 mm *vs* 4.6 ± 3.6 mm, respectively, $P < 0.001$). Comparisons of CWL endoscopy findings for SC and non-SC lesions by univariate analysis revealed that the incidence of redness (72% *vs* 41%, respectively, $P < 0.001$) and a flat or depressed type of lesion (58% *vs* 44%, respectively, $P = 0.013$) was significantly higher in the SC group. Using non-magnified NBI endoscopy, the incidence of a brownish area was significantly higher for SC lesions (79% *vs* 57%, respectively, $P < 0.001$). On magnified NBI endoscopy, the incidence of IBE (68% *vs* 33%, $P < 0.001$) and microvascular proliferation (82% *vs* 51%, $P < 0.001$), dilation (90% *vs* 76%, $P =$

0.002), and irregularity (82% *vs* 31%, $P < 0.001$) was also significantly higher for the SC compared with the non-SC lesions. Multivariate analysis revealed that the incidence of redness ($P = 0.022$) on CWL endoscopy and IBE ($P < 0.001$) and microvascular irregularities ($P < 0.001$) on magnified NBI endoscopy was significantly higher in SC than non-SC lesions. Redness alone exhibited significantly higher sensitivity and significantly lower specificity for the diagnosis of SC compared with redness plus IBE and microvascular irregularities (72% *vs* 52%, $P = 0.002$; and 59% *vs* 92%, $P < 0.001$, respectively). The accuracy of redness plus IBE and irregularities for the diagnosis of SC was significantly greater than using redness alone (82% *vs* 62%, respectively, $P < 0.001$).

CONCLUSION: Redness, IBE, and microvascular irregularities appear to be closely related to SC lesions. Magnified NBI endoscopy may increase the diagnostic accuracy of CWL endoscopy for SC.

Key words: Narrow-band imaging; Magnified endoscopy; Endoscopic diagnosis; Pharynx; Pharyngeal cancer; Superficial carcinoma; Squamous cell carcinoma; Dysplasia

Peer reviewer: Marko Duvnjak, MD, Department of Gastroenterology and Hepatology, Sestre Milosrdnice University Hospital, Vinogradska cesta 29, 10 000 Zagreb, Croatia

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INTRODUCTION

Most epithelial malignancies of the head and neck are squamous cell carcinomas (SCCs)[1]. The most important known risk factors for these carcinomas are tobacco use and alcohol consumption, direct exposure to which may result in the synchronous or metachronous development of SCC and dysplasia (a precancerous lesion) in the upper aerodigestive tract, especially in the oropharynx and hypopharynx, and the esophagus[1,2]. This was reported more than 50 years ago as part of the concept of "field cancerization"[2].

In the esophagus, the clinical utility of Lugol chromoendoscopy for the early detection of superficial neoplasms has been established, and this technique is now widely used[3]. Consequently, the rate of detection of early stage esophageal SCC (ESCC) is increasing. Endoscopic treatment of tumors at the earliest stages, particularly mucosal carcinomas, has resulted in considerable improvements in prognosis[4]. However, although early treatment increases the long-term survival of patients with ESCC, in accordance with the concept of field cancerization, the incidence of secondary head and neck SCCs in patients with ESCC has also increased[5]. Among the secondary head and neck SCCs associated with ESCC, oropharyngeal and hypopharyngeal SCCs (OHSCCs) are the most common and adversely affect the improved prognosis of ESCC[5].

Despite its usefulness in screening for superficial neoplasms in the esophagus, Lugol chromoendoscopy cannot be used to screen for OHSCCs because of the accompanying irritation due to iodine and the risk of aspiration. Early stage OHSCCs show an obscure appearance and most affected patients have few symptoms, making the early detection of OHSCCs difficult. Most such lesions are detected at advanced stages, thereby resulting in an extremely poor prognosis[6]. Furthermore, even if the advanced lesions can be removed, dysphagia or aphonia following surgery for advanced OHSCCs causes a marked deterioration in the patients' quality of life[5,6]. Accordingly, a new approach that facilitates the detection and diagnosis of early stage OHSCCs is needed.

The narrow-band imaging (NBI) system is a revolutionary optical image-enhanced technology that uses narrow bandwidth NBI filters. Magnifying endoscopy combined with NBI clearly visualizes the surface microvascular structures in different organs because NBI illumination consists of two limited wavelength ranges that are well absorbed by hemoglobin[7]. Recent studies have reported the usefulness of NBI for the early detection and diagnosis of OHSCCs using gastrointestinal endoscopy or rhinolaryngoscopy[8-10]. However, to the best of our knowledge, there have been no studies to date that have investigated the relationships between detailed findings of conventional white light (CWL) endoscopy, magnified NBI endoscopy, and histology in superficial pharyngeal lesions. There is also scant information currently available in the literature regarding the differential diagnosis of non-neoplasia, dysplasia, and carcinoma using endoscopy.

The process underlying the carcinogenesis of OHSCCs has been proposed to be a dysplasia-carcinoma sequence similar to that seen in ESCC, proceeding from mild or moderate dysplasia (low-grade dysplasia) to severe dysplasia [high-grade dysplasia (HGD)] and SCC[11]. Previous epidemiological follow-up studies of esophageal squamous neoplasms have suggested that the relative risk of HGD developing into invasive SCC is comparable to that of SCC *in situ*[11,12]. In contrast, a low-grade dysplasia indicates a significantly lower risk of malignant transformation[11]. Among squamous neoplasias, HGD appears to be a good candidate for intervention, as with SCC[13].

The aim of our present study was to investigate the relationships between the endoscopic and histologic findings of superficial lesions in the oropharynx and hypopharynx, and to evaluate the diagnostic utility of NBI for superficial squamous neoplasms showing HGD or SCC.

MATERIALS AND METHODS

The macroscopic appearance of superficial lesions was evaluated and lesions were classified as either elevated (< 5 mm in height), flat, or depressed (not ulcerous). Between March 2005 and April 2009, CWL and non-magnified/magnified NBI endoscopies were performed in 1010 consecutive patients for the evaluation of the oropharyngeal and hypopharyngeal region. The endoscopic findings for 455 superficial lesions in the oropharynx and hypopharynx in 342 patients were evaluated prospectively in our present study. Biopsy samples were obtained for all 455 superficial lesions, although 10 lesions from seven patients were excluded from analysis because insufficient material was obtained by biopsy to enable a histological diagnosis to be made. The findings for 445 superficial oropharyngeal and hypopharyngeal lesions in 335 patients were therefore analyzed in the present study.

Superficial lesions that demonstrated HGD or SCC histologically were defined as superficial carcinomas (SC). Endoscopic findings of SC lesions were compared with those of non-SC lesions in the oropharynx and hypopharynx to reveal the endoscopic features of SC lesions. Moreover, we investigated the diagnostic utility of NBI for SC lesions. The present analyses were performed with local ethics committee approval and all patients provided informed consent prior to their inclusion in the study.

Endoscopic procedures

All patients were orally administered 20 000 U pronase (Pronase MS; Kaken Pharmaceutical Products Inc., Tokyo, Japan) prior to pharyngeal anesthesia to eliminate saliva and mucus. Pharyngeal anesthesia was achieved using four to five pump-spray applications of lidocaine (Xylocaine; AstraZeneca, Osaka, Japan). All endoscopic inspections were performed in conscious, sedated patients after the administration of pethidine hydrochloride (35-70 mg; Opystan; Mitsubishi Tanabe Pharma, Osaka, Japan) and flunitrazepam (0.2-0.8 mg; Rohypnol; Chugai Pharmaceutical, Tokyo, Japan).

Endoscopic examinations were performed using a high-resolution and zoom gastrointestinal endoscope (GIF-Q240Z or GIF-H260Z; Olympus Medical Systems, Tokyo, Japan) with a maximum magnification of × 90 on a 19 inch (48.3 cm) monitor. The light source unit (Olympus Medical Systems) has two rotary filters in front of a xenon bulb, one for CWL and the other for NBI. Thus, it is easy to switch from the CWL to NBI mode by simply pushing the control knob on the endoscope. A black rubber attachment (Olympus Medical Systems) was mounted on the tip of the scope to enable the endoscopist to adjust the focus easily and to maintain the focal distance between the tip of the scope and the lesion surface at 2 mm during magnified observation.

Three endoscopic techniques, namely CWL and non-magnified and magnified NBI, were performed for each patient by one of three endoscopists (NY, YY or KG). First, the oropharynx and hypopharynx were inspected thoroughly using CWL endoscopy, after which the endoscope was withdrawn to the oral cavity and a switch made from the CWL to NBI mode. The oropharynx and hypopharynx were then examined using non-magnified NBI endoscopy. For lesions that had not been detected by CWL endoscopy and were first detected using non-magnified NBI endoscopy, the NBI mode was switched back to the CWL mode and the lesions were evaluated using CWL endoscopy. All lesions were then inspected using magnified NBI endoscopy. Finally, tissue samples were obtained endoscopically from the lesions using biopsy forceps (Radial Jaw 3; Boston Scientific, Natick, MA). All endoscopic images were preserved in digital form.

After completion of each endoscopic inspection, each endoscopic finding of CWL, non-magnified NBI, or magnified NBI was recorded immediately on a case report form.

Definitions of endoscopic findings

CWL endoscopy: Endoscopic findings for each superficial lesion were evaluated with regards to color, delineation, and macroscopic appearance. Superficial lesions were determined to be either "redness" or "white or isochromatic" in terms of color, "well-delineated" or "poorly delineated", and either "elevated" or "flat or depressed" macroscopically. Each of these parameters was classified based on the predominant finding.

Non-magnified NBI endoscopy: Superficial lesions exhibiting a well-demarcated brownish area by non-magnified NBI endoscopy were classified as having a "brownish area"[8].

Magnified NBI endoscopy: In a preliminary study[14], we found that the "brownish area" observed by non-magnified NBI endoscopy can comprise any one or a combination of the following observations on magnified NBI endoscopy: (1) an intervascular brownish epithelium (IBE); (2) microvascular dilation; and (3) microvascular proliferation. IBE is the presence of brown-colored epithelium between superficial microvessels in a lesion that differs from the whitish surrounding squamous epithelium. Microvascular dilation is defined as the presence of a group of superficial microvessels, the calibers of which are more than twice the caliber of the surrounding reference microvessels. Microvascular proliferation is defined as the presence of a group of superficial microvessels with a greater density than that of the surrounding reference microvessels. In addition, microvascular irregularities were assessed using magnified NBI endoscopy, where "irregularities" were defined as the presence of a group of superficial microvessels showing marked variations in caliber and/or highly variable forms compared with the surrounding reference microvessels.

Figure 1 schematically summarizes the features of superficial lesions and the surrounding mucosa seen using magnified NBI endoscopy. A representative image

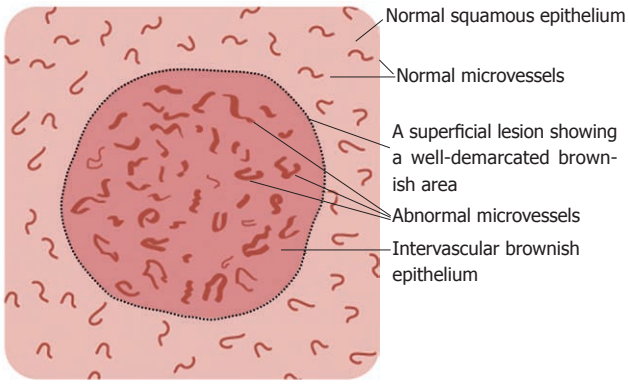

Figure 1 Schema of the magnified endoscopic features of a superficial lesion and its surrounding mucosa as detected by narrow-band imaging. The superficial lesion shows an intervascular brownish epithelium and abnormal microvessels exhibiting dilation, proliferation and irregularities.

of an SC lesion is shown in Figure 2.

Histologic assessment

Endoscopic biopsy specimens of the 445 superficial lesions examined in this study were subjected to histologic assessment. Tissue samples were placed on custom-made black filter paper (Toyo Roshi Kaisha, Tokyo, Japan) and fixed in 40 g/L formaldehyde for 24 h. Black filter paper makes it easier to flatten out the whitish specimens, which makes it easier to prepare vertical microsections and make a more accurate diagnosis of the histological grade of the intraepithelial neoplasm. All specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Histologic diagnoses were made by a pathologist (MI) specializing in gastrointestinal pathology who was unaware of the endoscopic findings and were based on the revised Vienna classification[13].

Statistical analysis

Continuous variables are expressed as the mean ± SD and were analyzed using the Student's *t* test. The χ^2 test and logistic regression were used to analyze categorical data. $P < 0.05$ was considered significant. All analyses were performed using STATA 10.0 software (STATA, College Station, TX).

RESULTS

Table 1 lists the clinicopathologic features of our current cohort of 335 patients comprising 445 superficial lesions in the oropharynx and hypopharynx. These 445 lesions were divided histologically into two groups, namely non-SC (normal epithelium, inflammation, hyperplasia, papilloma, "other", and low-grade dysplasia) and SC (HGD and SCC). The category of "other" included hyperkeratosis ($n = 2$), regenerative atypia ($n = 1$), atrophy ($n = 1$), xanthoma ($n = 1$), and melanosis ($n = 1$). Most patients in both groups were male and there were no significant differences found in age, male:female ratio, or in the location of the lesions between the two groups. However, the lesion diameter was significantly greater in the SC group ($P < 0.001$).

Table 1 Clinicopathologic features of patients with pharyngeal superficial lesions

	Total (n = 445)	Non-SC (n = 333)	SC (n = 112)	P value
Age (yr)	63 ± 9	63 ± 10	64 ± 8	
Male:female ratio	16:1	14:1	27:1	
Lesion location (n)				
Oropharynx/hypopharynx	269/176	206/127	63/49	
Lesion diameter (mm)	6.2 ± 5.6	4.6 ± 3.6	11.0 ± 7.6	< 0.001[1]
Histology (n)		Normal: 40 Inflammation: 115 Hyperplasia: 50 Papilloma: 33 Other[2]: 6 LGD: 89	HGD: 35 SCC: 77	

Where appropriate, data are given as the mean ± SD. [1]Student's *t* test. [2]"Other" histologic diagnoses included hyperkeratosis ($n = 2$), regenerative atypia ($n = 1$), atrophy ($n = 1$), xanthoma ($n = 1$), and melanosis ($n = 1$). HGD: High-grade dysplasia; LGD: Low-grade dysplasia; SC: Superficial carcinoma; SCC: Squamous-cell carcinoma.

Table 2 lists the results of univariate analysis of the relationships between the endoscopic findings in the two study groups. Univariate analysis of the CWL endoscopy findings identified significant differences in the color and macroscopic appearance of lesions between the SC and non-SC groups. Redness was more commonly seen for lesions in the SC than in the non-SC group (72% *vs* 41%, respectively, $P < 0.001$). In addition, lesions in the SC group were significantly more likely to appear flat or depressed (58% *vs* 44%, respectively, $P = 0.013$). In both groups, approximately three-quarters of the lesions appeared to be well delineated, with no significant difference detected.

On non-magnified NBI endoscopy, the incidence of a brownish area was significantly higher in the SC group than in the non-SC group (79% *vs* 57%, respectively, $P < 0.001$). On magnified NBI endoscopy, the incidence of IBE was also significantly higher in the SC group (68% *vs* 33%, respectively, $P < 0.001$). Furthermore, the incidences of microvascular proliferation (82% *vs* 51%, $P < 0.001$), dilation (90% *vs* 76%, $P < 0.001$), and irregularities (82% *vs* 31%, $P < 0.001$) were again significantly greater in the SC group.

The results of multivariate logistic regression analysis are listed in Table 3. Significant differences between the non-SC and SC groups were identified for redness ($P = 0.022$) on CWL endoscopy, and for IBE ($P < 0.001$) and microvascular irregularities ($P < 0.001$) on magnified NBI. The diagnostic sensitivity, specificity, and accuracy using redness alone and redness plus IBE and microvascular irregularities are indicated in Table 4. The redness of lesions on CWL endoscopy had 72% sensitivity, 59% specificity, and 62% accuracy for a diagnosis of super-

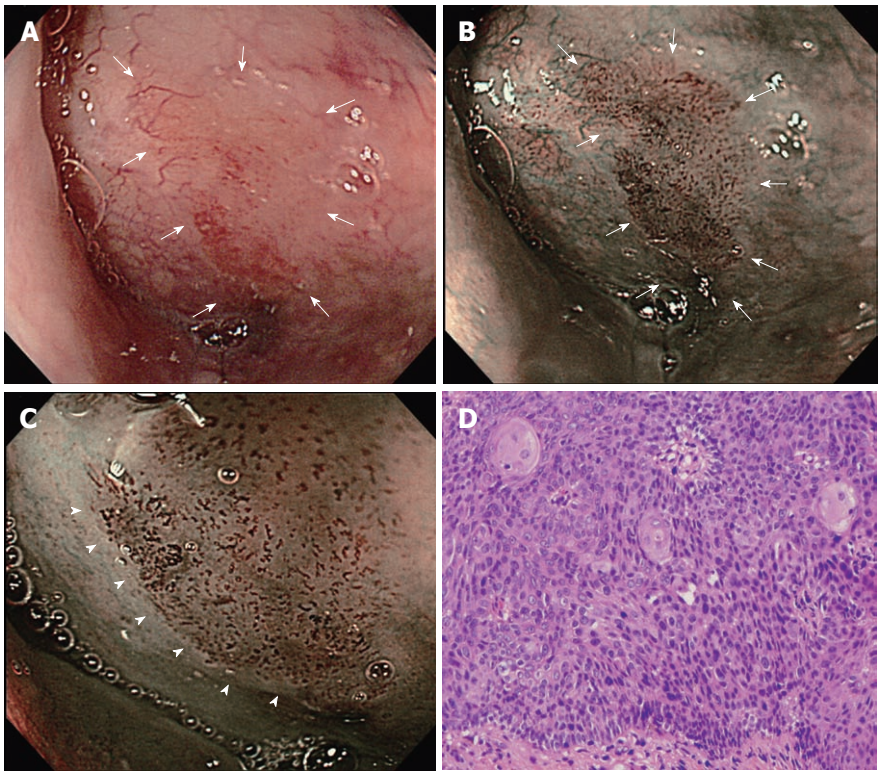

Figure 2 Representative images of a superficial carcinoma. A: Conventional gastrointestinal endoscopy image revealing a flat and poorly delineated lesion with a slight redness at the piriform sinus of the right hypopharynx (arrows); B: The superficial carcinoma lesion is revealed as a well-demarcated brownish area by non-magnified narrow-band imaging (NBI) endoscopy (arrows); C: Magnified NBI endoscopy visualizes the brownish area of the superficial carcinoma and shows it to be comprised of intervascular brownish epithelium and superficial microvessels of a dark brown color exhibiting dilation, proliferation and irregularities. Arrowheads indicate the demarcation line of the lesion; D: Histology of an endoscopically biopsied superficial carcinoma specimen demonstrating squamous cell carcinoma *in situ* with keratinization (hematoxylin and eosin stain; original magnification × 50).

Table 2 Univariate analysis of relationships between endoscopic findings and histology

Endoscopy	Endoscopic findings	Non-SC (n = 333)	SC (n = 112)	OR	95% CI	P value
CWL (Non-magnified)	Color (redness; %)	41	72	3.69	2.31, 5.89	< 0.001[1]
	Delineation (well delineated; %)	66	69	1.13	0.71, 1.79	
	Macroscopic type (flat or depressed; %)	44	58	1.73	1.12, 2.67	0.013[1]
NBI						
(Non-magnified)	Brownish area (%)	57	79	2.79	1.69, 4.61	< 0.001[1]
(Magnified)	IBE (%)	33	68	4.22	2.67, 6.67	< 0.001[1]
	Microvascular					
	Proliferation (%)	51	82	4.41	2.60, 7.49	< 0.001[1]
	Dilation (%)	76	90	2.9	1.48, 5.68	0.002[1]
	Irregularities (%)	31	82	10.27	6.01, 17.56	< 0.001[1]

[1]Univariate logistic regression analysis between superficial carcinoma (SC) and non-SC groups. CWL: Conventional white light endoscopy; NBI: Narrow-band imaging; IBE: Intervascular brownish epithelium; OR: Odds ratio; CI: Confidence interval.

ficial carcinoma in the oropharynx and hypopharynx. If IBE and irregularities on magnified NBI endoscopy were taken into account in addition to the redness of lesions, the diagnostic yield was 52% for sensitivity, 92% for specificity, and 82% for accuracy. The sensitivity for redness alone was significantly higher than that for redness plus IBE and microvascular irregularities (P = 0.002). Specificity and accuracy for redness plus IBE and microvascular irregularities were significantly higher than values for redness alone (P < 0.001 and P < 0.001, respectively).

There were no complications, such as massive hemorrhaging or aspiration, associated with the endoscopic examinations undertaken in the present study cohort.

DISCUSSION

Advances in endoscopic techniques enabling accurate diagnoses to be made in the oropharynx and hypopharynx are crucially needed. At present, endoscopic biopsy in the pharyngeal region may be quite challenging due to the gag reflex and the complicated structure of the region.

Table 3 Multivariate analysis of relationships between endoscopic findings and histology

Endoscopy	Endoscopic findings	Non-SC (n = 333)	SC (n = 112)	OR	95% CI	P value
CWL (Non-magnified)	Color (redness; %)	41	72	2.16	1.12, 4.16	0.022[1]
	Macroscopic type (flat or depressed; %)	44	58	0.84	0.47, 1.51	
NBI						
(Non-magnified)	Brownish area (%)	57	79	0.63	0.26, 1.56	
(Magnified)	IBE (%)	33	68	3.39	1.88, 6.11	< 0.001[1]
	Microvascular					
	Proliferation (%)	51	82	1.64	0.81, 3.35	
	Dilation (%)	76	90	0.91	0.39, 2.13	
	Irregularities (%)	31	82	7.51	4.02, 14.06	< 0.001[1]

[1]Multivariate logistic regression analysis between superficial carcinoma (SC) and non-SC groups. CWL: Conventional white light endoscopy; NBI: Narrow-band imaging; IBE: Intervascular brownish epithelium; OR: Odds ratio; CI: Confidence interval.

Table 4 Diagnostic yields of the endoscopic features of pharyngeal superficial carcinoma

	Endoscopic findings		
	Redness	Redness plus IBE and microvascular irregularities	P value[1]
% Sensitivity (95% CI)	72 (63-80)	52 (42-61)	0.002
% Specificity (96% CI)	59 (53-64)	92 (88-94)	< 0.001
% Accuracy (99% CI)	62 (58-65)	82 (78-85)	< 0.001

[1]Pearson's χ^2 test. IBE: Intervascular brownish epithelium; CI: Confidence interval.

Indeed, in our present study we failed to obtain sufficient biopsy material for the histologic assessment of 10 lesions. Thus, progress in endoscopic diagnostics in the pharyngeal region will be of greater clinical significance than for other regions of the gastrointestinal tract.

Previous studies have revealed that a brownish area with a proliferation of dilated microvessels on NBI endoscopy facilitates the endoscopic detection of OHSCCs at an early stage[8-10]. However, the differences that are evident on NBI and CWL endoscopy and that can differentiate between non-SC and SC lesions in the oropharynx and hypopharynx remain unclear. The results of the present study suggest that there are several endoscopic features that are closely related to SC lesions that could be used in the differential diagnosis of SC and non-SC lesions. Our univariate analysis revealed that SC lesions are associated with a brownish area and microvascular proliferation and dilation. These results are similar to those of a previous study that reported that all malignant lesions appear as well-demarcated brownish areas with proliferation of dilated microvessels[8].

However, the multivariate analysis performed in the present study revealed that redness, IBE, and microvascular irregularities seem to be independent factors related to SC lesions, rather than a brownish area or microvascular dilation and proliferation. In our non-SC group, most lesions (61%) were found to be inflammatory lesions (n = 115) or low-grade dysplasias (n = 89), which are often found to have a brownish area (80% and 67%, respectively) or to exhibit microvascular proliferation (63% and 62%, respectively) and dilation (89% and 78%, respectively). The relatively high rates of these endoscopic features in inflammatory lesions and low-grade dysplasias may have affected the outcome of the multivariate analysis in the present study.

The sensitivity of redness by CWL endoscopy alone for SC lesions was significantly higher than that combined with IBE and microvascular irregularities by magnified NBI endoscopy. Conversely, the specificity and accuracy of redness plus IBE and microvascular irregularities for SC lesions were significantly higher than redness alone. These results suggest that magnified NBI endoscopy may complement CWL endoscopy in the diagnosis of SC lesions.

Ishihara *et al*[15] have reported that in the esophagus, the criterion of a brownish epithelium (with the same definition used for IBE in the present study) was an independent factor significantly associated with high-grade intraepithelial neoplasia consisting of HGD and intramucosal SCC. IBE seems to be a key finding associated with superficial high-grade neoplasms. Histologically, the appearance of IBE could be attributed to increased intraepithelial cell density or changes in the intraepithelial cells themselves as a result of malignant transformation. Further investigations in the future are needed to identify the mechanisms underlying IBE.

There are some notable limitations to the present study. First, the three endoscopic procedures performed in each patient were performed by the same endoscopist. Thus, the possibility exists that the subsequent assessment of endoscopic images may have been influenced by information bias and a carryover effect from prior endoscopies. Moreover, it remains unclear whether non-magnified or magnified NBI endoscopy provides an additional benefit over CWL endoscopy alone. Second, our histological diagnoses were based on biopsy samples obtained during the endoscopic procedure. The histologic findings for these samples may therefore not be representative of the histology of the entire lesion.

The image resolution of a gastrointestinal endoscope is greater than that of a rhinolaryngoscope. Because of the growing interest in the pharyngeal region by gastroenterologists, the number of cases of OHSCCs detected

and treated by gastrointestinal endoscopy is also increasing[16,17]. However, the field of view obtained using gastrointestinal endoscopy is limited compared with the comprehensive examinations performed by otorhinolaryngologists, using rhinolaryngoscopy as well as direct visual inspection. It thus remains difficult for gastrointestinal endoscopists to detect or treat tumors of the tongue, the floor of the oral cavity, and the nasopharynx. Hence, to improve the prognosis and quality of life of patients with head and neck SCCs and/or esophageal SCCs, gastrointestinal endoscopists and otorhinolaryngologists need to collaborate in the future to further develop endoscopic diagnosis and treatment methods for these conditions.

The endoscopic features of redness, IBE, and microvascular irregularities seem to be significantly associated with SC lesions in the oropharynx and hypopharynx. The diagnostic accuracy of redness plus IBE and microvascular irregularities for SC lesions was found to be greater than the accuracy of redness alone. Therefore, magnified NBI endoscopy may increase the diagnostic accuracy of CWL endoscopy for SC lesions.

COMMENTS

Background

Prolonged tobacco use and alcohol consumption may result in the synchronous or metachronous development of squamous cell carcinoma (SCC), both in the pharynx and the esophagus. The prognosis of patients with esophageal SCC has improved due to the early detection of these lesions as a result of the widespread adoption of an upper gastrointestinal endoscopy with Lugol staining. However, an early detection of oropharyngeal and hypopharyngeal SCCs (OHSCCs) remains difficult because Lugol staining is not possible during a screening endoscopy for OHSCCs. Moreover, OHSCCs at an early stage show an obscure appearance and manifest few symptoms. Hence, most OHSCCs are detected at advanced stages, resulting in an extremely poor prognosis, including that of patients with esophageal SCCs. Identifying the endoscopic features of superficial OHSCCs is thus essential for both early detection and differential diagnosis.

Research frontiers

The narrow-band imaging (NBI) system is a revolutionary optical image-enhanced technology that uses narrow bandwidth NBI filters. Magnifying endoscopy combined with NBI clearly visualizes the surface microvascular structures in different organs as NBI illumination consists of two limited wavelength ranges that are well absorbed by hemoglobin. NBI endoscopy can visualize a hemoglobin rich lesion such as a superficial squamous neoplasia as a brownish area with no use of Lugol staining.

Innovations and breakthroughs

Recent studies have reported the usefulness of NBI for the early detection and diagnosis of superficial OHSCCs using gastrointestinal endoscopy or rhinolaryngoscopy. However, there has been no report to date that has compared the endoscopic features of non-neoplastic lesions and pharyngeal squamous neoplasias using magnified NBI as well as conventional white light (CWL) endoscopy. This is the first report to investigate the relationships between detailed findings of CWL endoscopy, magnified NBI endoscopy, and histology in an analysis of superficial pharyngeal lesions. Moreover, we have for the first time evaluated the diagnostic utility of magnified NBI endoscopy for superficial OHSCCs compared with CWL endoscopy.

Applications

This study may help to reveal endoscopic features of superficial carcinomas in the pharynx and the accuracy of endoscopy with CWL, NBI, or magnified NBI for diagnosing superficial carcinoma. Identifying these endoscopic features will lead to an enhanced detection rate of these lesions and will be useful in making a differential diagnosis.

Terminology

NBI is a revolutionary optical image-enhanced technology that allows people to see an obscure tumor as a brownish area and observe the detailed structure of superficial microvessels when this technology is combined with magnified endoscopy. Superficial carcinoma is histologically defined as a high-grade dysplasia (HGD) as well as an SCC as HGD seems to have the same clinical implications as SCC *in situ*.

Peer review

The topic itself is interesting for otorhinolaryngologists as well as for gastroenterologists. The NBI technique can still be considered as a novel method and therefore is a valuable source of new and interesting studies such as this one. The authors outlined the limitations of this study, because many researchers do not do so. The abstract and key words are appropriate. The abstract gives clear insight into the materials and diagnostic procedures used. The materials and methods section is also appropriately detailed and will enable replication of the methodologies used in this study. The discussion is well organized, systematic and clearly written. The cited references are relevant and appropriate. The figures are nicely incorporated in the text and appropriately presented. The language used is good and clear. It is a well structured article.

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BRIEF ARTICLE

Increased fibrosis progression rates in hepatitis C patients carrying the prothrombin G20210A mutation

Nitsan Maharshak, Philippe Halfon, Varda Deutsch, Hava Peretz, Shlomo Berliner, Sigal Fishman, Shira Zelber-Sagi, Uri Rozovski, Moshe Leshno, Ran Oren

Nitsan Maharshak, Sigal Fishman, Shira Zelber-Sagi, Moshe Leshno, Ran Oren, Department of Gastroenterology and Liver diseases, Tel Aviv Sourasky Medical Center, affiliated to the Sackler School of Medicine, Tel Aviv University, 64239 Tel Aviv, Israel
Philippe Halfon, Department of Virology, Laboratoire Alphabio, 23 Rue de Friedland, Hôpital Ambroise Paré, 13006 Marseille, France
Varda Deutsch, Uri Rozovski, The Hematology Institute, Tel Aviv Sourasky Medical Center, affiliated to the Sackler School of Medicine, Tel Aviv University, 64239 Tel Aviv, Israel
Hava Peretz, Clinical Biochemistry Laboratory, Tel Aviv Sourasky Medical Center, affiliated to the Sackler School of Medicine, Tel Aviv University, 64239 Tel Aviv, Israel
Shlomo Berliner, Internal Medicine "E", Tel Aviv Sourasky Medical Center, affiliated to the Sackler School of Medicine, Tel Aviv University, 64239 Tel Aviv, Israel
Shira Zelber-Sagi, School of Public Health, University of Haifa, Mount Carmel, 31905 Haifa, Israel
Author contributions: Berliner S and Oren R devised the study concept; Maharshak N, Halfon P, Deutsch V, Peretz H, Berliner S, Fishman S, Zelber-Sagi S, Rozovski U, Leshno M and Oren R designed the study; Maharshak N, Halfon P, Deutsch V, Peretz H, Fishman S, Zelber-Sagi S, Rozovski U, Oren R- acquired the data; Maharshak N, Zelber-Sagi S, Rozovski U, Leshno M and Oren R analyzed and interpreted the data; Maharshak N wrote the manuscript.
Correspondence to: Nitsan Maharshak, MD, Department of Gastroenterology and Liver diseases, Tel Aviv Sourasky Medical Center, affiliated to the Sackler School of Medicine, Tel Aviv University, 6 Weizmann St, 64239 Tel Aviv, Israel. nitsan_maharshak@walla.com
Telephone: +972-3-6974282 Fax: +972-3-6974622
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Abstract

AIM: To examine whether hepatitis C virus (HCV)-infected patients who carry hypercoagulable mutations suffer from increased rates of liver fibrosis.

METHODS: We analyzed DNA samples of 168 HCV patients for three common hypercoagulable gene mutations: prothrombin 20210 (PT20210), factor V Leiden (FV Leiden) and methylene tetrahydrofolate reductase (MTHFR). The patients were consecutively recruited as part of the prospective "Fibroscore Study" in France. The effect of the various mutations on the rate of fibrosis was analyzed statistically and was correlated with epidemiological, clinical and biochemical data such as grade and stage of liver biopsies, patients' risk factors for liver cirrhosis, and timing of infection.

RESULTS: Fifty two of the patients were categorized as "fast fibrosers" and 116 as "slow fibrosers"; 13% of the "fast fibrosers" carried the PT20210 mutation as compared with 5.5% of the "slow fibrosers", with an odds ratio of 4.76 (P = 0.033; 95% CI: 1.13-19.99) for "fast" liver fibrosis. Carriage of MTHFR or FV Leiden mutations was not associated with enhanced liver fibrosis.

CONCLUSION: Carriage of the PT20210 mutation is related to an increased rate of liver fibrosis in HCV patients.

Key words: Hepatitis C virus; Liver fibrosis; Hypercoagulation; Prothrombin 20210

Peer reviewers: Hisato Nakajima, MD, Department of Gastroenterology and Hepatology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan; Fumio Imazeki, MD, Department of Medicine and Clinical Oncology, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

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INTRODUCTION

Cirrhosis is a major cause of morbidity and mortality in patients who suffer from chronic hepatitis C virus (HCV) infection. Up to 24% of patients will develop cirrhosis during their lifetime[1]. Various characteristics, such as male gender, older age at infection, alcohol consumption, obesity and concurrent hepatitis B or human immunodeficiency virus (HIV) infection enhance the rate of liver fibrosis[2,3]. Unfortunately, it is still largely impossible to predict who is more prone to fibrosis, and, thus, careful follow-up or treatment is required for most patients.

Hypercoagulable states have been hypothesized to play a role in organ fibrosis. In various inflammatory states, such as those of the lung or kidney, thrombosis and fibrin formation result in organ injury. It has been recently proposed that hypercoagulable states may be an additional contributing factor to liver fibrosis through several mechanisms, such as thrombotic events in small venous blood vessels in the liver and stimulation of hepatic stellate cells by thrombin[4]. Moreover, increased fibrin deposition has been demonstrated in animal models of liver fibrosis[5]. These observations have been strengthened by a study conducted by Anstee *et al*[6], which explored a mouse model of liver fibrosis and demonstrated that the extent of fibrosis was much higher in mice carrying the factor V Leiden (FV Leiden) mutation.

Primary hypercoagulable states, such as FV Leiden and prothrombin 20210 (PT20210), result from mutations in genes that encode proteins of the coagulation cascade[7]. FV Leiden results from a G1691A single nucleotide polymorphism gene mutation that leads to an amino acid substitution of arginine for glutamine at position 506 of the protein, which is one of the cleavage sites of activated protein C (APC). The mutated protein is more resistant to APC cleavage, and, as a result, the negative feedback on the coagulation cascade is impaired. Thus, the FV Leiden mutation is responsible for venous thromboembolisms at a high prevalence of 1%-8.5%[8]. Another common mutation involves the elevation of plasma prothrombin levels due to a G→A transition in nucleotide 20210 in the prothrombin gene. The prevalence of heterozygosity for this mutation among Caucasian populations is 1%-6%[9]. A third common mutation that results in hypercoagulation is a genetic variant of the *methylene tetrahydrofolate reductase* (*MTHFR*) gene, which leads to an elevation in homocysteine levels and an increased risk of venous and arterial thrombosis. Epidemiological data from humans have shown that APC resistance resulting from FV Leiden heterozygosity is related to an increased rate of fibrosis in HCV patients[4,10], as opposed to carriage of the PT20210 mutation, which has not been found to be a contributing factor to liver cirrhosis[10]. Hyperhomocysteinemia and MTHFR C677T mutations have been found to play roles in liver steatosis in HCV patients and, thus, indirectly play a role in the progression of liver fibrosis[11].

In this work, we examined whether a mutation in one of these genes contributes to accelerated liver fibrosis in French HCV patients.

MATERIALS AND METHODS

Patients

In this retrospective study, we analyzed data that were collected from HCV-infected patients. The first 168 consecutive patients were included from the "Fibroscore Study", which was a French national, multicenter, prospective, and cross-sectional study of Caucasian patients that was performed by researchers who are well known for their specific expertise in HCV in five centers in the southeast region of France, including Saint-Joseph Hospital and La Conception Hospital (Marseille), Archet Hospital (Nice), Hyeres Hospital and the Arnault Tzanck Institute (St. Laurent du Var). All patients who suffered from chronic HCV infection, as documented by a positive test screen for HCV RNA in serum, were included in this study. Signed informed consent was obtained from all of the patients before their inclusion. Liver biopsy and biochemical markers were performed the same day. Liver biopsy was performed at each center and analyzed by the resident pathologist. For all patients, ultrasound examination was performed before liver biopsy.

Information relating to the patients' demographics, risk factors, virological status, clinical examinations, clinical data [age at exposure to the virus, alcohol consumption and body mass index (BMI)] and biological data (virus genotype) was prospectively recorded at each center on the day of biopsy.

Patients were excluded if they suffered from another proven liver disease, such as autoimmune hepatitis or alcoholic liver disease, or if they had positive serology for hepatitis B or HIV. Patients for whom the date of infection was unknown were excluded as well.

The stored DNA samples from the 168 recruited patients were analyzed for the three mutations that are responsible for hypercoagulable states, that is, FV Leiden, PT20210 and MTHFR.

This study was approved by the local Helsinki ethics committees.

Definitions of slow and fast fibrosis

Fibrosis rates were calculated by dividing the fibrosis stage by the number of years of infection. We employed Poynard's fibrosis progression model in order to define our patients' fibrosis rate status and classified them as "fast fibrosers" or "slow fibrosers"[12]. According to the model,

Table 1 Patient characteristics by fibrosis rates (by Poynard)

	Slow fibrosers (n = 116)	Fast fibrosers (n = 52)	P value
Female (%)	37%	31%	0.43
Age (yr)	49.8 ± 10.5	43.9 ± 7.1	< 0.001
Age at infection (yr)	25.1 ± 10.8	25.2 ± 7.7	0.97
Infection duration (yr)	24.7 ± 8	18.7 ± 8.1	< 0.001
Alcohol intake ⩾ 50 g/d	7.1%	10.4%	0.53
BMI (kg/m^2)	23.8 ± 2.9	23.4 ± 3.1	0.5
Genotype (%)			0.49
1	74.3%	62.8%	
2	2.8%	2.3%	
3	16.5%	23.3%	
4	6.4%	11.6%	
Inflammatory score, grade (units)	1.3 ± 0.68	1.81 ± 0.69	< 0.001
Fibrosis stage (%)			< 0.001
0	14.7%	0%	
1	45.7%	9.6%	
2	29.3%	5.8%	
3	8.6%	48.1%	
4	1.7%	36.5%	

BMI: Body mass index.

the means of the fibrosis progression rates, as predicted by age and infection duration, are as follows: (1) patients who were infected at an age of less than 20 years were expected to develop cirrhosis after 40 years of infection; (2) patients who were infected in their third or fourth decade progress to cirrhosis after 35 years of infection; (3) patients in the fifth decade of life were expected to develop cirrhosis after 20 years of infection; and (4) patients who were older than 50 years were expected to become cirrhotic after 15 years of infection.

Histopathology

Liver biopsy examinations were performed at each center and analyzed by the local pathologist, wherein the fibrosis stage and activity grading were evaluated according to the METAVIR scoring system. Fibrosis was staged on a scale of 0-4: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

The grading of activity that was assessed by the METAVIR system (based on the intensity of necroinflammatory activity, largely on necrosis) was scored as follows: A0, no histological activity; A1, mild activity; A2, moderate activity and A3, severe activity.

In order to assess liver biopsy quality, Regev quality criteria were used (fragment length of 15 mm or more, five or more portal tracts and one fragment). A biopsy that is between 10 and 15 mm in length and has less than five portal tracts or is fragmented is considered to be a fair quality biopsy, whereas a biopsy is considered to be of poor quality when it is less than 10 mm in length.

DNA collection and gene analysis

DNA samples were isolated from the peripheral blood of all patients. DNA extraction was performed using the QIAamp DNA blood kits and silica-membrane-based DNA purification (Qiagen, Germany).

Table 2 Frequency of hypercoagulation mutations (%)

Mutation type	Wild type	Heterozygote	Homozygote
Factor V Leiden (n = 159)	96.2	3.8	0
Prothrombin 20210 (n = 156)	92.3	7.1	0.6
MTHFR (n = 163)	35.6	50.9	13.5

Percentages of patients carrying each of the mutations by genotype. MTHFR: Methylene tetrahydrofolate reductase.

Analyses of gene mutations of FV Leiden, prothrombin G20210A and MTHFR C677T were performed *via* real-time polymerase chain reaction, using fluorescent hybridization probes (Dyn Diagnostics Roche, Israel) in a LightCycler instrument (Roche Diagnostics, Basel, Switzerland), specified as follows: FV Leiden: anchor: '5-LC-Red640-TGT CCT TGA AGT AAC CTT TCA GAA ATT CTG-PH, sensor WT: '5-GGC GAG GAA TAC AGG TAT-FL; PTG20210A: anchor: '5-LC-Red640-TGC TCC CAG TGC TAT TCA TGG AC-PH, sensor WT: '5-GAC TCT CAG CGA GCC TCA-FL; and MTHFR: anchor: '5-LC-Red640-CGC AGC TTT TCT TTG AGG CTG ACA-PH, sensor WT: '5-CGG GAG CCG ATT TCA TCAQ-FL.

Statistical analysis

All statistical analyses were performed using SPSS, version 17 (SPSS Inc., Chicago, Illinois, United States). The rate of fibrosis was calculated as the ratio of the fibrosis score to the duration of infection at the time of biopsy. This value was used for a univariate analysis of variance (ANOVA) in order to calculate whether the factors were significantly associated with the rate of fibrosis, and in a linear regression model to calculate the influence of each variable on the fibrosis rate. Fibrosis rates were subdivided into "slow fibrosers" or "fast fibrosers" for the construction of a multivariate logistic regression model that served to calculate the odds ratio (OR) for fast fibrosis.

Demographic differences between the "fast" and "slow" fibrosers were assessed using independent sample t test and ANOVA, whereas χ^2 test or Fisher's exact test were employed when appropriate, as designated in the tables.

RESULTS

One hundred and sixty-eight consecutive patients with available liver biopsies were recruited in this study. The average fibrosis rate in the entire cohort was 0.11 ± 0.17 fibrosis units per year. Demographic and disease-related data categorized by fibrosis rate are presented in Table 1. Patients who were categorized as "fast fibrosers" were significantly younger, had shorter disease duration, consumed more alcohol, and had a higher disease grade and stage according to histological analyses. HCV genotypes did not statistically differ between the two groups.

The frequencies of the three mutations that were analyzed in this cohort are specified in Table 2.

Table 3 Percentage of hypercoagulation gene mutation carriage by rate of fibrosis (by Poynard)

Gene	Mutation carriage	Percent of slow fibrosers	Percent of fast fibrosers	*P* value
PT20210	Wild type	94.5	86.9	0.18[1]
	Heterozygote	5.5	10.9	
	Homozygote	0	2.2	
Factor V Leiden	Wild type	95.5	98	0.67
	Heterozygote	4.5	2	
	Homozygote	0	0	
MTHFR	Wild type	35.6	36	0.80
	Heterozygote	52	48	
	Homozygote	12.4	16	

[1]Heterozygote and homozygote categories were unified for Fisher's exact test. PT20210: Prothrombin 20210; MTHFR: Methylene tetrahydrofolate reductase.

Table 4 Prothrombin 20210 mutation rates in various "fibrosis rate" models

Rate of fibrosis	PT20210 mutation in slow fibrosers (%)	PT20210 mutation in fast fibrosers (%)	*P* value[3]
Rate of fibrosis by Poynard	5.50	13	0.18
0.13 units/yr[1]	5.10	15.80	0.07
0.133 units/yr[2]	5	16.70	0.03

Various cut-offs of rate of fibrosis were employed on our cohort to differentiate between fast and slow fibrosers. [1]Rate of fibrosis according to Fishman *et al*[22]; [2]Rate of fibrosis according to Wright *et al*[10]; [3]*P* value calculated by Fisher's exact test. PT20210: Prothrombin 20210.

PT20210 carriers

Six patients (5.5% of 110 patients) of the "slow fibrosers" group were carriers of the PT20210 mutation, whereas, in the "fast fibrosers" group, 5 patients (10.9% of 46 patients) and one patient were heterozygous and homozygous, respectively, for the mutation (Table 3).

The occurrence of the PT20210 mutation among the "slow fibrosers" and "fast fibrosers" was not significantly different when the fibrosis rate was calculated according to the Poynard model; however, when we tested other cut-offs that have been used in the literature to differentiate slow and fast fibrosers, the difference became statistically significant (Table 4). Nevertheless, we used the Poynard model to define the slow and fast fibrosis groups in all of our calculations.

In a univariate analysis using the "rate of fibrosis" as the dependent variable and the PT20210 mutation status as the independent variable, while controlling for age, gender, BMI, amount of alcohol consumption, age of infection, and inflammation grade, we found that PT20210 status had a statistically significant association with the fibrosis rate ($P = 0.002$).

In order to explore the influence of PT20210 status, age, gender, age of infection, amount of alcohol consumption, BMI, and inflammation grade, we constructed a linear regression model with the above variables as the predictive variables, and the rate of fibrosis as the dependent variable (Table 5). These variables accounted for 44.8% of the variance in the fibrosis rate ($R^2 = 0.448$). The PT20210 status accounted for 9% of the fibrosis rate ($R^2 = 0.093$, $P = 0.002$).

Table 5 Linear regression analysis of rate of fibrosis

Predictive variable	R^2	*P* value
PT20210 status	0.093	0.002
Age	0.022	0.033
Gender	0.016	0.042
Age of infection	0.167	< 0.001
BMI	Not contributing	
Alcohol consumption	Not contributing	
Inflammation grade	0.121	< 0.001

PT20210: Prothrombin 20210; BMI: Body mass index.

Table 6 Multivariate analysis of the association between rate of liver fibrosis (slow fibrosers *vs* fast fibrosers) predicted by prothrombin 20210 mutation and various known parameters

Parameter	OR (95% CI)	*P*-wald[1]
Age	0.91 (0.86-0.96)	< 0.001
PT20210	4.76 (1.13-19.99)	0.033
Inflammation grade[2]	7.42 (3.07-17.95)	< 0.001

Variables removed from the model: gender, BMI and alcohol consumption. [1]The Wald test was used to assess the significance of each logit; [2]Inflammation was categorized: grades 0 and 1 compared with grades 2 and 3. PT20210: Prothrombin 20210; OR: Odds ratio; CI: Confidence interval.

In order to calculate the adjusted OR of the variables that affected liver fibrosis, we constructed a multivariate logistic regression model in a stepwise method. The variants that were included were PT20210 status, age, gender, BMI, alcohol consumption, and inflammation grade. The age of infection was not included, as it is used to define which patients are "fast fibrosers" in Poynard's model. We found that the presence of the PT20210 mutation corresponded with an OR of 4.76 ($P = 0.033$; 95% CI: 1.13-19.99) for "fast" liver fibrosis (Table 6).

Recent studies[13] have suggested that genotype 3 might be associated with the rate of fibrosis in hepatitis C patients. In order to account for this, we constructed an additional multivariate logistic regression model. This model included all universal variables that are known to affect the HCV fibrosis rate as well as genotype 3 status and the presence of the PT20210 mutation. Genotype 3 status was not a statistically significant predictor ($P = 0.25$), as opposed to PT20210, which remained in the model with an OR of 4.02 ($P = 0.048$; 95% CI: 1.01-16.00) (data not shown).

FV Leiden and MTHFR carriers

No significant association was found between FV Leiden carriage and fibrosis rate. Five patients (4.5% out of 110 patients with available gene analyses) of the "slow fibrosers" group were heterozygous for this mutation, as was only one patient (2% of 49 patients) of the "fast

fibrosers" group (Table 3).

Similarly, MTHFR was not associated with fibrosis rate in HCV patients (Table 3). Of the "slow fibrosers," 59 patients were heterozygous (52% of 113 patients with available gene analyses), whereas 14 (12.4%) patients were homozygous to the mutation. Among the "fast fibrosers", 24 (48% of 50 patients) patients were heterozygous for the mutation and 8 (16%) patients were homozygous for the mutation.

DISCUSSION

We have shown that carriage of the PT20210 mutation is related to an increased incidence rate of fibrosis in hepatitis C patients, whereas MTHFR or Factor V Leiden mutations are non-contributory.

The mean fibrosis rate in the "fast fibrosers" group was 0.23 ± 0.27 fibrosis units/year, which translates to 17 years of disease duration from infection to stage 4 cirrhosis. This rate was substantially faster than that of the "slow fibrosers" group (0.057 ± 0.037 fibrosis units/year), although classic contributing factors, such as BMI or alcohol consumption, did not substantially differ between the two groups.

In theory, enhanced coagulation may be an important factor in liver fibrosis. This hypothesis is supported by animal model studies, and, accordingly, a probable association between FV Leiden carriage and enhanced liver fibrosis in HCV patients was proposed[4,10]. The association of liver fibrosis with MTHFR carriage has not been directly examined, and the carriage of PT20210 has exhibited only an insignificant trend towards increased fibrosis in a single prior study[10].

In order to confirm that our results do not represent a type 1 statistical error, we employed three different cutoffs that have been previously used in the literature to differentiate fast from slow rates of liver fibrosis (Table 4) and eventually used the one that was the most stringent (the Poynard rate of fibrosis) in our calculations. Moreover, when the rate of fibrosis was used as a continuous variable (in the linear regression and univariate analysis models), the carriage of the PT20210 mutation was associated with faster fibrosis and explained up to 9% of the observed fibrosis rates when known factors that may affect fibrosis rate (age, age of infection, gender, alcohol consumption, BMI, and inflammation grade, which is more controversial) were controlled for.

Thus, although our cohort was relatively small, our findings suggest an impact of PT20210 mutation carriage on liver fibrosis in HCV patients. Interestingly, multiple mutations in more than one of the examined genes did not increase the fibrosis rate. We have not found a correlation between fibrosis rate and FV Leiden or MTHFR mutations. The relatively small number of patients who carried the FV Leiden mutation in our cohort may account for the lack of agreement between our findings and previous publications. With regard to MTHFR, one has to take into account folic acid and homocysteine levels, which were not examined in our cohort and may result in a non-hypercoagulation phenotype despite a procoagulation genotype. An additional limitation of our study may be the fact that none of the recruited patients had a clinical history of a hypercoagulable state, such as deep vein thrombosis. This may reflect a non-intentional selection bias and may have resulted in an underestimation of the hypercoagulation mutations in our cohort. Nevertheless, the percentage of patients who carried the PT20210 mutation in our cohort was at the high end of the range that has been reported in European cohorts, where the prevalence of the PT20210 mutation in the control groups is usually between 1%-3%, but may be as high as 6.5%[14]. PT20210 prevalence increases as one shifts from northern to southern Europe, and it is more prevalent in Caucasians as opposed to other ethnicities[15]. These two factors apply to our cohort and may at least partially explain the prevalence of PT20210 mutation carriage. Among the "slow fibrosers" group, 5.5% of patients carried the mutation, and this figure is within the reported prevalence of the PT20210 mutation in southern Europe.

The combined frequency of PT20210 among the entire cohort was above 7%; however, this statistic includes the "fast fibrosers" group, which we found to have a high frequency of PT20210 mutation carriage (13%). This signifies the importance of our findings. The PT20210 mutation was much more common in the "fast fibrosers" group, and, thus, the results of this study strengthen the notion that hypercoagulation causes faster fibrosis in HCV patients. In addition, the high percentage of PT20210 carriage in our cohort may partially explain why our results reached statistical significance, whereas Wright *et al*[10] demonstrated only a trend for a contribution of the PT20210 mutation to the fibrosis rate in HCV patients. In their cohort, although the number of patients was larger (n = 287), the prevalence of PT20210 mutation was only 4.5%.

Consistent with previous studies, we found that the age of infection and gender are related to fibrosis rate, whereas the HCV genotype had no effect on the rate of fibrosis, wherein the latter of these two has been controversial in the literature[2,13]. In contrast, inflammatory grade, which is considered to be a controversial factor regarding fibrosis rate, was determined to be a significant contributor to the fibrosis rate in our study. Because patients who suffered from alcoholic liver disease were excluded, only a small number of patients (< 8%) consumed large amounts of alcohol. This may account for the fact that we did not observe the well-known effect of alcohol consumption on liver fibrosis.

Although HCV patients who carry hypercoagulable gene mutations may account for only 5%-10% of all patients, millions of patients belong to this group around the world. Moreover, apart from the hypercoagulable states that relate to PT2010 and FV Leiden and were discussed here, other disease states may result in hypercoagulablity and perhaps in increased rates of liver fibro-

sis. Among these is the anti-phospholipid syndrome that has long been in debate with respect to its frequency and its effects in HCV patients. Specifically, anti-cardiolipin antibodies have been found in a sizable portion of HCV patients and are considered to be one of several autoimmune phenomena that are associated with this disease[16,17]. The implications of this disease on coagulation in this context are still under debate[18-21].

Ultimately, an analysis of gene mutations for hypercoagulability and the detection of hypercoagulable states in these patients may assist the clinician in the risk stratification of HCV patients according to fibrosis risk. Patients who are at high risk of liver fibrosis may be recommended to receive earlier anti-HCV therapy. With regard to the risk stratification of HCV patients according to the prevalence of the PT20210 mutation, a more specific and accurate method to stratify these patients may be found by assessing their prothrombin plasma levels, which may correlate better with the fibrosis rate.

This study lends additional support to the hypothesis that coagulation processes are involved in the pathogenesis of fibrosis in the liver. These accumulating reports should encourage the design of a larger prospective cohort study that will examine the impact of the various hypercoagulation states on liver fibrosis in HCV patients in particular, and in other fibrosis states of the liver in general. A better understanding of the pathophysiology of liver fibrosis may facilitate the development of novel therapeutic interventions that will help slow the rate of fibrosis.

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COMMENTS

Background

Cirrhosis is a major cause of morbidity and mortality in patients suffering from chronic hepatitis C virus (HCV) infection. Although various factors are known to affect liver fibrosis, it is still impossible to predict who will suffer from an increased rate of liver fibrosis among these patients. Recently, it has been suggested that HCV patients who possess hypercoagulation mutations [such as factor V Leiden (FV Leiden) mutation] may have an increased liver fibrosis rate.

Research frontiers

Hypercoagulable states have been hypothesized to play a role in organ fibrosis. In various inflammatory states, such as those of the lung or kidney, thrombosis and fibrin formation result in organ injury. It has been recently proposed that hypercoagulable states may be an additional contributing factor to liver fibrosis through several mechanisms, such as thrombotic events in small venous blood vessels in the liver, and hepatic stellate cell stimulation by thrombin. Increased fibrin deposition has also been demonstrated in animal models of liver fibrosis. These observations have been strengthened by a mouse model of liver fibrosis that demonstrated that the extent of fibrosis was much higher in mice carrying the FV Leiden mutation.

Innovations and breakthroughs

The study is the first to find a significant impact of an additional common hypercoagulation mutation, namely prothrombin G20210A (PTG20210A), on the rate of liver fibrosis in HCV patients.

Applications

The work strengthens the notion that hypercoagulation states affect the rate of liver fibrosis. If proven true in larger, prospective trials, HCV patients should be assessed for hypercoagulation mutations and should be regarded as a susceptible group for early cirrhosis, and receive early treatment against HCV. Additionally, better understanding of the role of hypercoagulation states in fibrosis may aid in the development of new therapies for organ fibrosis.

Terminology

Cirrhosis is a state of liver fibrosis with major morbidity and mortality in patients who suffer from liver diseases, in general, and particularly from hepatitis C. Hypercoagulation states such as PTG20210A, FV Leiden and methylene tetrahydrofolate reductase, are pathological conditions that cause an enhanced activity of the coagulation system and may expose patients to thrombotic events.

Peer review

The authors reported the association of fibrosis progression in chronic hepatitis C patients with PTG20210A mutation. This paper is interesting and informative.

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BRIEF ARTICLE

Experimental study of destruction to porcine spleen *in vivo* by microwave ablation

Fei Gao, Yang-Kui Gu, Jing-Xian Shen, Chang-Lun Li, Xiong-Ying Jiang, Jin-Hua Huang

Fei Gao, Yang-Kui Gu, Jing-Xian Shen, Chang-Lun Li, Xiong-Ying Jiang, Jin-Hua Huang, State Key Laboratory of Oncology in South China, Guangzhou 510060, Guangdong Province, China

Fei Gao, Yang-Kui Gu, Jing-Xian Shen, Chang-Lun Li, Xiong-Ying Jiang, Jin-Hua Huang, Medical Imaging and Interventional Center, Cancer Center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China

Author contributions: Gao F designed the study, performed the majority of experiments and wrote the manuscript; Gu YK also performed the majority of experiments; Shen JX, Li CL and Jiang XY provided analytical tools and were also involved in editing the manuscript; Huang JH provided the collection of all the material in addition to providing financial support for this work.

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Correspondence to: Jin-Hua Huang, MD, Medical Imaging and Interventional Center, Cancer Center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China. hjinh@mail.sysu.edu.cn
Telephone: +86-20-87343272 Fax: +86-20-87343272

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Abstract

AIM: To discuss the safety, feasibility and regularity of destruction to porcine spleen *in vivo* with congestion and tumescence by microwave ablation (MWA).

METHODS: Ligation of the splenic vein was used to induce congestion and tumescence *in vivo* in five porcine spleens, and microwave ablation was performed 2-4 h later. A total of 56 ablation points were ablated and the ablation powers were 30-100 W. The ablation time (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min) was performed at a power of 60 W. After ablation, the ablation size was measured in pigs A, C, D and E and spleen resection. In pig B, the ablation size was measured and 2 ablation points were sent for pathology analysis and all tissues were sutured following ablation. Pig B was killed 1 wk later and the ablation points were sent for pathology analysis. Bleeding, tissue carbonization surrounding electrodes, and pathological changes were observed, and the effect on destruction volume relative to different ablation powers, times and positions was analyzed.

RESULTS: The incidence of bleeding (only small amounts, < 20 mL) in the course of ablation was 5.4% (3/56) and was attributed to tissue carbonization surrounding electrodes, which also exhibited an incidence of 5.4% (3/56). The destruction volume was influenced by different ablation powers, times and points. It showed that the ablation lesion size increased with increased ablation time, from 1 to 10 min, when the ablation power was 60 W. Also, the ablation lesion size increased with the increase of ablation power, ranging from 30 to 100 W when the ablation time was set to 3 min. A direct correlation was seen between the destruction volume and ablation time by the power of 60 W (r = 0.97542, P < 0.0001, and also between the destruction volume and ablation powers at an ablation time of 3 min (r = 0.98258, P < 0.0001). The destruction volume of zone Ⅱ (the extra-2/3 part of the spleen, relative to the first or second class vascular branches), which was near the hilum of the spleen, was noteably larger than the destruction volume of zone Ⅰ (the intra-1/3 part of the spleen) which was distal from the hilum of the spleen (P = 0.0015). Pathological changes of ablation occurring immediately and 1 wk after MWA showed large areas of coagulation. Immediately following ablation, intact spleen tissues were observed in the areas of coagulation necrosis, mainly around arterioles, and there were no obvious signs of hydropsia and inflammation, while 1 wk following the ablation, the coagulation necrosis was well distributed and complete, as many nuclear fragmentations were detected, and there were obvious signs of hydropsia and inflammation.

CONCLUSION: *In vivo* treatment of congestion and tumescence in the spleen using microwave ablation of water-cooled antenna is a safe and feasible method that is minimally invasive.

Key words: Experimental study; Microwave ablation; Porcine spleen; *In vivo*; Water-cooled antenna

Peer reviewer: Yukihiro Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, 615-8256 Kyoto, Japan

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INTRODUCTION

Minimally invasive methods for treatment of hypersplenism, such as partial splenic embolization (PSE) and radiofrequency ablation (RFA), have been developed in recent years. PSE has been widely used, but is not optimal due to a high recurrence rate and the finding that the embolism volume varies based on the surgeon's experience and the estimation of contrast medium's flow rate, making it difficult to accurately evaluate embolism volume in real time[1]. In recent years, RFA has been used for the treatment of hypersplenism, since it has greater control over the destruction volume of the spleen; however, RFA runs a high risk of bleeding. In this study, we propose microwave ablation (MWA) as an effective method for controlling the destruction volume of the spleen. MWA has the added advantages of higher rate of temperature increase, high thermal efficiency, stable and controllable thermal field, good hemostasis effect, and good blood vessel coagulation[2,3]. This study describes the destruction to porcine spleen *in vivo* using microwave ablation with water-cooled antenna.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by Cancer Center, Sun Yat-sen University.

Materials

Five healthy Tibet pigs (assigned A, B, C, D and E), one female and four males aged 6-8 mo (weighing 32-39 kg) were selected for this study.

Methods

All the pigs were fasted for 12 h before operation and received atropine i.m. (0.5 mg; 30 min before operation), Su Mian Xin i.m. (1 mL/kg; 20 min before operation), and ketamine i.m. (8 mg/kg; 20 min before operation). After successful induction of anesthesia, the animal was fixed on the operation table. After abdominal skin preparation and sterilization, the skin, hypoderma, fat, muscle and peritoneum were cut open for complete exposure of the spleen. Then the hilum of the spleen was separated and the splenic artery and splenic vein were exposed. Next, the splenic vein was ligated using blood vessel forceps. Microwave ablation was performed when congestion and tumescence were seen, which was 2-4 h after ligation of the splenic vein. The ablation size was measured in pigs A, C, D and E after ablation and spleen resection. In pig B, the ablation size was measured and 2 ablation points were sent for pathology analysis and all tissues were sutured following ablation. Pig B was killed 1 wk later and the ablation points were sent for pathology analysis (Figure 1).

Device parameters

Specifications of water-cooled antenna: diameter 1.7 mm, model Ⅲ with high-power (made by Qi Ya Medical Treatment Facility Limited Company, Nanjing, China; batch No. 090226003EX); Emission Facility: made by Qi Ya Medical Treatment Facility Limited Company, Nanjing, China; Frequency: 2450 MHz; Output power: 0-120 W; Precision of temperature control: ± 0.1 ℃; Discharge waveform: continuous wave; operating conditions: main voltage AC 220% ± 10%, environmental temperature 5 ℃-40 ℃.

Ablation points

There were a total of 56 ablation points (8 in pig A, 12 in B, 14 in C, 13 in D and 9 in E). The ablation powers were 30, 40, 50, 60, 70, 80, 90 and 100 W. The ablation time (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min) was performed at a power of 60 W. The whole spleen was divided into two sections: zero Ⅰ, which was adjacent to the hilum of the spleen (the intra-1/3 part of the spleen); and zero Ⅱ, which was distal from the hilum of the spleen (the extra-2/3 part of the spleen), relative to the first or second class vascular branches.

Observation index

The incidence of bleeding of the pin hole, tissue carbonization surrounding electrodes, ablation volume (multiplied by long diameter and short diameter), and the pathological changes of ablation points and the surrounding tissues were observed using hemotoxylin and eosin.

Statistical analysis

Statistical analysis was performed using SAS 8.0 (not all the carbonization points were included).

The correlation analysis between the ablation volume (zero Ⅰ and zero Ⅱ) and the ablation time (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min) was performed at a power of 60 W.

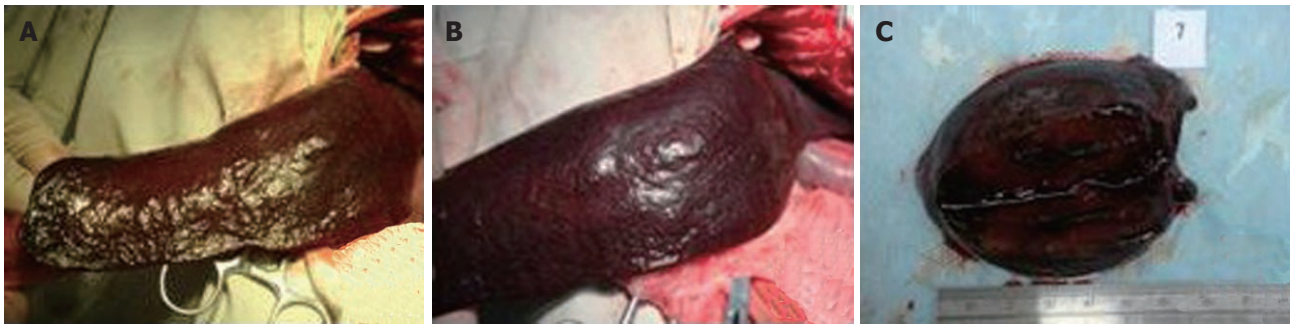

Figure 1 Procedures of experimental study of destruction to porcine spleen *in vivo* by microwave ablation. A: The appearance of the spleen before ligation of splenic vein; B: Splenomegalia was seen 2 to 4 h after ligation of the splenic vein; C: The ablation size was measured after microwave ablation.

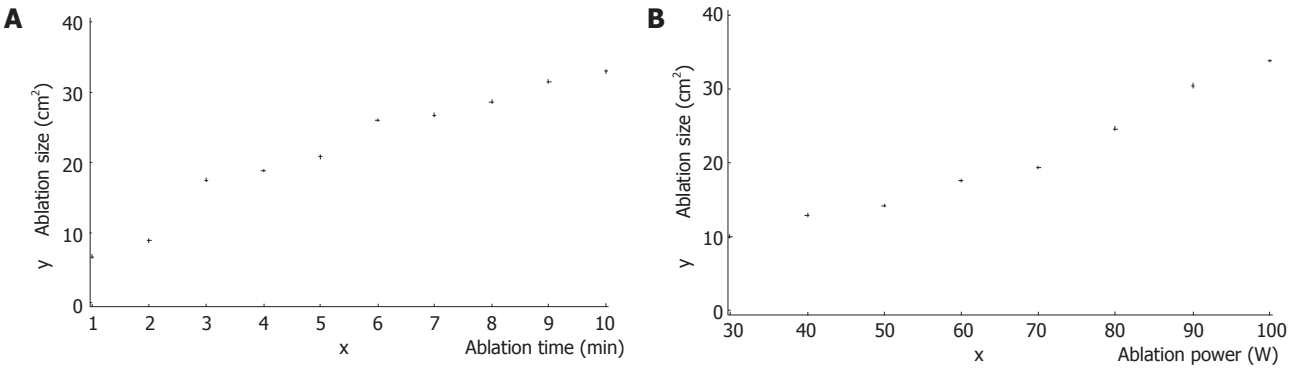

Figure 2 The ablation lesion size was correlated with the ablation time and power. A: $r = 0.97542$, x = 5.5 ± 3.028, y = 23.4 ± 9.023, $P < 0.0001$; B: $r = 0.98258$, x = 65.0 ± 24.495, y = 18.47 ± 8.47, $P < 0.0001$.

The correlation analysis between the ablation volume and the ablation power (30, 40, 50, 60, 70, 80, 90 and 100 W) was performed using an ablation time of 3 min. For each correlation analysis, the data was represented by scatterplot and the value of r was calculated.

The t test of independent samples was performed to compare the ablation range of zero I (9 points of 3 min by 60 W) and that of zero Ⅱ (8 points of 3 min by 60 W).

RESULTS

Incidence of bleeding of the pin hole and tissue carbonization

The incidence of bleeding was 5.4% (3/56), only small amounts (< 20 mL) were caused by tissue carbonization surrounding the electrodes. The carbonization incidence rate was also 5.4% (3/56).

Relationship between ablation lesion size and time

As shown in Figure 2A, the ablation range had a direct correlation with ablation time by the power of 60 W (r = 0.97542, x = 5.5 ± 3.028, y = 23.4 ± 9.023, P = 0.0001). The ablation lesion size increased with increased ablation time, from 1 to 10 min, when the ablation power was 60 W.

Relationship between ablation lesion size and power

As shown in Figure 2B, the ablation range was directly correlated with the power (30-100 W), at an ablation time of 3 min (r = 0.98258, x = 65.0 ± 24.495, y = 18.47 ± 8.47, P = 0.0001). The ablation lesion size increased with the increase of ablation power, ranging from 30 to 100 W when the ablation time was set to 3 min.

Comparison between the ablation lesion size of zone Ⅰ and zone Ⅱ (60 W, 3 min)

The ablation lesion size of zone Ⅱ (the extra-2/3 part of the spleen, relative to the first or second class vascular branches), which was near the hilum of the spleen was significantly larger than that of zone I (the intra-1/3 part of the spleen) which was distal from the hilum of the spleen (10.763 ± 2.001 *vs* 16.569 ± 4.112, P = 0.0015, t = -3.81, P = 0.0015).

Pathological changes of ablation points and the surrounding tissues

As shown in Figure 3, pathological changes occurred immediately and up to 1 wk after the ablation, and were marked by a large area of coagulation necrosis. Immediately following ablation, intact spleen tissues were observed in the areas of coagulation necrosis, mainly around arterioles, and there were no obvious signs of hydropsia and inflammation. One week following the ablation, the coagulation necrosis was well distributed and complete, as many nuclear fragmentations were detected, and there were obvious signs of hydropsia and inflammation.

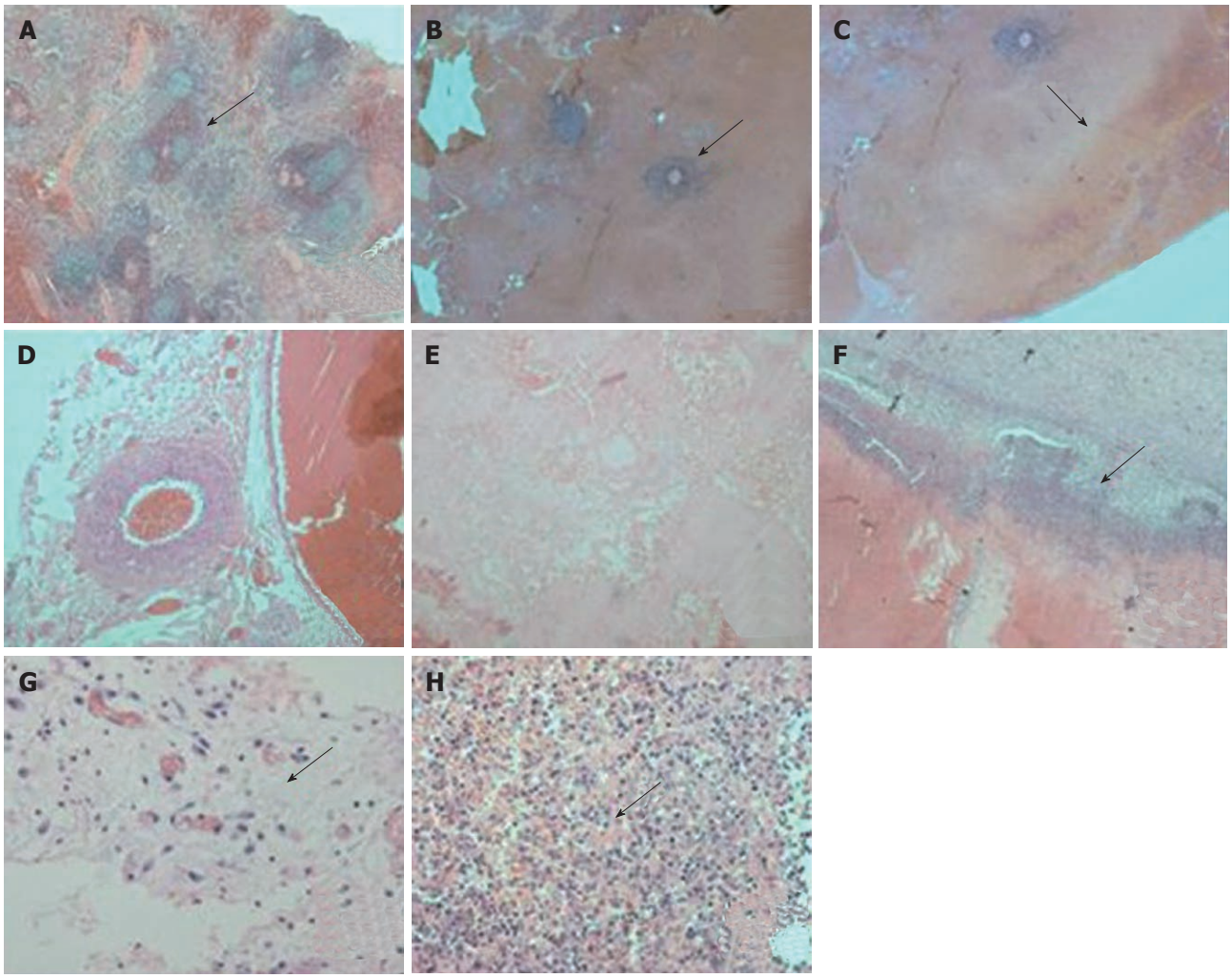

Figure 3 The pathological changes after microwave ablation. A: Normal spleen tissue (the arrow indicates the splenic corpuscle), hemotoxylin and eosin (HE) × 20; B-D: The pathological changes immediately after microwave ablation (MWA); B: There was some remaining spleen tissue around arterioles in the areas of coagulation necrosis (↑), HE × 20; C: The ablation borderline was not very clear (↑), HE × 20; D: There were no obvious signs of hydropsia or inflammatory reaction, HE × 40; E-H: Pathological changes 1 wk after MWA; E: The coagulation necrosis was well-distributed and complete, HE × 20; F: The ablation borderline was clearer 1 wk after ablation (↑), HE × 20; G: There were some signs of hydropsia and inflammation (↑), HE × 40; H: Many nuclear fragmentations could be seen clearly (↑), HE × 40.

DISCUSSION

Advantage of MWA in the treatment of splenomegalia and hypersplenism

With the development of minimally invasive therapy, new methods such as PSE and RFA are being optimized for the treatment of hypersplenism. Many research studies and clinical practices have proved that PSE has good therapeutic effects in the treatment of hypersplenism[4-7]. However, it has a high recurrence rate and the embolism volume varies based on the surgeon's experience and the estimation of the contrast medium's flow rate. For this reason, PSE is usually influenced by subjective factors, making it difficult to accurately evaluate embolism volume in real time[1]. Recently, some studies reported that RFA can effectively control the destruction volume of the spleen and overcome the limitation posed by PSE[8,9]. With RFA, coagulative necrosis, not liquefaction necrosis, occurred in the spleen after thermo-ablation, and the probability of splenic abscess was obviously decreased. Furthermore, the recurrence rate of hypersplenism was obviously decreased because the heat-sink effect can damage the vascular endothelium and lead to thrombogenesis, which can lead to spleen consolidation, interstitial fibrosis and decreased splenic recirculation.

MWA is one method of thermo-ablation and can also control the destruction volume of the spleen. MWA has the added advantages of a rapid rise in temperature, high thermal efficiency, stable and controllable thermal field, good hemostatic effect, and effective blood vessel coagulation[2,3]. Shibata *et al*[10] first compared MWA with RFA in pig liver and found that the temperature of the area surrounding the MWA electrode was significantly higher than the temperature of the area surrounding the RFA electrode; the MWA electrode achieved superior results to the RFA electrode with respect to the diameter of the coagulated area and the temperature of the area in which the electrode was inserted, at the specified times. Yu *et al*[11] also compared the effectiveness of MWA and RFA using a single internally cooled probe

in a hepatic porcine model and the results showed that MWA had higher potential for complete destruction of liver tumors than RFA. Crocetti *et al*[12] designed a study to compare the feasibility, safety, and effectiveness of MWA versus RFA of lung tissue at histopathology in a rabbit model and found the feasibility and safety of MWA and RFA are similar in a lung rabbit model, but MWA produced a greater damage to peripheral small vessels inducing thrombosis. So, MWA has the advantage in the treatment of splenomegalia and hypersplenism and shows good prospects in the treatment of spleen diseases[13]. Some reports have also shown that MWA improves the peripheral blood conditions[13,14]. Further experimental and clinical research should investigate the relationship between the destructive proportion of the spleen and the improvement of the peripheral blood conditions. Regarding the type of cooled antennas, two types, air-cooled and water-cooled, have been used for microwave ablation to create as large ablation patterns as possible[15]. Firstly, cooling water significantly reduces the temperature near the antenna, as confirmed by a previous study[16]. This approach avoids carbonization near the antenna, since the coagulation region is limited by the carbonized tissues. Therefore, a water-cooled antenna makes it possible to create the maximum ablation pattern. Secondly, a water-cooled antenna could effectively reduce the adhesion between antenna and tissue, and reportedly reduces patient pain during treatment and reduces the risk of infection after the operation[16].

Safety of microwave ablation

The main complication of MWA treatment is bleeding of the pin hole, because the spleen tissue is soft and fragile and it has many close-set blood sinuses. In the current study, we report that the incidence of bleeding was 5.4% (3/56), only small amounts (< 20 mL), and that the bleeding was controlled by further ablation of the pin hole, suggesting that MWA is a safe method. Compared to RFA, MWA has higher thermal efficiency and better blood vessel coagulation[17,18]. So, the risk of pin hole errhysis is lower than that occurring with RFA, especially since the pin hole coagulation after MWA further decreases the probability of bleeding. In this study, the risk of pin hole bleeding is likely increased due to splenic congestion and tumescence, and the splenic sinus expanding 2-4 h after splenic vein ligation. In the cases of splenomegalia and hypersplenism caused by long-term portal hypertension, in which there is endangium hyperplasia and arteriole and veinule obstruction, MWA may run a lower risk of bleeding. Animal models of splenomegalia and hypersplenism caused by portal hypertension should be developed to test this hypothesis.

Regarding the relation between the carbonization surrounding electrodes and bleeding, the current study presumes that the crushing and avulsion of carbonization tissues to the spleen tissues without ablation, such as in the course of needle withdrawal, can lead to bleeding, especially since the spleen has an abundant blood supply. So, cutting down the carbonization incidence rate is an effective method for reducing the risk of bleeding, which can be regulated based on ablation power.

In this study, the carbonization incidence rate was 5.4% (3/56). Because the spleen has an abundant blood supply, the tissues of coagulative necrosis are easy to adhere to the pinhead after dehydration, and then carbonization occurs, which decreases the heat conduction and affects the ablation range. Further statistical analysis was not performed because there were few carbonization cases in this study, presumably due to the higher speed of water circulation and ice water circulation, which could decrease the carbonization incidence rate.

Effect of the ablation point on lesion size

Some reports find that thermo-ablation targeted next to blood vessels affects the ablation range, and that the extent of this effect is closely related to the caliber of the blood vessel[19-23], but there are no related reports describing the effect of the point of MWA in spleen ablation. In the current study, it is presumed that the blood vessels adjoining the hilum of the spleen are always branches of the first class, and that the caliber is larger and the blood flow rate is faster, and therefore they can convey the heat acquired by the spleen easily and have a predictable affect on the ablation range. The blood vessels distal from the hilum of spleen are always branches of the second and third class, with a smaller caliber and slower blood flow rates, and therefore they cannot convey the heat acquired by the spleen easily and consequently have little effect on ablation lesion size. In this study, the ablation lesion size of zone I was obviously smaller than that of zone Ⅱ by the power of 60 W in the same time period.

Effect of ablation time and power to the ablation lesion size

This study found that the ablation lesion size increased with increased ablation time, from 1 to 10 min, when the ablation power was 60 W. Also, the ablation lesion size increased with the increase of ablation power, ranging from 30 to 100 W when the ablation time was set to 3 min. So, by adjusting ablation time and power, MWA can accurately control the destruction volume of the spleen in cases of splenomegalia and hypersplenism, and effectively overcome the limitations in existing treatments, such as a reduced therapeutic effect caused by a smaller destruction volume and more complications associated with excess destruction volume.

Some studies that use PSE for the treatment of hypersplenism caused by portal hypertention[1,24] report that 50%-70% is the best embolism proportion. The best destruction proportion of MWA needs further research. Different studies report different results on the best ablation power and time. Hope *et al*[25,26] found that 45 W was the best ablation power and 10 min was the best ablation time for in vivo experimental treatment of porcine kidney using microwave ablation. Hines-Peralta *et al*[27]

found the ablation range increased with the increase in ablation time and power when used for *in vivo* and *ex vivo* experimental treatment of porcine liver using microwave ablation (50-150 W; 2-20 min). The current study is similar to that of Hines-Peralta, and the best ablation power and time should be further studied using more ablation points.

Pathological changes after microwave ablation

Similar to other reports[28,29], in the current study pathology marked by coagulation necrosis of a large area was noted both immediately and 1 wk after MWA. Immediately following MWA, some remaining spleen tissue was observed around arterioles, within areas of coagulation necrosis, and there were no obvious signs of hydropsia and inflammation. However, 1 wk after MWA, coagulation necrosis was well-distributed and complete, with clear detection of many nuclear fragmentations and hydropsia and inflammation. The remaining spleen tissues detected around arterioles immediately following MWA was likely due to the heat conveying. This tissue mainly consisted of lymphocytes which had integrated cytolemma, cell organelles and cell nuclei. Though the current study did not assess pathological changes occurring beyond 1 wk post-ablation, it is possible that there is extended hydropsia and inflammation.

The differences between the pathology changes immediately and 1 wk later in this study were the changes around arterioles in the ablation area and the hydropsia and inflammatory reaction beside which was the normal tissue reaction after thermal ablation. The disaggregation and necrosis noted in this study was probably related to the finding that the remaining cells around the arterioles were surrounded by coagulation necrosis and ischemic necrosis due to a loss of blood supply. The difference between the pathological changes occurring immediately versus 1 wk after MWA is also reflected in the change of the ablation borderline. Specifically, the ablation borderline was clearer 1 wk post-ablation, and included many nuclear fragmentations with surrounding inflammatory cells that were trachychromatic. Here we show an optimized method for treatment of hypersplenism. Results from this study have the potential to enhance therapeutic treatment of hypersplenism.

COMMENTS

Background

The incidence of hypersplenism is currently rising faster in China and current treatments have lots of limitations. Microwave ablation (MWA) of water-cooled antenna is expected to be a safe and feasible method to treat this disease that is also minimally invasive.

Research frontiers

MWA is a good method of thermo-ablation which has more advantages of a rapid rise in temperature, high thermal efficiency, stable and controllable thermal field, good hemostatic effect, and effective blood vessel coagulation than radiofrequency ablation (RFA) in tumor treatment. However, whether MWA can be used in spleen ablation and how to control the destruction volume of the spleen has not been unequivocally addressed. In this study, the authors demonstrate that MWA is a safe and feasible method that is minimally invasive in spleen ablation and the appropriate volume of ablation of spleen can be obtained by controlling the ablation time and power.

Innovations and breakthroughs

Recent reports have highlighted the value of RFA in the treatment of hypersplenism. However, MWA has more advantages, such as a rapid rise in temperature, high thermal efficiency, stable and controllable thermal field, good hemostatic effect, and effective blood vessel coagulation than RFA. This is the first study to report MWA as a safe and feasible method in spleen ablation by animal experiment. Furthermore, this studies would suggest the regularity of the spleen damage *in vivo* using MWA and confirm the efficacy of microwave ablation from the pathological point of view.

Applications

By understanding the regularity of spleen damage *in vivo* using MWA, this study may represent a future strategy for therapeutic intervention in the treatment of patients with hypersplenism.

Peer review

The authors examined the safety, feasibility and regularity of destruction to porcine spleen *in vivo* with congestion and tumescence by microwave ablation. It reveals that MWA is safe in spleen ablation and the destruction volume of the spleen can be easily controlled. The results are interesting and may represent a treatment option for hypersplenism.

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BRIEF ARTICLE

Key details of the duodenal-jejunal bypass in type 2 diabetes mellitus rats

Li-Ou Han, Li-Hong Zhou, Su-Jun Cheng, Chun Song, Chun-Fang Song

Li-Ou Han, Chun Song, Chun-Fang Song, Department of General Surgery, Harbin Medical University First Affiliated Hospital, Harbin 150001, Heilongjiang Province, China
Li-Hong Zhou, Su-Jun Cheng, Department of Endocrinology, Harbin Medical University First Affiliated Hospital, Harbin 150001, Heilongjiang Province, China
Author contributions: Song CF and Song C designed the research and contributed equally to this work; Han LO, Zhou LH and Cheng SJ performed the research; Han LO and Zhou LH analyzed the data and wrote the paper.
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Correspondence to: Dr. Chun-fang Song, Department of General Surgery, Harbin Medical University First Affiliated Hospital, 23 Youzheng St, Harbin 150001, Heilongjiang Province, China. songchunfang3210@126.com
Telephone: +86-451-85555735 Fax: +86-451-85555735
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Abstract

AIM: To investigate which surgical techniques and perioperative regimens yielded the best survival rates for diabetic rats undergoing gastric bypass.

METHODS: We performed Roux-en-Y gastric bypass with reserved gastric volume, a procedure in which gastrointestinal continuity was reestablished while excluding the entire duodenum and proximal jejunal loop. We observed the procedural success rate, long-term survival, and histopathological sequelae associated with a number of technical modifications. These included: use of anatomical markers to precisely identify Treitz's ligament; careful dissection along surgical planes; careful attention to the choice of regional transection sites; reconstruction using full-thickness anastomoses; use of a minimally invasive procedure with prohemostatic pretreatment and hemorrhage control; prevention of hypothermic damage; reduction in the length of the procedure; and accelerated surgical recovery using fast-track surgical modalities such as perioperative permissive underfeeding and goal-directed volume therapy.

RESULTS: The series of modifications we adopted reduced operation time from 110.02 ± 12.34 min to 78.39 ± 7.26 min ($P < 0.01$), and the procedural success rate increased from 43.3% (13/30) to 90% (18/20) ($P < 0.01$), with a long-term survival of 83.3% (15/18) ($P < 0.01$).

CONCLUSION: Using a number of fast-track and damage control surgical techniques, we have successfully established a stable model of gastric bypass in diabetic rats.

Key words: Duodenal-jejunal bypass; Type 2 diabetes mellitus; Minimally invasive surgery; Fast-track surgery; Damage control surgery; Permissive underfeeding; Goal-directed volume therapy

Peer reviewers: Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma – Sapienza, Viale del Policlinico 155, Roma 00161, Italy; Dr. İbrahim Sakçak, 6th Surgery Clinic, Numune Teaching and Education Hospital, Ankara 061000, Turkey

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INTRODUCTION

When gastric bypass has been used to treat morbid obesity in patients with concomitant type 2 diabetes mellitus

(T2DM), some of these patients have seen their diabetes resolve even before they have lost weight[1-3]. Available data suggest that reduced caloric intake alone is not sufficient to explain the observed short-term metabolic and endocrine effects[4,5]. Recently, Rubino *et al*[6] demonstrated that a duodenal-jejunal bypass (DJB) procedure that preserved gastric volume could directly benefit T2DM patients, suggesting that duodenal-jejunal exclusion, rather than the restriction or reduction of gastric volume, is the critical factor in surgical treatment of T2DM.

Understanding the mechanisms underlying this surprising phenomenon would enhance our understanding of the pathophysiology of T2DM, and potentially require us to revise our approach to treating this disease[7]. Therefore, as a first step, we chose to apply an existing rat model for DJB in fat-fed/STZ rats. In such a model, a range of surgical, perioperative, and other factors would be expected to influence the effect of bypass surgery on diabetes. However, there are few data regarding the influence of particular surgical modalities or non-surgical aspects of care on diabetic rats. Therefore, in this study, we carefully compared a number of both surgical techniques and perioperative management regimens for their ability to improve the success rate in diabetic rats underlying DJB, including minimally invasive procedural designs and the use of damage control and fast-track surgical modalities.

MATERIALS AND METHODS

Laboratory animals

All animal use was in compliance with the regulations set out by the institutional animal research committee at Harbin Medical University. Male Wistar rats, 8 wk of age and weighing between 200 and 230 g, were purchased from CAAS Harbin Veterinary Research Institute [Approval NO. SCXK (HLJ) 2006-009]. The rats were given a high-fat diet for 8 wk, followed with an intraperitoneal injection of streptozotocin (30 mg/kg). After 72 h of treatment, rats with a non-fasting blood glucose level above 300 mg/dL (16.7 mmol/L), as measured by an electronic glucometer (One Touch Ultra, Lifescan, Johnson and Johnson, Milpitas, CA), were considered diabetic[8,9].

Experimental protocol

Wistar rats (n = 50) were randomly assigned for either conventional DJB (n = 30) or modified DJB (n = 20). The modification consisted of an improved surgical technique and postoperative care regimen, each of which was based on a prior analysis of the histopathological sequelae in failed cases of treatment with conventional DJB. Rats surviving more than 4 wk postoperatively were considered to constitute an operative success. The operative duration was recorded for rats in both groups, as was the mean plasma glucose level (PGL) both preoperatively and 4 wk postoperatively.

Table 1 Postoperative rehydration formula (total volume = 115 mL)

Component	Formula 1	Formula 2
0.9% sodium chloride	50 mL	50 mL
10% potassium chloride	3 mL	3 mL
5% glucose	0 mL	60 mL
10% glucose	30 mL	-
25% glucose	30 mL	-
Regular insulin	2 IU	-
Cimetidine	2 mL (0.2 g)	2 mL (0.2 g)
Cefoperazone sodium/sulbactam sodium	1.0 g	1.0 g

Conventional DJB

After fasting (12 h) and water deprivation (4 h), rats were anesthetized with 0.5% pelltobarbitalum natricum (30 mg/kg). After a median longitudinal incision was made in the upper abdomen, a Roux-en-Y duodenal-jejunal bypass that preserved the gastric volume while excluding the entire duodenum and proximal jejunal loop was constructed. First, after transecting the pylorus, the distal duodenal end was closed. The jejunum was transected 8 cm distal to Treitz's ligament, and the distal jejunal loop was connected *via* an end-to-end anastomosis to the free end of the pylorus[10]. Gastrointestinal continuity was reestablished *via* an end-to-side anastomosis between the proximal end of the jejunum and the distal jejunal loop, 12 cm distal to the gastrojejunal anastomosis. Consecutive sutures were used for the anastomoses, while full-thickness intermittent sutures were used for the closure of abdominal incisions.

Postoperatively, rats were housed separately in metabolic cages that were warmed for 6 h by a 60-watt incandescent lamp. Fluids were administered daily as subcutaneous injections into the back of the neck, providing a daily total of 18.7 kcal/kg, with a 5:1 ratio of glucose to insulin (Table 1, Formula 1). Enteral nutrition with Nutrison® [Nutricia Pharmaceutical (Wuxi) Co., Ltd., Wuxi, China], given three to four times daily, was begun after the first bowel movements (usually 48 h postoperatively), and provided a total daily caloric intake of 120 kcal/kg and a nitrogen content of 0.8 g/kg. Rats were subsequently given free access to water and semisolid food, and were finally graduated to regular rodent chow (the First Affiliated Hospital of Harbin Medical University Animal Center, Harbin, China). Throughout the postoperative period, rats were carefully monitored for general well-being, urine output, the nature and amount of feces, feeding profile, incision healing, and blood glucose.

Modified DJB

Rats were fasted for 12 h, but were given free access to water bottles containing a 50 g/L solution of glucose in normal saline. Before anesthesia, animals received subdermal injections (2 mL into the flexor side of the hindlimbs) of both sodium chloride (0.9%) and glucose (5%). Twenty minutes after subdermal injection of atro-

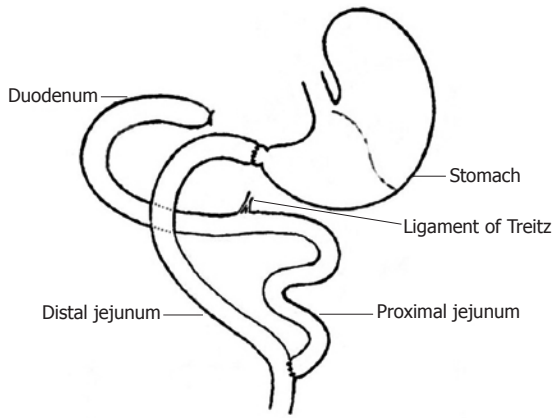

Figure 1 Schematic diagram of Roux-en-Y duodenal-jejunal bypass. The duodenum was transected 2-3 mm distal to the pylorus, and the distal duodenal end was ligated and closed. After the jejunum was transected 8 cm distal to Treitz's ligament, an end-to-end anastomosis was achieved between the distal jejunal loop and the proximal duodenum. The proximal jejunal loop was then anastomosed end-to-side to the distal jejunal loop at 12 cm distal to the gastro-jejunal anastomosis.

pine (0.1 mg/kg), baseline anesthesia was initiated with an intraperitoneal injection of 0.5% pelltobarbitalum natricum (20 mg/kg). Intermittent inhalation of ether was used to maintain anesthetic duration and depth during the operation. Sputum was periodically suctioned throughout anesthesia to maintain airway patency. A median longitudinal incision was begun at the xiphoid and caudally extended approximately 3 cm along the upper abdominal linea alba. The incision was then retracted using a blepharostat. Retraction of the hepatic lobes revealed the gastric pylorus and the initial portion of the duodenum, which was elevated with the notched forceps. The vascular branches running vertically to the pancreatic head, adjacent to the pylorus, were explored to carefully define the transection site. To avoid later damaging the biliary tract, the common bile duct and its point of convergence with the duodenum were also visualized. The first part of the duodenum was retracted caudally to expose the underlying colon, below which Treitz' s ligament was identified. In the Roux-en-Y procedure, the first part of the duodenum was transected 2-3 mm distal to the pylorus and the distal end of the duodenum was closed with a ligature, which both preserved gastric volume and excluded the entire duodenum and proximal jejunal loop. The procedure was otherwise identical to the conventional DJB (Figure 1).

Technical precautions

Although the skin incision was extended to the xiphoid so as to fully expose the surgical field, care was taken to avoid pneumothorax. Prehemostasis of the pyloroduodenal transection was achieved by ligating the major pyloric vascular branches of the gastroduodenal arteries with 7-0 minimally invasive sutures (Warwick Medical Supplies Co., Ltd. Hangzhou China). Treitz's ligament was carefully identified, and the intended site of jejunal transection, 8.0 cm distal to this structure, was marked with 7-0 sutures. To match the caliber of each side of the anastomosis, the jejunal segment to be transected was exteriorized for 3-5 min and subsequently restored. The duodenal segment adjacent to the intended division line was circled with 5-0 minimally invasive sutures (Warwick Medical Supplies Co., Ltd. Hangzhou, China), which were drawn through the vascular branches between the duodenum and the pancreatic head. Before transection of the duodenum, the segment 2-3 mm distal to the pylorus was dissected using a microhemostat. The distal duodenal end was ligated using preplaced 5-0 sutures to close the transection. The jejunum and its mesentery were transected in a similar manner.

Intermittent full-thickness anastomoses were created using 6-0 minimally invasive sutures (Warwick Medical Supplies Co., Ltd. Hangzhou China), with care taken to avoid intestinal distortion. The first stitch of the gastro-jejunal anastomosis was made at the mesenteric margin using varus sutures, with 2-3 cm suture tails retained for marking and traction. The second and third stitches were placed to connect the midpoint of the posterior wall and the contramesenteric margin. The first part of duodenum was reversed, using notched forceps, to expose the posterior wall of the anastomosis, which was anastomosed at a stitch interval of 0.5 mm and a margin interval of 1.0 mm. The anterior wall was anastomosed similarly. The jejunojejunal anastomosis was completed in the reverse sequence.

Closure of the mesenteric hole was achieved using end-to-end stitches in the avascular mesentery, with care taken to avoid ligating vessel arches and thereby compromising the anastomotic blood supply. The surgical field was intermittently rinsed with warm saline to compensate for any fluid loss from the laparotomy, as well as to prevent drying damage to the exposed portion of the intestines. Before closing the abdomen, the blood supply of each anastomosis was routinely inspected, and the intestinal loops were appropriately sequenced. The abdominal incision was closed with full-thickness stitches using 2-0 minimally invasive sutures (Yangzhou Huaxia Medical Devices CO., Ltd. Yangzhou, China). Throughout the procedure, the ambient temperature was maintained at 25 ℃, using an electric blanket if necessary, to keep the rat body surface dry and prevent intraoperative hypothermia.

Postoperative care

Several modifications were made to the standard regimen of postoperative care for DJB in rats[11,12]. First, only half the volume of fluid normally given by infusion was administered on the day of surgery, and it was augmented with insulin-free 5% glucose (12 mL/kg) (Table 1, Formula 2). Routine fluid infusion (50 mL/kg per day) was given on the following day (Table 1, Formula 1). Enteral nutrition using Nutrison® [Nutricia Pharmaceutical (Wuxi) Co., Ltd., Wuxi, China], was begun 48 h postop-

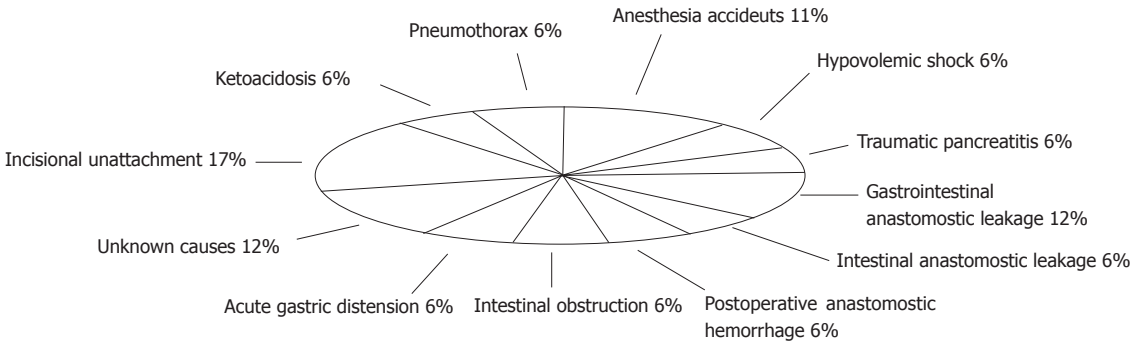

Figure 2 Causes of mortality in the conventional duodenal-jejunal bypass group.

eratively, when the majority of rats had resumed bowel movements.

Statistical analysis

All qualitative data were expressed as percent. Fisher's exact probability test was used to compare the difference in survival rate between rats in the conventional and modified DJB groups. All quantitative data were expressed as mean ± SD. Intragroup PGL values were compared using the paired Student's *t* test, while operative duration and mean PGL were compared between the 2 groups using the independent two-sample Student's *t* test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Procedural success rate and operative duration

The preliminary study allowed us to become familiar with the anatomical structures involved in the procedure and to become proficient in performing the required microsurgical anastomoses. In the conventional DJB group [mean operative duration time (OT) 110.02 ± 12.34 min], 13 rats survived, representing a success rate of 43.3% (13/30). Judging from the histopathological sequelae, the causes of mortality in this group potentially included anesthetic accidents (n = 2, 11%), hypovolemic shock (n = 1, 6%), pneumothorax (n = 1, 6%), traumatic pancreatitis (n = 1, 6%), ketoacidosis (n = 1, 6%), postoperative anastomotic hemorrhage (n = 1, 6%), acute gastric distension (n = 1, 6%), gastrointestinal anastomotic leakage (n = 2, 12%), intestinal anastomotic leakage (n = 1, 6%), intestinal obstruction (n = 1, 6%), and incisional unattachment (n = 3, 17%). Two rats died of unknown causes within 24 h of surgery (Figure 2). In the modified DJB group, the mean OT was reduced to 78.39 ± 7.26 min (P < 0.01). Of the 20 rats in this group, only 2 died, due to anastomotic leakage or intestinal obstruction. The success rate in the modified DJB group (18/20, 90.0%) was significantly higher than that in the conventional DJB group (P < 0.01).

Mean PGL

In the conventional DJB group, the preoperative and 4-wk-postoperative mean PGL levels were 16.69 ± 1.69 mmol/L and 7.46 ± 0.49 mmol/L (P < 0.01), respectively, while the corresponding values in the modified DJB group were 17.02 ± 1.51 mmol/L and 7.23 ± 0.39 mmol/L (P < 0.01). There was no significant difference in mean postoperative PGL between the 2 groups (P > 0.05). However, 4 wk after the operation, the levels of PGL in conventional or modified DJB groups were significantly decreased compared with the preoperative preoperative (P < 0.01).

DISCUSSION

Obese patients with T2DM have been reported to show an improvement in diabetic symptoms after being treated for obesity with DJB. Although the DJB animal model offers a first step toward understanding the mechanism of this phenomenon, it is technically challenging and associated with high procedure-related mortality. In the current study, we modified a number of technical details of both the procedure and the postoperative care regimen that resulted in a reduced operative duration, while yielding a higher success rate, with postoperative PGL levels comparable to those obtained with the standard procedure.

Anesthetic management is an important consideration when performing animal surgery. The anesthetic pelltobarbitalum natricum has potent effects in suppressing breathing and cardiac function. Unfortunately, its pharmacological effects vary greatly from rat to rat, giving it a very narrow safety range. Rats anesthetized with this drug most often die as a consequence of excessive airway secretion. For this reason, we chose to use a combined general anesthetic protocol, beginning with a low-dose of pelltobarbitalum natricum and then controlling the depth and duration of anesthesia using intermittent administration of inhaled ether[13]. Airway management relied on both premedication with atropine, to inhibit airway secretion, and the intraoperative suction of sputum to maintain airway patency.

The success rate in DJB is critically dependent on choosing the correct site of transection and anastomosis. In the modified DJB procedure, the first part of the

duodenum was transected 2-3 mm distal to the pylorus, and the two sides of the prospective gastrojejunostomy were matched in both diameter and layer in order to simplify the anastomotic manipulation and prevent anastomotic stricture. We took care in a number of areas when choosing the jejunal transection site. First, a well-vascularized jejunal loop was transected midway between two main branches of the mesenteric vessel arches, and when necessary, intestinal canals were stitched to reach the major branches of mesenteric vessel arches to ensure that the anastomoses were sufficiently perfused and thus protected from anastomotic leakage or stenosis. In addition, the transected jejunal loop was exteriorized to decrease intestinal tension and allow us to better identify segments that were matched in diameter. The site of anastomosis was chosen based on the mobility of the associated jejunal mesentery, so as to minimize the tension these structures might exert on the subsequent gastrojejunostomy. Although a continuous stitch would have reduced the operative duration, it was also judged likely to increase the risk of postoperative hemorrhage and stenosis at the anastomosis. Supporting this, in the conventional DJB group, hemorrhage from the continuously stitched anastomoses was responsible for one case of postoperative mortality, while intermittent full-thickness sutures had no such sequelae.

We used a number of minimally invasive techniques in an effort to reduce complications from hemorrhage, surgical damage, and infection. First, the anterior pyloric vessels were pre-ligated to provide active hemostasis. Careful identification of anatomical structures and dissection along surgical planes also contributed to preventing excessive hemorrhage and secondary injury[14]. As a relatively constant anatomical marker, Treitz's ligament was carefully identified, to minimize the surgical invasiveness on the intestines. Simple closure of the distal duodenal end with 5-0 ligature significantly decreased the volume of blood lost, and also reduced operative duration without risking duodenal leakage. To reduce the risk of postoperative traumatic pancreatitis, we took care to avoid contact with the pancreas. For the anastomotic reconstruction, the use of 6-0 minimally invasive sutures and notched forceps lessened intestinal contusion, protected the blood supply to the anastomosis, and significantly lessened anastomotic leakage by minimizing mechanical and ischemic injuries. We took particular care to diminish any action, which could contribute to peritoneal contamination.

Damage Control Surgery philosophy is the key to ensure a successful operation[15,16]. Although frequently ignored as a prognostic factor, hypothermia can initiate a lethal triad of complicating factors, as it aggravates metabolic acidosis and compromises coagulation[17,18]. Intraoperative hypothermia can be caused by heat loss from the open peritoneal cavity as well as by anesthesia and fluid infusion[19]. Hypothermic rats are also susceptible to ventricular arrhythmia, infection, and an elevated catabolic rate[20]. Prolonged hypothermia increases the incidence of multiple organ dysfunction syndrome (MODS) and mortality [21]. In the conventional DJB group, the two deaths that occurred within the first postoperative 24 h might have been due to MODS that was itself secondary to hypothermia. Appropriate damage control techniques in the context of the animal duodenal-jejunal model include reducing both the overall procedure time and the period in which the peritoneal cavity lies open, as well as ensuring postoperative active heat preservation. In this study, the techniques used to shorten the OT included the rapid and accurate identification of Treitz's ligament, hemorrhage control by careful dissection along anatomical planes, simple closure of the distal duodenal end, and interrupted closure of the abdominal incision in full-thickness.

Fast-track techniques were used in our modified DJB. Preoperative oral administration of glucose-saline solution to fasted rats prevents hypoglycemia and attenuates postoperative insulin resistance[22,23]. Postoperative stress can be addressed by permissive underfeeding of enteral nutrition, with the low-calorie supply best given in the short term to better meet metabolic requirements and optimize blood glucose[11,24]. Stress hyperglycemia on the operative day should be prevented by strict restriction of glucose intake, with 5% glucose given at a dose of 12 mL/kg to prevent postoperative hypoglycemia. To maintain nitrogen balance in the longer term, the number of calories supplied in enteral nutrition can be progressively increased until a state of balanced enteral nutrition is reached.

Another fast-track surgical measure is the strict restriction of fluid and electrolytes, as overdosing of electrolyte solution will delay the resumption of gastrointestinal activity[25-27]. Marjanovic *et al*[12] reported that restricted rehydration favored the initial healing of intestinal anastomoses, while over-rehydration increased both the severity of peripheral edema and the incidence of complications. In addition to the perioperative use of insulin in T2DM rats, appropriate fluid therapy is also critical to prevent acute metabolic disturbance such as ketosis. Therefore, we suggested using an individualized regimen of goal-directed volume therapy (GDT) to appropriately restrict rehydration[28]. Our results demonstrated that the composition and volume of the fluid infused according to a GDT regimen was able to optimally balance input and output and to effectively decrease the incidence of acute metabolic disturbance. Additionally, in the case of ketoacidosis and hyperosmotic dehydration, adequate rehydration should be given along with insulin, and the animal should be closely monitored.

In conclusion, we successfully established a sound duodenal-jejunal bypass model in diabetic rats that is based on minimally invasive techniques and fast-track surgical management. Our findings suggest that duodenal-jejunal bypass is a potential treatment alternative for diabetic patients who are especially unresponsive to other modalities of medical intervention, provided

that surgical invasiveness is similarly minimized and the principles of fast-track and damage control surgery are observed.

COMMENTS

Background

When gastric bypass is used to treat morbid obesity in patients with concomitant type 2 diabetes mellitus (T2DM), some of these patients have seen their diabetes resolve even before they have lost weight. Available data suggest that reduced caloric intake alone is not sufficient to explain the observed short-term metabolic and endocrine effects. Recently, Rubino *et al* demonstrated that a duodenal-jejunal bypass (DJB) procedure that preserved gastric volume could directly benefit T2DM patients, suggesting that duodenal-jejunal exclusion, rather than the restriction or reduction of gastric volume, is the critical factor in surgical treatment of T2DM. Therefore, as a first step, the authors chose to apply an existing rat model for DJB in animals rendered diabetic by administration of streptozotocin. In such a model, a range of surgical, perioperative, and other factors would be expected to influence the effect of bypass surgery on diabetes. However, there is little data regarding the influence of particular surgical modalities or non-surgical aspects of care on diabetic rats.

Research frontiers

Obese patients with type 2 diabetes mellitus have been reported to show an improvement in diabetic symptoms after being treated for obesity with DJB. Although the DJB animal model offers a first step toward understanding the mechanism of this phenomenon, it is technically challenging and associated with high procedure-related mortality. In the current study, the authors carefully compared a number of both surgical techniques and perioperative management regimens for their ability to improve the success rate in diabetic rats underlying DJB, including minimally invasive procedural designs and the use of damage control and fast-track surgical modalities.

Innovations and breakthroughs

In the current study, the authors modified a number of technical details of both the procedure and the postoperative care regimen (including anesthetic management, the correct site of transection and anastomosis, a number of minimally invasive techniques, hypothermia and fast-track techniques) yielding a higher success rate, and reduced fasting blood glucose levels in diabetic rats. The authors successfully establish a sound duodenal-jejunal bypass model in diabetic rats that is based on minimally invasive techniques and fast-track surgical management.

Applications

In this study the authors successfully establish a sound duodenal-jejunal bypass model in diabetic rats. The findings suggest that duodenal-jejunal bypass is a potential treatment alternative for diabetic patients who are especially unresponsive to other modalities of medical intervention, provided that surgical invasiveness is similarly minimized and the principles of fast-track and damage control surgery are observed.

Terminology

DJB, the operation consists of a stomach-preserving bypass of a short segment of proximal small intestine. DJB may be associated with a sleeve resection of the stomach to reduce potential for marginal ulcerations and increase the weight loss effect if performed in mildly or severely obese patients. Fast-track surgery, a new method of application of preexisting procedures in pre-intra and post surgical phase pre-written and carried out in a multi-disciplinary way in order to obtain a rapid recovery after operation.

Peer review

It is a very well conducted animal model study. The results are interesting and very useful for the study of this subject. Morbidly obese patients undergoing gastric by-pass is lost in T2DM can be more easily treated. In this respect, it is an important study.

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CASE REPORT

Thalidomide induces mucosal healing in Crohn's disease: Case report

Márcio Rios Leite, Sandra Sousa Santos, André Castro Lyra, Jaciane Mota, Genoile Oliveira Santana

Márcio Rios Leite, André Castro Lyra, Genoile Oliveira Santana, Department of Gastroenterology, Prof. Edgard Santos University Hospital, Federal University of Bahia, Salvador, Bahia 40110-060, Brazil
Sandra Sousa Santos, Jaciane Mota, Department of Gastrohepatology, São Rafael Hospital, Monte Tabor Foundation, São Marcos Avenue, 2152, Salvador, Bahia 41256-900, Brazil
Author contributions: Rios Leite M and Santana GO contributed equally to this work; Sousa Santos S and Mota J reviewed the chart and designed the figures; Rios Leite M wrote the paper; Lyra AC and Santana GO reviewed the paper.
Correspondence to: Márcio Rios Leite, MD, Department of Gastroenterology, Prof. Edgard Santos University Hospital, Federal University of Bahia, Augusto Viana Street, Salvador, Bahia 40110-060, Brazil. marciorios leite@hotmail.com
Telephone: +55-71-82023372 Fax: +55-71-32357048
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Abstract

Crohn's disease is a chronic inflammatory disorder of the gastrointestinal tract that is defined by relapsing and remitting episodes. Tumor necrosis factor alpha (TNF-α) appears to play a central role in the pathophysiology of the disease. Standard therapies for inflammatory bowel disease fail to induce remission in about 30% of patients. Biological therapies have been associated with an increased incidence of infections, especially infection by *Mycobacterium tuberculosis* (*Mtb*). Thalidomide is an oral immunomodulatory agent with anti-TNF-α properties. Recent studies have suggested that thalidomide is effective in refractory luminal and fistulizing Crohn's disease. Thalidomide costimulates T lymphocytes, with greater effect on CD8+ than on CD4+ T cells, which contributes to the protective immune response to *Mtb* infection. We present a case of Crohn's disease with gastric, ileal, colon and rectum involvement as well as steroid dependency, which progressed with loss of response to infliximab after three years of therapy. The thorax computed tomography scan demonstrated a pulmonary nodule suspected to be *Mtb* infection. The patient was started on thalidomide therapy and exhibited an excellent response.

Key words: Anti-tumor necrosis factor alpha; Crohn's disease; Mucosal healing; *Mycobacterium tuberculosis*; Thalidomide

Peer reviewer: Dr. Jörg C Hoffmann, MA, Priv, Doz, MD, Chief of the Medizinischen Klinik I, mit Schwerpunkt Gastroenterologie, Diabetologie, Rheumatologie und Onkologie, St. Marien-und St Annastiftskrankenhaus, Salzburger Straße 15, D67067 Ludwigshafen, Germany

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract that is defined by relapsing and remitting episodes[1]. The inflammatory process is characterized by increased production of proinflammatory cytokines[2]. Tumor necrosis factor alpha (TNF-α) plays a central role in the pathogenesis of the disease[3].

Over the past few decades, medical therapy for CD has progressed significantly with immunomodulating agents such as azathioprine, 6-mercaptopurine, methotrexate, tacrolimus and cyclosporin, and biological agents such as infliximab, adalimumab and certolizumab[1]. Nevertheless, the use of biological therapies has been associated with an increased incidence of infections, especially

infection by *Mycobacterium tuberculosis* (*Mtb*)[4].

Thalidomide is an oral immunomodulatory agent with anti-TNF-α properties[5,6]. Thalidomide increases the IFN-γ level and modulates several other cytokines as well, especially interleukin (IL)-2 and IL-12[2]. Recently, an open-label trial assessing the treatment of refractory CD reported response rates of 64% and 70% after a 12-wk course of thalidomide[7,8].

Thalidomide costimulates T lymphocytes, with greater effect on CD8+ than on CD4+ T cells, which contributes to the protective immune response to *Mtb* infection[9,10]. Results from four case reports, one clinical trial and one placebo-controlled trial suggest the use of thalidomide for central nervous system tuberculosis (TB) not responding to standard therapy is beneficial. Thalidomide should not be used for routine treatment, but it may be useful as a "salvage therapy" in patients with tuberculosis meningitides and tuberculomas that are not responding to anti-TB drugs or to high-dose corticosteroids[11].

We present the case of a 24-year-old CD patient, with gastric, ileal, colon and rectum involvement, as well as steroid dependency, which progressed with diminished response to infliximab after three years of therapy. A thoracic computed tomography scan revealed a pulmonary nodule suspected to be *Mtb* infection. He was started on thalidomide therapy and exhibited an excellent response.

CASE REPORT

A 24-year-old male was first diagnosed with CD at the age of 10 when he presented fever, arthralgia of large joints (knees and ankles), bloody diarrhea (more than 10 bowel movements/d) and a Harvey-Bradshaw Index of 13. Endoscopy showed involvement of gastric corpus, ileum and all segments of the colon and rectum.

Initially, he was prescribed 1 mg/kg per day steroid, 100 mg/d azathioprine and 2.5 g/d sulfasalazine by a pediatric gastroenterologist. After eight years, he presented signs of active inflammatory bowel disease, with imaging revealing CD activity in gastric tissue, ileal tissue, and all segments of the colon and rectum (Harvey-Bradshaw Index 15). He was started on therapy with infliximab 5 mg/kg and continued on 100 mg/d azathioprine. After 2 mo, the patient presented significant clinical improvement with reduced stool frequency of 3 bowel movements/d and remission of joint inflammation. Endoscopic evaluation showed reduction of inflammatory disease activity in all segments of the colon and rectum, as well as healing of gastric lesions. The Harvey-Bradshaw Index was 4 after infliximab treatment.

Six months after the first dose of infliximab, the patient relapsed with bloody diarrhea in 6-8 bowel movements/d and a Harvey-Bradshaw Index of 12. The infliximab dose was increased to 10 mg/kg, which controlled the disease activity and led to a Harvey-Bradshaw index of 2. After 3 years, the patient presented with worsening of disease. Imaging studies revealed inflammatory activity in the gastric body, sigmoid and descending colon (Figure 1A). Infliximab was discontinued, and the azathioprine dose was increased to 150 mg/d in combination with mesalazine 1.2 g/d. We opted not to introduce adalimumab due to the presence of a cavitary pulmonary nodule in the left upper lobe measuring 0.6 × 0.8 cm^2 (Figure 2A). At that time, we suspected infectious pneumonia (fungal/mycobacterial), eosinophilic granuloma or vasculitis. As the lung nodule was not accessible to percutaneous or bronchoscopy biopsy, we opted for a conservative approach. Because we did not have a definitive diagnosis of pulmonary disease and because the patient presented with all the symptoms of inflammatory bowel disease, including diarrhea, abdominal pain, weight loss, arthralgia and a Harvey-Bradshaw Index of 15, we opted to introduce thalidomide.

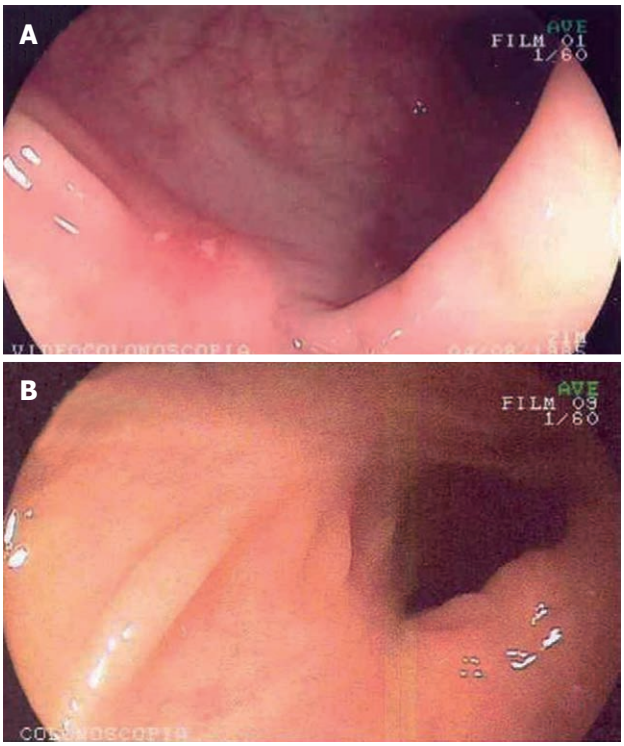

Figure 1 Colonoscopy. A: Colon showing scattered aphthous ulcers, with some confluence; B: Colon showing scar areas.

Thalidomide was started at 100 mg/d, and the patient's symptoms resolved, with initial improvement of abdominal pain and diarrhea between 1 and 4 mo (Harvey-Bradshaw Index 8) and complete remission of symptoms at 6 mo of therapy (Harvey-Bradshaw Index 3). The patient was asymptomatic, presenting 2-3 regular bowel movements/d and pasty stools, without blood or mucus. He denied abdominal pain, nausea and vomiting and complained of neuropathy. His weight increased by 10 kg. Laboratory tests showed normal inflammatory activity (CRP 3; ESR 2). Colonoscopy revealed scar tissue in sigmoid colon, descending, transverse, and ascending colon; and cecum; the ileocecal valve and terminal ileum showed ulceration, with thick fibrin interspersed with normal mucosa (Figure 1B). Control computerized tomography showed small (0.5 cm) calcified pulmonary nodules in the

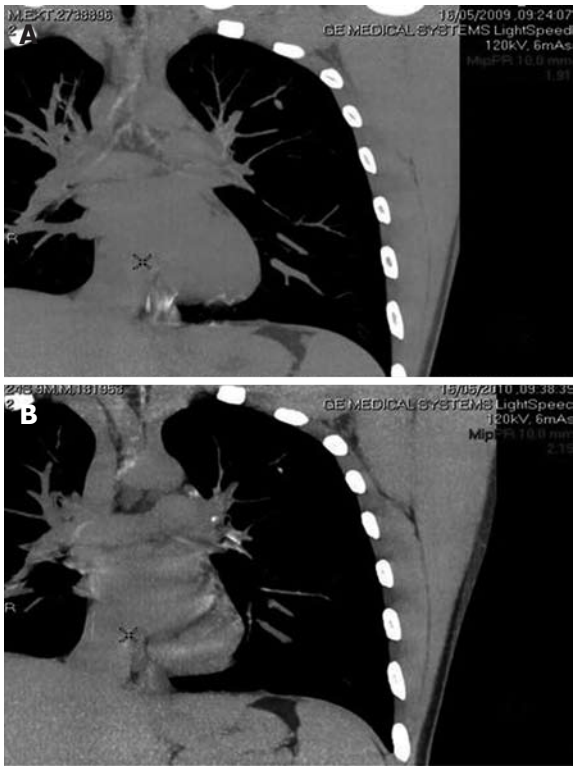

Figure 2 Chest computed tomography. A: Small nodule, measuring 0.8 to 0.6 cm, in the upper lobe of the left lung; B: Small calcified nodule located in the apico-posterior lobe of the left lung, measuring 0.5 cm in diameter.

apico-posterior segment, which were suggestive of primary *Mtb* infection (Figure 2B).

DISCUSSION

Standard therapies for inflammatory bowel disease fail to induce remission in about 30% of patients[12]. Recent studies have suggested that thalidomide at doses varying from 50 to 300 mg/d is effective in refractory luminal and fistulizing CD[10,11]. The response appears to be fairly rapid, and the remission rate is 20%-40% after 12 wk of thalidomide therapy[13].

Considering the present case, a patient with long-standing CD with extensive involvement of the digestive tract who failed to respond to infliximab after 3 years of therapy and had a pulmonary nodule possibly resulting from *Mycobacterium tuberculosis*, it was necessary to offer an effective and safe therapy to avoid infection. New therapies are needed in the context of patients with inflammatory bowel disease and tuberculosis diagnosed or suspected before or after biological treatment. Thalidomide appears to be a good option in this situation, as in selected cases of *Mtb* infection of the central nervous system.

Wettstein and Meagher were the first authors to describe thalidomide therapy in a woman with complicated CD. The patient was treated with all of the current medication at the time, including total parenteral nutrition, metronidazole and cyclosporine, without success. Resolution of the disease was achieved using 300 mg thalidomide per day and maintenance of 100 mg per day until the date of publication of the case[14]. Vasiliauskas *et al*[8] published an open-label trial that evaluated the tolerance and efficacy of low dose thalidomide for the treatment of moderate to severe steroid-dependent CD in 12 males with CDAI $\geqslant$ 250 and $\leqslant$ 500. The patients were treated with thalidomide 50 mg/d during the first 6 mo, increasing to 100 mg/d until the completion of 1 year of treatment. Disease activity (CDAI) improved consistently in all patients during the first 4 wk, with a response rate of 58% and a remission rate of 17%. After 12 mo, 70% of patients had responded, and 20% achieved remission. All patients were able to reduce steroid dosage to less than 50% of the initial dose; 48% of patients discontinued the use of steroids[11]. Our patient also used low doses of thalidomide and is still in remission after 2 years of therapy without adverse events, which demonstrates that low doses of thalidomide appear to be well tolerated and effective over a period exceeding 12 wk.

Ehrenpreis *et al*[7] reported an open-label trial of 22 patients with refractory CD (CDAI > 200 and/or perianal disease) treated with thalidomide 200 mg/d (18 patients) or 300 mg/d (4 patients). Nine patients with luminal disease and 13 with fistulas were included. Sixteen patients completed 4 wk of treatment (12 clinical responses, 4 remissions). Of the patients who completed 12 wk of treatment, 14 met the criteria for clinical response, nine achieved remission. Mean CDAI before and after treatment was 371 (95-468) and 175 (30-468), respectively ($P < 0.001$)[10]. Of note, none of these previous studies described the results of thalidomide usage after a long period of treatment. We describe here the case report of a 24-year-old male maintained on thalidomide therapy for more than 2 years without loss of response.

Bariol *et al*[15] evaluated the safety and efficacy of thalidomide for symptomatic inflammatory bowel disease. Eleven patients (6 with CD, 4 with UC and 1 with indeterminate colitis) were treated with thalidomide 100 mg/d for 12 wk. Two patients were withdrawn from therapy after three weeks because of mood disorders. Of the remaining 9 patients, 8 responded to treatment (5 DC, 2 UC and 1 IC). The frequency of stools fell from 4.3 to 2.3 per day ($P = 0.0012$); stool consistency improved from 2.1 to 1.2 ($P = 0.02$); and mean CDAI decreased from 117 to 48 ($P = 0.0008$). Endoscopic inflammation, histological inflammation, CRP and ESR decreased significantly ($P = 0.011$, $P = 0.03$, $P = 0.023$ and $P = 0.044$, respectively). However, levels of serum TNF-α did not change[15]. Thalidomide acts partly by accelerating the degradation of TNF-α mRNA, resulting in decreased production of this cytokine by activated mononuclear cells. It has also been shown to inhibit NF-κB activation, thus suppressing transcription of other important inflammatory cytokines, such as IL-12 both *in vitro* and *in vivo*[16,17]. Thalidomide increases expression of the Th2-type cytokine IL-4 and decreases the helper to suppress T-cell ratio, effects that are likely to contribute to its efficacy in CD[18]. Furthermore,

it has been shown to interfere with angiogenesis and to downregulate cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), thought to be important in lymphocyte homing mechanisms in inflammatory bowel disease[19]. These multiple alternative mechanisms of action may be involved in thalidomide's effectiveness in patients with severe disease refractory to conventional and biological agents, as was the case for the patient presented in this report.

Kane *et al*[20] reported a series of cases where thalidomide was a "salvage" therapy for patients with delayed hypersensitivity to infliximab. Four patients (two with active luminal disease and two with fistulous disease) were treated with thalidomide 200 mg/d and were evaluated monthly for 12 wk. One patient with a single perianal fistula had complete closure, and another had improvement in five perianal fistulas in 4 wk and closure in 12 wk. One patient had a CDAI decrease of 250 points in 4 wk. One patient was withdrawn from the study because of sedation after the first week of treatment. Reported side effects were sedation (4/4), hypertension (1/4) and peripheral neuropathy (1/4)[20]. Due to thalidomide's toxicity it is recommended only in selected cases of refractory CD, as in our patient, when the benefits outweigh the risk of peripheral neuropathy, and there is strict and full adherence to the program of birth control, avoiding the risk of teratogenicity.

To our knowledge, this is the first case report of a patient presenting with a pulmonary nodule that could be *M. tuberculosis* infection who was treated with thalidomide to control severe CD after loss of response to biological therapy, with a favorable evolution. Therefore, thalidomide might be considered a therapeutic option in similar clinical situations.

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AG490: An inhibitor of hepcidin expression *in vivo*

Shu-Ping Zhang, Zhe Wang, Li-Xin Wang, Si-Jin Liu

Shu-Ping Zhang, Zhe Wang, Li-Xin Wang, Si-Jin Liu, State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
Author contributions: Zhang SP, Wang Z and Wang LX performed experiments; Zhang SP and Liu SJ analyzed the data; Liu SJ designed the study and wrote the manuscript.
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Correspondence to: Si-Jin Liu, Professor, State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. sjliu@rcees.ac.cn
Telephone: +86-10-62849330 Fax: +86-10-62849330
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Abstract

A liver-produced hormone, hepcidin, appears to be the key player in iron metabolism. The overexpression of hepcidin is the underlying cause of anemia of inflammation. The identification of compounds inhibiting hepcidin expression could ameliorate anemia associated with inflammation. In the current study, we have demonstrated for the first time that AG490 significantly abolishes hepcidin expression in mice. Our work represents a novel approach to suppress hepcidin expression for treatment of anemia of inflammation and anemias occurring under other conditions.

Key words: AG490; Hepcidin; Anemia; Inflammation

Peer reviewer: Loes van Keimpema, MSc, PhD, Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB, Nijmegen, The Netherlands

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TO THE EDITOR

Hepcidin, mainly secreted by hepatocytes, is the hormone with a central role in regulating iron homeostasis (reviewed in[1]). Hepcidin suppresses iron absorption from the duodenum and iron egress from macrophages by promoting degradation of ferroportin protein (reviewed in[1]). Thus, the hepcidin-ferroportin interaction controls iron content in serum and iron distribution in tissues, and hepcidin level plays a primary role in regulating this interaction. Hepcidin level is predominantly modulated by erythropoietic activity, iron content, and inflammatory stimuli. Both acute and chronic inflammation states lead to anemia of inflammation (AI), which represents a prevalent type of anemia worldwide (reviewed in[2]). The overexpression of hepcidin is the underlying cause of AI. Therefore, identifying selective compounds inhibiting hepcidin expression could ameliorate anemia of inflammation or anemia associated with other chronic conditions (such as tumors).

A recent study has demonstrated that AG490 compound significantly reduced hepcidin expression *in vitro*[3]. This study provided evidence that AG490 suppressed hepcidin transcription by inhibiting the JAK/STAT signaling pathway in mouse hepatocytes[3]. AG490 is a tyrosine kinase inhibitor which has been extensively used for inhibiting JAK2/STAT3. However, no *in vivo* study has been performed to investigate the inhibitory effect of AG490 on hepcidin production. Here, we are the first to show that AG490 significantly inhibits hepcidin expression in mice.

AG490 (from Calbiochem), freshly prepared in 15% ethanol, was injected intraperitoneally into 6-mo-old female BALB/c mice. For an acute treatment, AG490 was given once (15 mg/kg), and mice were then sacrificed

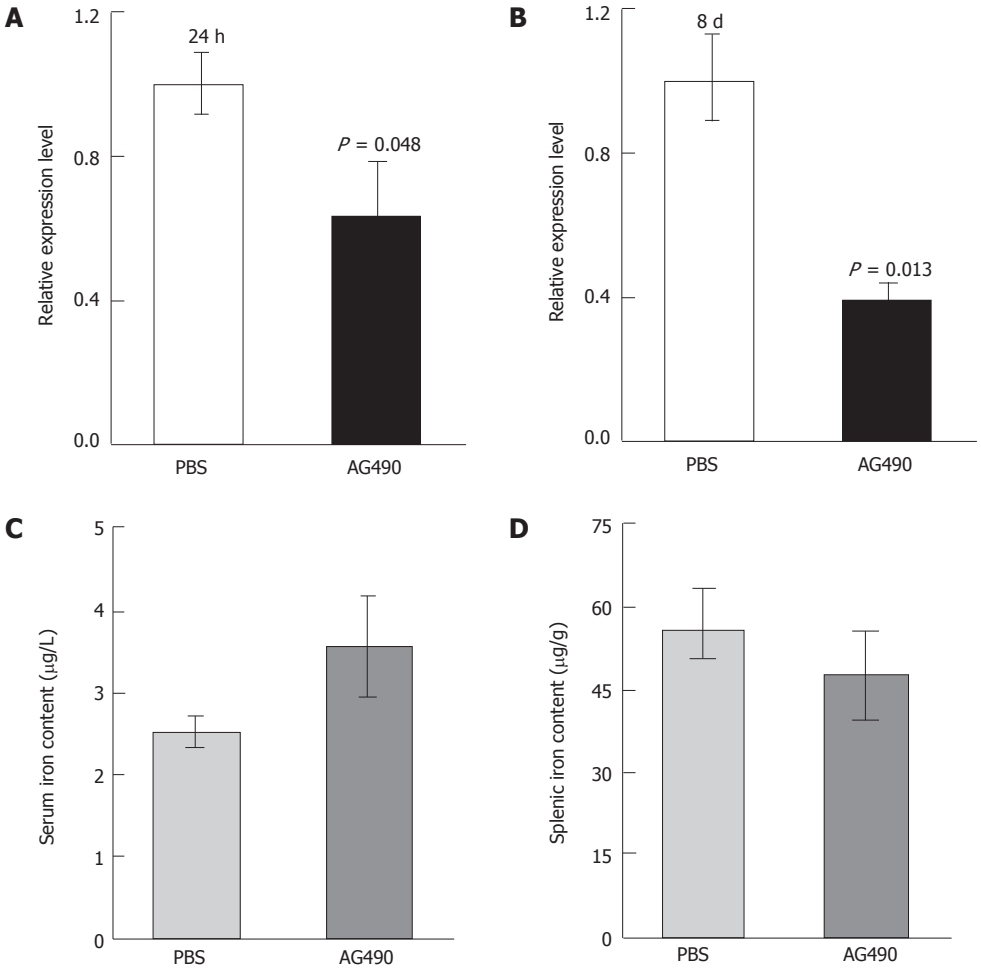

Figure 1 Reduced hepcidin expression upon AG490 treatment. The relative expression level of hepcidin was assessed by quantitative real-time polymerase chain reaction analysis and normalized with β-actin in liver samples from mice treated with AG490 after 24 h (A) and 8 d (B). Hepcidin expression in the phosphate buffer solution (PBS) control mice was defined as 1. Serum and spleen iron content is shown in (C) and (D), respectively, for mice undergoing treatment with AG490 or PBS for 8 d. Results are presented as mean ± SE (n = 9 for A, and n = 3-4 for B, C and D). The SPSS Statistics 17.0 software package was utilized to analyze the data. The difference between two groups was assessed using the independent t test, and $P < 0.05$ was considered statistically significant.

24 h later. For a chronic treatment, AG490 was administered every 4 d for a total of two times at the same dose, and mice were sacrificed on day 8. Control mice received an equal volume of phosphate buffer solution in 15% ethanol. At the end point of the experiments, 50 mg liver and spleen samples from each mouse were collected for tissue iron assay and another batch of 50 mg liver samples were saved for total RNA extraction. A sample of 100 μL serum for each mouse was used for serum iron examination. Iron and hepcidin quantitative real-time polymerase chain reaction assays were carried out as previously described[4,5].

Upon acute and chronic treatment with AG490, we did not observe any abnormality with regard to mouse diet or activities, and no toxicity to various organs was demonstrated through histological examination. After 24 h of treatment with AG490, hepcidin expression from hepatocytes was reduced by 37% compared to control mice ($P < 0.05$, Figure 1A); however, iron content in serum and spleen was not significantly altered (data not shown). Hepcidin expression was further downregulated after two injections over a period of 8 d: the relative expression level in the AG490-treated mice was reduced by 60% compared to control mice ($P < 0.05$, Figure 1B). As a result, serum iron was increased by about 40% in the AG490-treated mice compared to control mice (Figure 1C); there was a corresponding reduction for the splenic iron content in the AG490-treated mice compared to control mice (Figure 1D). These observations together suggested that AG490 efficiently attenuated hepcidin production from the liver to increase intestinal iron absorption and macrophagic iron egress.

Iron acquisition and distribution to tissues in mammals are strictly regulated in order to keep systemic iron homeostasis coordinated[6,7]. Iron level and its homeostasis are closely linked to inflammatory responses. Sequestration of iron presumably limits the uptake of iron by invading microbes and thus enhances resistance to infection; however, infection and inflammation increase hepcidin expression, which consequently leads to AI[8]. Thus, inhibitors such as AG490 might be beneficial to improve anemia caused by inflammation or other chronic diseases by reducing hepatic hepcidin production. Similar to our findings, a recent study indicated that heparin also

has a potent inhibitory effect on hepcidin expression *in vitro* and *in vivo*[9]. Additionally, AG490 or its variants (such as WP1066) have been documented to treat cancers by diminishing active JAK2 signaling[10-12].

To summarize, AG490 represents a prospective approach to attenuate hepcidin-mediated biological actions in order to enhance iron uptake through enterocytes and iron release from macrophages in anemias, and hepcidin repression induced by AG490 *in vivo* reveals a promising and potentially specific therapeutic means to suppress hepcidin expression in AI or other chronic conditions such as cancers.

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Jan Bures, MD, PhD, Professor, University Department of Gastroenterology, Charles University, Faculty of Medicine, University Teaching Hospital, Sokolska 581, 50005 Hradec Kralove, Czech Republic

Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, No. 301, Nok-Ji Building, Seoul 136-701, South Korea

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Marcela Kopacova, MD, PhD, Associate Professor, 2nd Department of Internal Medicine, Charles University Teaching Hospital, Sokolska 581, 500 05 Hradec Kralove, Czech Republic

Emiko Mizoguchi, MD, PhD, Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

Dr. Luca Morelli, MD, UO, Anatomy and Histology, Ospedale S. Chiara, Largo Medaglie d'Oro 9, Trento 38100, Italy

Mercedes Susan Mandell, MD, PhD, Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

Sabine Mihm, Professor, Department of Gastroenterology, Georg-August-University, Robert-Koch-Str.40, Göttingen D-37099, Germany

Patrick O'Dwyer, MB, BCh, BAO, FRCS, MCh, FRCS (Glasg), University Department of Surgery, Western Infirmary, Glasgow, G11 6NT, United Kingdom

Dr. Richard Parker, MB, ChB, MRCP (London), Department of Gastroenterology, University Hospital of North Staffordshire, Newcastle Road, Stoke on Trent, North Staffordshire, ST4 6QG, United Kingdom

Dr. John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent, ME20 7NH, United Kingdom

Dr. Marco Scarpa, PhD, Gastroenterology Section, Department of Surgical and Gastro-enterological Sciences, University of Padova, via Giustiniani 2, Padova 35128, Italy

Yukihiro Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan

Dr. Hsu-Heng Yen, MD, Departmenet of Gastroenterology, Changhua Christian Hospital, 135 Nanhsiao Street, Changhua 500, Taiwan, China

Dr. Pingchang Yang, MD, PhD, Department of Pahtology and Molecular Medicine, McMaster University, BBI-T3330, 50 Charlton Ave East, Hamilton, L8N 4A6, Canada

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AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

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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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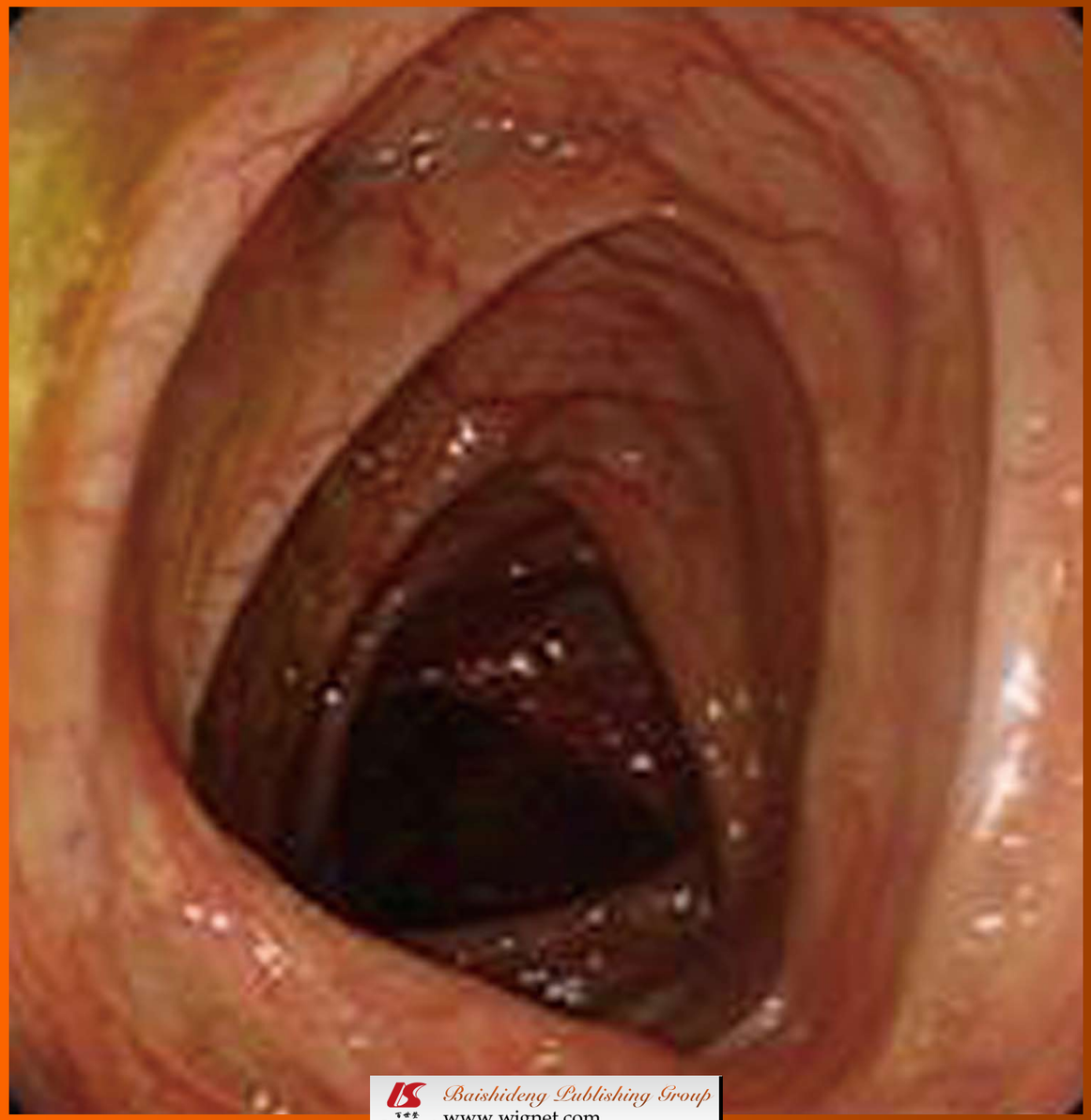

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EDITORIAL

Neurogenic bowel dysfunction in patients with spinal cord injury, myelomeningocele, multiple sclerosis and Parkinson's disease

Richard A Awad

Richard A Awad, Experimental Medicine and Motility Unit, Gastroenterology Service U-107, Mexico City General Hospital, 06726 México, DF, México
Author contributions: Awad RA solely contributed to this paper.
Correspondence to: Richard A Awad, MD, MSc, Head and Professor, Experimental Medicine and Motility Unit, Gastroenterology Service U-107, Mexico City General Hospital, Dr. Balmis No. 148, Col. Doctores, 06726 México, DF, México. awadrichardalexander@prodigy.net.mx
Telephone: +52-55-50043806 Fax: +52-55-50043806
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Abstract

Exciting new features have been described concerning neurogenic bowel dysfunction, including interactions between the central nervous system, the enteric nervous system, axonal injury, neuronal loss, neurotransmission of noxious and non-noxious stimuli, and the fields of gastroenterology and neurology. Patients with spinal cord injury, myelomeningocele, multiple sclerosis and Parkinson's disease present with serious upper and lower bowel dysfunctions characterized by constipation, incontinence, gastrointestinal motor dysfunction and altered visceral sensitivity. Spinal cord injury is associated with severe autonomic dysfunction, and bowel dysfunction is a major physical and psychological burden for these patients. An adult myelomeningocele patient commonly has multiple problems reflecting the multisystemic nature of the disease. Multiple sclerosis is a neurodegenerative disorder in which axonal injury, neuronal loss, and atrophy of the central nervous system can lead to permanent neurological damage and clinical disability. Parkinson's disease is a multisystem disorder involving dopaminergic, noradrenergic, serotoninergic and cholinergic systems, characterized by motor and non-motor symptoms. Parkinson's disease affects several neuronal structures outside the substantia nigra, among which is the enteric nervous system. Recent reports have shown that the lesions in the enteric nervous system occur in very early stages of the disease, even before the involvement of the central nervous system. This has led to the postulation that the enteric nervous system could be critical in the pathophysiology of Parkinson's disease, as it could represent the point of entry for a putative environmental factor to initiate the pathological process. This review covers the data related to the etiology, epidemiology, clinical expression, pathophysiology, genetic aspects, gastrointestinal motor dysfunction, visceral sensitivity, management, prevention and prognosis of neurogenic bowel dysfunction patients with these neurological diseases. Embryological, morphological and experimental studies on animal models and humans are also taken into account.

Key words: Neurogenic bowel dysfunction; Spinal cord injury; Myelomeningocele; Multiple sclerosis; Parkinson's disease; Central nervous system; Enteric nervous system

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INTRODUCTION

Exciting new features have been described concerning neurogenic bowel dysfunctions (NBD), including interactions between the central nervous system (CNS), enteric nervous system (ENS), neurotransmission of noxious and non-noxious stimuli, and the fields of gastroenterology and neurology. Patients with spinal cord injury (SCI), myelomeningocele (MMC), multiple sclerosis (MS) and Parkinson's disease (PD) present with autonomic dysreflexia[1], serious upper and lower NBD characterized by constipation[2], incontinence, severe gastrointestinal (GI) motor dysfunction[3] and altered visceral sensitivity[4]. SCI is associated with severe autonomic dysfunction, with bowel dysfunction as a major physical and psychological burden for these patients[5]. The outcome of MMC patients is fraught with multiple problems reflecting the multisystemic nature of the disease[6]. MS is a devastating autoimmune disease[7] with symptoms dependent on the clinical type and the site of lesions[8]. It has been considered a chronic, inflammatory disorder of the central white matter in which demyelination results in the ensuing physical disability. Recently, MS is viewed as a neurodegenerative disorder in which axonal injury, neuronal loss, and atrophy of the CNS can lead to permanent neurological and clinical disability, in which mitochondrial DNA defects are involved[9]. PD is considered as a disorder involving dopaminergic, noradrenergic, serotoninergic, and cholinergic systems, characterized by motor and non-motor symptoms[10]. Interestingly, in recent years it has become evident that PD affects several neuronal structures outside the substantia nigra, between which are the ENS. Recent reports have shown that the lesions in the ENS occur at a very early stage of the disease, even before the involvement of the CNS. This has led to the hypothesis that the ENS could be critical in the pathophysiology of PD, as it could represent a point of entry for a putative environmental factor to initiate the pathological process[11]. This review covers the data related to etiology, epidemiology, clinical aspects, pathophysiology, genetics, gastrointestinal motor dysfunction, visceral sensitivity, management, prevention, and prognosis of NBD patients with these neurological diseases. Embryologic, morphological and experimental studies on animal models and humans are also taken into account.

LITERATURE REVIEW

Search strategy

A Medline search was performed using the following subject headings: spinal cord injury, neural tube defects (NTD), myelomeningocele, multiple sclerosis, Parkinson's disease, animal models, and human. The date of the most recent search was February 28, 2011.

Selection criteria

Clinical, epidemiological, pathophysiological, motor dysfunction, visceral sensitivity and experimental studies on animal models and patients with SCI, MMC, MS, PD, as well as specific therapies for these neurological diseases involving bowel dysfunction were reviewed. Issues related to genetics, embryology, morphology, prevention and prognosis were also taken into account.

Data collection and analysis

A total of 177 articles were included in the analysis.

ETIOLOGY

SCI etiology is generally divided into traumatic and non-traumatic causes[12].

The onset of NTD occurs at 21-28 d of embryonic development[13]. MMC results from lack of closure of the neural tube during this stage[14]. Its etiology is complex, involving both genetic and environmental factors[15]. A maternal effect as well as a gender-influenced effect, have been suggested as part of its etiology[16]. Although there are more than 200 small animal models with NTD, most of them do not replicate the human disease phenotype. The candidate genes studied for risk association with spina bifida include those important in folic acid metabolism, glucose metabolism, retinoid metabolism, apoptosis, and those that regulate transcription in early embryogenesis[17].

MS is an etiologically unknown disease with no cure[7]. It is the leading cause of neurological disability in young adults, affecting over two million people worldwide. MS has been considered a chronic, inflammatory disorder of the CNS white matter in which demyelination results in the ensuing physical disability. Recently, MS has become increasingly viewed as a neurodegenerative disorder in which axonal injury, neuronal loss, and atrophy of the CNS can lead to permanent neurological damage and clinical disability[9].

GI dysmotility in PD has been attributed to the peripheral neurotoxin action[18]. Recently, it has been suggested that sporadic PD has a long prodromal period and several nonmotor features develop during this period. Hawkes *et al*[19] proposed that a neurotropic viral pathogen may enter the brain *via* nasal route with anterograde progression into the temporal lobe or *via* gastric route, secondary to the swallowing of nasal secretions. These might contain the neurotropic pathogen that, after penetration of the epithelial lining, could enter the axons of the Meissner plexus and, through transsynaptic transmission, reach the preganglionic parasympathetic motor neurons of the vagus nerve. This would allow retrograde transport into the medulla and from there into the pons and midbrain until the substantia nigra is reached[19]. A summary of suggested pathogenesis of GI disorders underlying PD is shown in Table 1.

EPIDEMIOLOGY

Traumatic SCI represents a significant public health problem

Table 1 Suggested pathogenesis of gastrointestinal disorders underlying Parkinson's disease

GI pathogenesis	Disorder
Peripheral neurotoxine action	Interstitial cells of Cajal involvement[18]
GI flora? Neurotropic viral pathogen	GI disorders[19]
GI flora? *Helicobacter pylori*	Modified l-dopa pharmacokinetics[102]
GI dysmotility: Early lesions in the enteric nervous system	GI dysfunction[11,163]
GI dysmotility: Disruption in parts of the CNS	Neurogenic dysphagia[54]
GI dysmotility: Lewy bodies in esophageal myenteric plexuses	Manometric abnormalities[97,98]
GI dysmotility: Reduction amplitude of peristaltic contractions	Decreased gastric motility[105]
GI dysmotility: Gastric pacemaker disturbances	Gastric dysrhythmias[106]
GI dysmotility: Loss of enteric dopaminergic neurons	Changes in colon motility[173]
Neurotransmitter dysfunction: Altered enteric nitrergic systems	Disturbed distal gut transit[95]
Neurohormone involvement: Neurotensin	GI disorders[103]
Levodopa	Altered oral phase of deglutition[96]
Monoamine dysfunction	Nonmotor symptoms[176]

GI: Gastrointestinal; CNS: Central nervous system.

worldwide[20]. Each year, 11 000 individuals are estimated to have SCI in the United States[21] with a mortality rate of 27.4 per million people. An annual incidence of 33.6 per million is reported in Greece and 19.5 per million in Sweden[22], while in Denmark the number of SCI patients is about 3000.

NTD is the second most common birth defect, with an incidence of 1/1000. MMC is the most common subtype (66.9%)[16]. NTD is rarely reported in black Americans and Japanese, but is not so rare in Cameroon and sub-Saharan black Africans, with an incidence of 1.9 cases per 1000 births[23]. In Switzerland, the incidence of NTD in children is 0.13 per thousand, corresponding to 9-10 affected newborns each year[15], while in Thailand, the incidence is 0.67 per 1000 births[24]. NTD is reported in adolescents aged 15-18 years[25] and in young adults aged 20-23 years[26].

MS affects young and middle-aged people[27], the mean age at disease onset is 30.7 ± 6.4 years, and it is believed that pregnancy, postpartum status and vaccines[8], as well as infection with Epstein-Barr virus[28], may influence the onset and course of the disease. An increase in females and an almost universal increase in the prevalence and incidence have been reported, challenging the theory of a geographical gradient of incidence in Europe and North America[29]. It affects 100 000 people in the United Kingdom[30], with a prevalence of 30.9/100 000 in Herzegovina[31]. An association between the risk of MS and the season of birth suggested that decreased exposure to the sunshine in the winter leading to low vitamin D levels during pregnancy is an area that needs further research[32].

PD is the second most common neurodegenerative disease after Alzheimer's disease[11], affecting one million people in the United States each year[33], and 20% of the population aged > 65 years in Mexico[34]. It is described in sporadic and familial forms[35] (at least 2 individuals are affected within 2-3 consecutive generations of a family).

DIAGNOSIS, CLINICAL DATA AND SYMPTOMS

Neurophysiologic testing of the sacral reflex is useful in the diagnosis of sacral lower motor neuron lesions, and increased elicitability of the penilo-cavernosus reflex is reported in patients with chronic SCI[36]. Patients with SCI may present[4] with brain anatomical changes of loss of motor control, chronic neuropathic[37] and abdominal pain[38], urinary[39]and sexual dysfunction[40], decubiti[41], neurogenic immune depression syndrome[42], and an increased risk of having a depressive disorder[43]. Spinal cord lesions affect colorectal motility, anorectal sensation, anal sphincter function, and cause neurogenic constipation[44]. Defecation is abnormal in 68% of cases, digital stimulation is required by 20%, suppositories by 10% and enemas by 28% of cases. Time spent in each defecation is more than 30 min in 24% cases. In children aged four years or older, daily fecal incontinence occurred in 14% and weekly incontinence in 14% cases[45]. SCI patients usually do not perceive the normal desire for defecation, rather describing it as abdominal distension, hardened or cool abdomen, hardening of the legs, abdominal pain, chills and dizziness, itching of the head, and a feeling of pain at the sacrum level[4]. Additionally, SCI subjects may develop autonomic dysreflexia in response to noxious stimulus[46]. Cardiovascular dysregulation, characterized by paroxysmal high blood pressure episodes, is the most prominent feature and is precipitated by manual emptying of rectal contents and by gastric and bowel distension[47]. Regarding the gravity of this issue, an NBD score (0-6 very minor, 7-9 minor, 10-13 moderate and 14 severe)[48], an international bowel function basic[49] and extended[50] SCI data set, as well as an international standard to document the remaining autonomic function after SCI[40] have been developed.

Prenatal screening with α-fetoprotein and ultrasonography have allowed the prenatal diagnosis of NTD in current obstetric care[51]. In an animal model with naturally occurring spina bifida (curly tail/loop tail mouse), using standard enzyme linked immunosorbent assay techniques, detection of amniotic fluid levels of the neurofilament heavy chain, glial acidic fibrillary protein and S100B, seems to provide important information for balancing the risks and benefits, both to mother and child, of in utero

surgery for MMC[52]. Colorectal problems are common in children with MMC and their impact on the quality of life becomes more severe as the child grows up.

Diagnosis of MS is made according to the McDonald and the Poser criteria, with the McDonald criteria showing a higher sensitivity for diagnosis[53]. Bowel symptoms are reported to be common in MS, including constipation (29%-43%) and fecal incontinence (over 50%), and 34% of patients spending more than 30 min a day managing their bowel movement[30]. Neurogenic dysphagia is also present[54]. Autonomic dysreflexia may occur in MS[55], characterized by hypertensive attacks, palpitations, difficulty in breathing, headaches and flushing[56]. Autonomic symptoms are disorders of micturition, impotence, sudomotor and GI disturbances, orthostatic intolerance as well as sleep disorders[57]. Neuropsychiatric symptoms include abnormalities in cognition, mood and behavior (major depression, fatigue, bipolar disorder, euphoria, pathological laughing and crying, anxiety, psychosis and personality changes). Major depression is a common neuropsychiatric disorder, with an approximate 50% lifetime prevalence rate[58]. Pediatric MS has been identified as an important childhood acquired neurologic disease[59].

GI diagnosis in PD[60] includes history, clinical examination, barium meal, breath test, stomach scintigraphy and colonic transit time[61]. Oropharyngeal dysphagia is recognized by difficulty in transferring a food bolus from the mouth to the esophagus or by signs and symptoms of aspiration pneumonia or nasal regurgitation[62]. PD is actually considered a neurodegenerative process that affects several neuronal structures outside the substantia nigra. Reports have shown that the lesions in the ENS occurred at a very early stage of the disease, even before CNS involvement[11]. GI symptoms are very important, as GI diseases may also display neurological dysfunction as part of their clinical picture[63]. PD patients have motor and non-motor fluctuations classified into three groups: autonomic, psychiatric, and sensory[64]. GI dysfunction is the most common non-motor symptom which comprises sialorrhea, swallowing disorders[65], dysphagia[66], acid regurgitation, pyrosis[67], early satiety, weight loss, constipation[68], incomplete rectal emptying, the need for assisted defecation and an increased need for oral laxatives[69].

PATHOPHYSIOLOGY

Genetic factors

Data was obtained from 1066 NTD families, 66.9% with MMC, suggesting a maternal effect, as well as a gender-influenced effect in the etiology of NTD[16]. Telomerase, the reverse transcriptase that maintains telomere DNA, is important for neural tube development and bilateral symmetry of the brain. However, it is reported that variants in the telomerase RNA component (TERC) are unlikely to be a major risk factor for the most common form of human NTD, lumbosacral MMC[70].

The association between a polymorphism in the *ABCB1* gene and PD has been observed. The ATP-binding cassette, sub-family B, member 1 (*ABCB1*) gene encoding P-glycoprotein (P-gp), has been implicated in the pathophysiology of PD due to its role in regulating the transport of endogenous molecules and exogenous toxins. ABCB1 polymorphisms thus constitute an example of how genetic predisposition and environmental influences may combine to increase the risk of PD[71]. On the other hand, extensive ENS abnormalities in mice transgenic for PD-associated α-synuclein gene mutations precede CNS changes. Most PD is sporadic and of unknown etiology, but a fraction is familial. Among familial forms of PD, a small portion is caused by missense (A53T, A30P and E46K) and copy number mutations in SNCA, which encodes α-synuclein, a primary protein constituent of Lewy bodies, the pathognomonic protein aggregates found in neurons in PD[72].

Gastrointestinal motor dysfunction and visceral sensitivity

Fecal incontinence in SCI, MMC and MS is mainly due to abnormal rectosigmoid compliance and recto-anal reflexes, loss of recto-anal sensitivity and loss of voluntary control of the external anal sphincter[73]. On the other hand, constipation is probably due to immobilization, abnormal colonic contractility, tone and recto-anal reflexes, or side effects from medication. SCI patients have a higher incidence of esophagitis and esophageal motor abnormalities[74], gastric stasis, paralytic ileus, abdominal distension[75], partial or complete loss of the sensations upon defecation, constipation[75], hemorrhoids[76], and need for assisted digital evacuation than controls[75]. Studies have shown a range of neurological alterations, such as low amplitude, slowly propagating abnormal peristaltic esophageal contraction[74], a decrease in phase Ⅲ of the interdigestive motor complex[77], reduction in gastric emptying[78], delayed GI transit, higher colonic myoelectric activity, reduced emptying of the left colon, and a suboptimal postprandial colonic response[79]. Visceral sensitivity testing according to Wietek *et al*[80] may be a future requirement, in addition to the American Spinal Injury Association (ASIA) criteria, in the assessment of the completeness of cord lesions in patients diagnosed with complete spinal cord transection, as some report the sensation of distension of the rectum. In our laboratory, with barostat methodology, we found that complete supraconal SCI patients preserve rectal sensation, and present with impaired rectal tone and impaired response to food. This data supports the fact that barostat sensitivity studies can complement ASIA criteria to confirm a complete injury. Our results also suggest that intact neural transmission between the spinal cord and higher centers is essential for noxious stimulus, but not for non-noxious stimuli, that patients with supraconal lesions may present PP visceral hypersensitivity, and that incontinence and constipation may not be related solely to continuity of the spinal cord[4,81]. Suttor *et al*[82], using

Table 2 Suggested pathogenesis of gastrointestinal disorders underlying spinal cord injury, myelomeningocele and multiple sclerosis

Disease	GI pathogenesis	Disorder
Spinal cord injury Myelomeningocele Multiple sclerosis	Abnormal rectosigmoid compliance Loss of recto-anal sensitivity Loss of voluntary control of the external anal sphincter	Fecal incontinence[73]
Spinal cord injury Myelomeningocele Multiple sclerosis	Immobilization, abnormal colonic contractility, side effects of medication	Constipation[94]
Multiple sclerosis	Paradoxical puborectalis contraction	Constipation[94]
Multiple sclerosis	Bladder distension	Autonomic dysreflexia[56]
Myelomeningocele	Severe constipation	Ventriculoperitoneal shunt malfunction[87]
Myelomeningocele	Visceral hypersensitivity	Constipation and impaired rectal tone and response to food[88]
Myelomeningocele	Higher spinal level of cord lesion, completeness of cord injury and longer duration of injury	Severe neurogenic bowel dysfunction[20]
Spinal cord injury	Noxious stimulus	Autonomic dysreflexia[46]
Spinal cord injury	Manual emptying of rectal contents and gastric and bowel distension	Cardiovascular dysregulation[47]

a dual barostat in six cervical SCI patients without NBD, reported that intact neural transmission between the spinal cord and higher centres is not essential for normal colorectal motor response from feeding to distension. Lumbosacral neuropathy was demonstrated in 90% of SCI subjects[83] using translumbar and trans-sacral motor-evoked potentials.

In MMC, studies have revealed swallowing disorders characterized by difficulty in bolus formation, nasopharyngeal and gastroesophageal reflux, tracheobronchial aspiration, and vocal cord paralysis[84], as well as a longer mean colonic transit time not related to the level of the spinal lesion[85] and reduction in anal sphincter pressure[86]. Ventriculoperitoneal shunt malfunction may occur in patients with MMC, and severe constipation that increases intra-abdominal pressure resulting in raised intracranial pressure, seems to be one of the causes[87]. Visceral sensitivity studies with the barostat reveal that constipated children with MMC present with impaired rectal tone, impaired response to food and postprandial visceral hypersensitivity[88].

GI dysfunction occurs in MS as in other neurologic diseases[63]. Slow gastric emptying rate[89], increased colonic transit time[90], absent PP colonic motor and myoelectric responses[91], altered maximal contraction pressures and anal inhibitory reflex threshold[92], impaired function of the external anal sphincter, and increased thresholds of conscious rectal sensation[93] have been reported. Paradoxical puborectalis contraction is common in MS patients with constipation[94] and it seems that autonomic dysreflexia occurs due to bladder distension[56]. A summary of suggested pathogenesis of GI disorders underlying spinal cord injury, myelomeningocele, and multiple sclerosis is shown in Table 2.

In PD, dysphagia, impaired gastric emptying and constipation may precede its clinical diagnosis for years[61]. ENS involvement could be critical as it may represent a point of entry for a putative environmental factor to initiate the pathological process[11]. On the other hand, the mechanisms related to enteric autonomic dysfunctions may involve the enteric dopaminergic or nitrergic systems. It has been reported that rats with a unilateral 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons develop marked inhibition of propulsive activity compared with sham-operated controls. Results suggest that disturbed distal intestinal transit may occur as a consequence of reduced propulsive motility, probably due to an impairment of a nitric oxide-mediated descending inhibition during peristalsis[95]. Neurogenic dysphagia may also appear in PD. It may be caused by a disruption in different parts of the CNS (supranuclear level, level of motor and sensory nuclei taking part in the swallowing process and peripheral nerve level) or a neuromuscular disorder[54]. It is also suggested that levodopa plays a role in the oral phase of deglution in PD[96]. Dysphagia is present in up to 50% of PD cases and seems to be correlated with manometric irregularities[97,98]. Castell *et al*[97] have described esophageal manometric abnormalities in 73% of PD patients characterized by complete aperistalsis or multiple simultaneous contractions (diffuse esophageal spasm) of the distal esophagus. They also reported repetitive proximal esophageal contractions[99], a very interesting finding supporting a previous report of a link between PD, achalasia[100], and scleroderma (e.g., PD and achalasia have Lewy bodies in the esophageal myenteric plexuses and the substantia nigra, as well as evidence of degeneration of the dorsal motor nucleus of the vagus), and esophageal manometric abnormalities were found in these three diseases. A link between PD and *Helicobacter pylori* (*H. pylori*)[101] has also been described, where HP eradication may improve the clinical status of infected patients with PD and motor fluctuations by modifying l-dopa pharmacokinetics[102]. Neurotensin, a 13 amino acid neurohormone located in the synaptic vesicles and released from the neuronal terminals in a calcium-dependent manner, is involved in the pathophysiology of PD and other neurodegenerative conditions[103]. Constipation and gastric atony are important

non-motor symptoms[104]. There is a trend toward a decreased gastric motility in PD patients as compared with healthy controls due mainly to a significant reduction in the amplitude of peristaltic contractions[105]; other authors have found gastric dysrhythmias indicating gastric pacemaker disturbances[106]. Slow transit in the colon has been reported[107], and using ano-rectal manometry, decreased basal anal sphincter pressures, prominent phasic fluctuations on squeeze pressure, and a hyper-contractile external sphincter response to the rectosphincteric reflex have been documented. It has also been suggested that dystonia of the external anal sphincter causes difficult rectal evacuation and the loss of dopaminergic neurons in the ENS may lead to slow-transit constipation[73].

MANAGEMENT

Managing SCI bowel function is complex, time consuming and remains conservative[75]. The use of manual evacuation[108], treatment with oral laxatives[108] and abdominal massage[109] have all been reported. Transanal irrigation is reported safe and can be used in most patients suffering from NBD[110], its results represent a lower total cost than conservative bowel management[111]; however, its rate of success is only 35% after 3 years[110]. Recent approaches include sacral neuromodulation[112] and dorsal penile/clitoral nerve neuromodulation for the treatment of constipation, as well as magnetic stimulation for NBD treatment[113]. Other options include colostomy, ileostomy, malone anterograde continence enema, and sacral anterior root stimulator implantation[114]. However, good quality research data is needed to evaluate the effects of these treatments for this condition.

For MMC patients with constipation, polyethylene glycol[44,115] and the use of transanal irrigation[116] seem to be effective, however, a majority of children found the procedure time consuming and did not help them to achieve independence at the toilet[117]. For incontinence, the approaches included intravesical[118] and transrectal electro-stimulation[119]; nevertheless these procedures lack well-designed controlled trials. For constipation and incontinence, biofeedback is used[120]. Surgical closure of MMC is usually performed in the early postnatal period, however, not all patients benefit from fetal surgery in the same way[121]. The management of cervical MMC is early surgical treatment with microneurosurgical techniques. Surgical excision of the lesions with intradural exploration of the sac to release any potential adhesion bands is safe and effective[122].

The current therapies for MS are few, symptom-related, and experimental[7]. In patients seen due to constipation, incontinence, or a combination of these symptoms a beneficial effect of biofeedback was attributed to some but not to all patients[123]. Other approaches include oral administration of probiotic bacteria, *Lactobacillus casei* and *Bifidobacterium breve*, which do not seem to exacerbate neurological symptoms[124]. An overactive bladder is successfully treated in 51% of cases with anticholinergic medication[125]. The use of agonists or antagonists of prostaglandin-receptors may be considered as a new therapeutic protocol in MS. The reason is that prostaglandins as arachidonic acid-derived autacoids play a role in the modulation of many physiological systems including the CNS, and its production is associated with inflammation, which is a feature in MS[126].

Levodopa, a prodrug of dopamine, is one of the main treatment options in PD[127]. However, in contrast to motor disorders, pelvic autonomic dysfunction is often refractory to levodopa treatment[128]. One point to bear in mind is that treatments should facilitate intestinal absorption of levodopa[128]. Current levodopa products are formulated with aromatic amino acid decarboxylase inhibitors such as carbidopa or benserazide to prevent the metabolism of levodopa in the GI tract and systemic circulation[127]. Food appears to affect the absorption of levodopa, but its effects vary with formulations and studies suggest that a high protein diet may compete with the uptake of levodopa into the brain, thus resulting in reduced levodopa effects[127]. Regarding disturbed motility of the upper GI-tract, hypersalivation is reported to be reduced by anticholinergics or botulinum toxin injections[61] while therapy for dysphagia includes rehabilitative, surgical, and pharmacologic treatments[129]. Regarding constipation, tegaserod improves both bowel movement frequency and stool consistency[130]. Mosapride citrate, a 5-HT4 agonist and partial 5-HT3 antagonist, in contrast to cisapride, does not block K (+) channels or D2 dopaminergic receptors[131]. Other prokinetics agents include metoclopramide, domperidone, trimebutine, cisapride, prucalopride, and itopride[132]. Polyethylene glycol[61], functional magnetic stimulation[133], and psyllium are also used[134]. However, the clinical significance of any of these results is difficult to interpret and it is not possible to draw any recommendation for bowel care from published trials, until well-designed controlled trials with adequate numbers of patients and clinically relevant outcome measures become available[134]. Recently, stem cells have been used as an alternate source of biological material for neural transplantation to treat PD. The potential benefits for this are relief of parkinsonian symptoms and a reduction in the doses of parkinsonian drugs employed. However, the potential risks include tumor formation, inappropriate stem cell migration, immune rejection of transplanted stem cells, hemorrhage during neurosurgery and postoperative infection[135].

PREVENTION AND PREDICTORS

An analysis of predictors of severe NBD in SCI shows that those with a cervical injury or a thoracic injury had a higher risk of severe NBD than those with a lumbar spine injury. Also those classified as ASIA a had a 12.8-fold higher risk of severe NBD than persons with ASIA D. Besides, a longer duration of injury (≥ 10 years) was considered as another risk factor of severe NBD. Moderate-to-severe depression was associated with reduced

bowel function. The results showed that a higher spinal level of cord lesion, completeness of cord injury and a longer duration of injury (≥ 10 years) could predict the severity of NBD in patients with SCI[20]. It is reported that clinical variables are not the best predictors of long-term mortality in SCI. Instead, the significant effect of poor social participation and functional limitations seem to persist after adjustment for other variables[136].

Folic acid supplementation reduced the incidence of NTD in several geographical regions. However, the incidence is still high and associated with a serious morbidity[137]. A study done in newborn babies with NTD and their mothers revealed an association between NTD and decreased hair zinc levels, so large population-based studies are recommended to confirm the association between zinc and NTD[138]. The prevalence of scoliosis in patients with MMC has been reported to be as high as 80%-90%. A study aiming to determine clinical and radiographic predictors of scoliosis in patients with MMC reported that the clinical motor level, ambulatory status, and the level of the last intact laminar arch are predictive factors for the development of scoliosis. It is suggested that in patients with MMC, the term scoliosis should be reserved for curves of > 20 degrees, it is also noteworthy that new curves may continue to develop until the age of fifteen years[139]. Other authors attempting to obtain a spine deformity predictor based on a neurological classification performed at five years of age report that group I (L5 or below) is a predictor for the absence of spinal deformity, group Ⅲ (L1-L2) or Ⅳ (T12 and above) is a predictor for spinal deformity and group Ⅳ is a predictor of kyphosis. This data confirms that future spinal disorders are expected in some patients, while no spinal deformity is expected in others[140]. Other reports indicate that the horizontal sacrum is an indicator of the tethered spinal cord in spina bifida aperta and occulta, as signs and symptoms indicative of a tethered spinal cord appear to correspond to increases in the lumbosacral angle[141]. It is also reported that behavior regulation problems in children with MMC are predicted by parent psychological distress, and that more shunt-related surgeries and a history of seizures predict poorer metacognitive abilities[142]. It seems that adults with MMC and shunted hydrocephalus may be at risk for decreased survival[143].

Inadequate serum vitamin D concentrations are associated with complications of some health problems including MS, which support a possible role for vitamin D supplementation as an adjuvant therapy[144]. In addition, it has been suggested that the favorable effect of sunlight ascribed to an increased synthesis of vitamin D may prevent certain autoimmune diseases, particularly MS. For this reason, limited sunbathing should be publically encouraged[145]. It has also been suggested that altering the composition of the gut flora may affect susceptibility to experimental autoimmune encephalomyelitis, an animal model of MS[146]. This data could have significant implications for the prevention and treatment of autoimmune diseases. In relation to this, an interesting new proposal shows that the GI tract is a vulnerable area through which pathogens (such as *H. pylori*) may influence the brain and induce MS, mainly *via* fast axonal transport by the afferent neurons connecting the GI tract to brain[147].

Symptoms such as dysphagia, impaired gastric emptying and constipation may precede the clinical diagnosis of PD by years and, in the future, these symptoms might serve as useful early indicators of the premotor stage[61]. Motor handicaps, such as rigor and action tremor, are independent predictors of solid gastric emptying[148]. It is currently recommended that the approach to PD should include strategies for detecting the disease earlier in its course and, eventually, intervening when the disease is in its nascent stage. The term Parkinson's associated risk syndrome has been coined to describe patients at risk for developing PD. These patients may have genetic risk factors or may have subtle, early non-motor symptoms including abnormalities in olfaction, GI function, cardiac imaging, vision, behavior, and cognition[149].

EMBRYOLOGICAL, MORPHOLOGICAL AND EXPERIMENTAL STUDIES AND ANIMAL MODELS

Embryology and morphology

Considerable insight into both normal neural tube closure and the factors possibly disrupting this process has been reported in recent years, yet, the mechanisms by which NTD arises as well as its embryogenesis remain elusive[150]. Normal brain development throughout childhood and adolescence is characterized by decreased cortical thickness in the frontal regions and region-specific patterns of increased white matter myelination and volume. Subjects with MMC show reduced white matter and increased neocortical thickness in the frontal regions, suggesting that spina bifida may reflect a long-term disruption of brain development that extends far beyond the NTD in the first week of gestation[151]. These variations in the diffusion metrics in MMC children are suggestive of abnormal white matter development and persistent degeneration with advancing age[152].

In rat fetuses with retinoic acid induced MMC, the normal smooth muscle and myenteric plexus development of the rectum and normal innervations of the anal sphincters and pelvic floor suggest that MMC is not associated with a global neuromuscular alteration in development of lower GI structures[153]. Besides, fetal surgery for repair of MMC allows normal development of anal sphincter muscles in sheep. Histopathologically, in the external sphincter muscles, the muscle fibers were dense, while in the internal sphincter muscles, endomysial spaces were small, myofibrils were numerous, and fascicular units were larger than those in unrepaired fetal sheep[154]. Studies in the development of the pelvic floor muscles of murine embryos with anorectal malformations, demonstrate that the embryos show an impaired

anatomic framework of the pelvis possibly caused by neural anomalous development, whereas muscle development proceeded physiologically. These results support the hypothesis that pelvic floor muscles may function in children with anorectal malformations, in whom neural abnormalities such as MMC have been ruled out, if the surgical correction is appropriately completed[155]. A mouse model was reported about the sharing of the same embryogenic pathway in anorectal malformations and anterior sacral MMC formation[156]. Indeed, some of the brain malformations associated with MMC in human patients are also found in the uncorrected fetal lamb model of MMC[157]. The late stage of gestation is important due to the presence of morphological changes. A study of in-utero topographic analysis of astrocytes and neuronal cells in the spinal cord of mutant mice with MMC revealed that at day 16.5 of gestation there is a deterioration of neural tissue in MMC fetuses, mainly in the posterior region, progressing until the end of gestation with a marked loss of neurons in the entire MMC placode. This study delineated the quantitative changes in astrocytes and neurons associated with MMC development during the late stages of gestation[158]. Data supported by other investigators show, in Curly tail/loop tail mouse fetuses, that around birth the unprotected neural tissue is progressively destroyed[159].

Traditionally, PD is attributed to the loss of mesencephalic dopamine-containing neurons; nonetheless, additional nuclei, such as the dorsal motor nucleus of the vagus nerve and specific central noradrenergic nuclei, are now identified as targets of PD[160]. Early in 1988, Wakabayashi[161] described the presence of Lewy bodies in Auerbach's and Meissner's plexuses of the lower esophagus, indicating that these are also involved in PD. Later on, the presence of α-synuclein immunoreactive inclusions in neurons of the submucosal Meissner plexus, whose axons project into the gastric mucosa and terminate in direct proximity to fundic glands, was reported[162]. The authors propose that these elements could provide the first link in an uninterrupted series of susceptible neurons that extend from the enteric tract to the CNS. The existence of such an unbroken neuronal chain lends support to the hypothesis that a putative environmental pathogen capable of passing the gastric epithelial lining might induce α-synuclein misfolding and aggregation in specific cell types of the submucosal plexus and reach the brain *via* a consecutive series of projection neurons. A recent study aimed at characterizing the neurochemical coding of the ENS in the colon of a monkey model of PD, showed that this element induces major changes in the myenteric plexus and to a lesser extent in the submucosal plexus of monkeys. This data reinforces the observation that lesions of the ENS occur in the course of PD and that this might be related to the GI dysfunction observed in this pathology[163].

Experimental approaches and animal models

Animal models used in MMC include an ovine model constituted by fetal lambs[164], fetal sheep[165], a Macaca mulatta model[166], a mice model[158], and a fetal rabbit model[167]. Several experimental approaches have been used. To study the correction of a MMC-like defect in pregnant rabbits, a spinal defect was surgically created in some of their fetuses at 23 d of gestation. The spinal defect was successfully repaired, and the fetal rabbit model was established for the study of intrauterine correction of an MMC-like defect[167]. A new gasless fetoscopic surgery for the correction of a MMC-like defect in fetal sheep served as an alternative to current techniques used for fetal endoscopic surgery[165]. A Macaca mulatta model was used for replicating MMC and to evaluate options for prenatal management, such as the collocation of an impermeable silicone mesh which protects the spine from amniotic liquid with results similar to skin closure[166]. In-utero analyses of astrocytes and neuronal cells in the spinal cord of mutant mice with MMC using the curly tail/loop-tail mice model have been reported. At day 16.5 of gestation, a deterioration of neural tissue in MMC fetuses was observed mainly in the posterior region, progressing until the end of gestation with a marked loss of neurons in the entire MMC placode. These results support the current concept of placode protection through in-utero surgery for fetuses with MMC[158]. Recently, the notion of prenatal neural stem cell delivery to the spinal cord as an adjuvant to fetal repair of spina bifida has been proposed[164].

The main animal model in MS was developed in mice and is called experimental autoimmune encephalomyelitis[7]. In this experimental model, it was reported that gut flora may influence the development of experimental autoimmune encephalomyelitis[146], and that despite reported blood-brain barrier disruption, CNS penetration for small molecule therapeutics does not increase in MS-related animal models[168]. The migratory potential, the differentiation pattern and long-term survival of neural precursor cells in this experimental autoimmune encephalomyelitis mice model were investigated. The results suggest that inflammation triggers migration whereas the anti-inflammatory component is a prerequisite for neural precursor cells to follow glial differentiation into myelinating oligodendrocytes[169]. A new exciting finding with this model is that a novel regulator of leukocyte transmigration into the CNS, denominated extracellular matrix metalloproteinase inducer (EMMPRIN), indeed regulates leukocyte trafficking through increasing matrix metalloproteinase activity. Amelioration of the clinical signs of experimental autoimmune encephalomyelitis by anti-EMMPRIN antibodies was critically dependent on its administration around the period of onset of clinical signs, which is typically associated with significant influx of leukocytes into the CNS. These results identify EMMPRIN as a novel therapeutic target in MS[170].

Several experimental approaches in PD deal with GI issues using diverse animal models as rats, mice and primates. The advent of transgenic technologies has contributed to the development of several new mouse models, many of which recapitulate some aspects of the

disease; however, no model has been demonstrated to faithfully reproduce the full constellation of symptoms seen in human PD[171]. As GI dysmotility in PD has been attributed in part to peripheral neurotoxin action, rats with salsolinol induced PD were studied to evaluate its effects on intramuscular interstitial cells of Cajal, duodenal myoelectrical activity and vagal afferent activity. The results suggest a direct effect of salsolinol on both interstitial cells of Cajal and the neuronal pathways for gastro-duodenal reflexes[18]. Delayed gastric emptying and ENS dysfunction in the rotenone model of PD suggested that enteric inhibitory neurons may be particularly vulnerable to the effects of mitochondrial inhibition by Parkinsonian neurotoxins and provide evidence that Parkinsonian GI abnormalities can be modeled in rodents[68]. Studies assessing the responses of myenteric neurons to structural and functional damage by neurotoxins *in vitro* reveal that neural responses to toxic factors are initially unique but then converge into robust axonal regeneration, whereas neurotransmitter release is both vulnerable to damage and slow to recover[172]. The prototypical parkinsonian neurotoxin, MPTP, as a selective dopamine neuron toxin in ENS and used in a mouse model, shows loss of enteric dopaminergic neurons and changes in colon motility[173] and its use in a primate animal model reveals changes in the myenteric plexus and, to a lesser extent, in the submucosal plexus. These models further reinforces the observation that lesions of the ENS occur in the course of PD which might be related to GI dysfunction observed in this pathology[163]. In order to determine the changes in the dopaminergic system in the GI tract, two kinds of rodent models were used. In one, 6-hydroxydopamine was microinjected into the bilateral substantia nigra of a rat. In the other, MPTP was injected intraperitoneally into mice. The results suggest that the different alterations of dopaminergic system observed in the GI tract of the two kinds of PD models might underline differences in GI symptoms in PD patients and might be correlated with the disease severity and disease process[174]. In a similar rat model, it is reported that a unilateral 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons led to a marked inhibition of propulsive activity compared with sham-operated controls, suggesting that disturbed distal gut transit, reminiscent of constipation in the clinical setting, may occur as a consequence of reduced propulsive motility, likely due to an impairment of nitric oxide-mediated descending inhibition during peristalsis[95]. Observations in Parkinsonian primates showed that when the implanted undifferentiated human neural stem cells survived, they had a functional impact as assessed quantitatively by behavioral improvement in this dopamine-deficit model[175]. Nonmotor symptoms of PD studied in an animal model with reduced monoamine storage capacity suggests that monoamine dysfunction may contribute to many of the nonmotor symptoms of PD, and interventions aimed at restoring monoamine function may be beneficial in treating the disease[176]. In a clinical approach, it was demonstrated that delay in gastric emptying did not differ between untreated, early-stage and treated, advanced-stage PD patients, suggesting that delayed gastric emptying may be a marker of the pre-clinical stage of PD[177].

CONCLUSION

This article reviews the current knowledge in all the fields of the neurological diseases with neurogenic bowel dysfunction, and the common issues in need of clarification. The hope is that with a full perspective of the situation, researchers can generate new ideas that can be useful for prevention, cure, or at least for the mean time, a better quality of life for the patient.

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EDITORIAL

Prevention of peritoneal adhesions: A promising role for gene therapy

Hussein M Atta

Hussein M Atta, Department of Surgery, Faculty of Medicine, Minia University, El-Minia 61519, Egypt
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Correspondence to: Hussein M Atta, MD, PhD, Professor, Department of Surgery, Faculty of Medicine, Minia University, Misr-Aswan Road, El-Minia 61519, Egypt. attahm@ems.org.eg
Telephone: +20-1-001407222 Fax: +20-2-22917077
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Abstract

Adhesions are the most frequent complication of abdominopelvic surgery, yet the extent of the problem, and its serious consequences, has not been adequately recognized. Adhesions evolved as a life-saving mechanism to limit the spread of intraperitoneal inflammatory conditions. Three different pathophysiological mechanisms can independently trigger adhesion formation. Mesothelial cell injury and loss during operations, tissue hypoxia and inflammation each promotes adhesion formation separately, and potentiate the effect of each other. Studies have repeatedly demonstrated that interruption of a single pathway does not completely prevent adhesion formation. This review summarizes the pathogenesis of adhesion formation and the results of single gene therapy interventions. It explores the promising role of combinatorial gene therapy and vector modifications for the prevention of adhesion formation in order to stimulate new ideas and encourage rapid advancements in this field.

Key words: Peritoneal adhesions; Tissue plasminogen activator; Gene therapy; Plasminogen activator inhibitor; Tissue inhibitor of metalloproteinase; Transforming growth factor β

Peer reviewer: Michael E Zenilman, MD, Clarence and Mary Dennis Professor and Chairman, Department of Surgery, SUNY Downstate Medical Center, PO Box 40, 450 Clarkson Avenue, Brooklyn, NY 11202, United States

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INTRODUCTION

Peritoneal adhesions are abnormal deposits of fibrous tissue that occur in the peritoneal cavity as a result of surgery or peritonitis, or their combination. Adhesions occur in more than 90% of patients following abdominal or pelvic surgery[1-3], and are only moderately reduced after laparoscopic surgical procedures compared to open surgery[4-8]. Adhesion reformation occurs postoperatively in 85% of patients, regardless of whether the adhesiolysis is performed *via* laparotomy or laparoscopy[9].

Intraperitoneal adhesions are a major source of morbidity, being the commonest cause of intestinal obstruction[10,11], secondary female infertility, and ectopic gestation[12,13]. They may also cause chronic abdominal and pelvic pain[14,15]. Small bowel obstruction is the most serious consequence of intra-abdominal adhesions. Retrospective studies have shown that 32%-75% of patients who require abdominal re-operation have adhesion-related intestinal obstruction[16,17].

Adhesions result in a large surgical workload and cost to health care systems. An epidemiological study in the United States showed that 282 000 hospital admissions in 1988 were due to adhesion-related disorders, and the cost

of in-patient adhesiolysis was $1.18 billion[18]. In 1994, 1% of all United States admissions involved adhesiolysis treatment, resulting in $1.33 billion in health care expenditure[19].

Adhesions and their associated complications are of rising medico-legal interest. Physicians worldwide need to be aware of the increasing burden of medico-legal claims arising from the complications of intra-abdominal adhesions. Successful medico-legal claims include cases of bowel perforation after laparoscopic division of adhesions, delays in the diagnosis of adhesion obstruction of the small bowel, infertility as a result of adhesions, and pain[20].

Currently, there is no effective method for preventing adhesion formation or reformation[21]. A better understanding of the pathogenesis of adhesion formation at the cellular and molecular level would undoubtedly help to develop more effective treatment strategies[3].

PATHOGENESIS

Vicious triad of trauma, hypoxia, and inflammation

The peritoneum is lined by mesothelial cells loosely attached to the basement membrane, which can readily be detached by the slightest trauma[22]. After injury to the peritoneum, a local inflammatory reaction causes increased vascular permeability in blood vessels supplying the damaged area, followed by an exudation of serosanguinous fluid rich in fibrin and inflammatory cells, ultimately leading to the formation of a fibrin matrix. Normally, the plasminogen activator activity (PAA), which resides in the mesothelial cells and submesothelial fibroblasts, degrades the fibrinous mass, resulting in healing of peritoneal surfaces (within three to five days) without adhesions. However, if the level of PAA is diminished, the fibrinous mass persists and the underlying fibroblasts migrate into the fibrinous mass. The fibroblasts then deposit extracellular matrix, including collagen and fibronectin, leading to adhesion formation. Over time, the adhesion may provide the framework for vascular ingrowth, during the process of angiogenesis[3,23,24].

The pathogenesis of adhesions involves three important trauma-induced processes (Figure 1): (1) trauma induces inhibition of the fibrinolytic and extracellular matrix (ECM) degradation systems[25,26]; (2) trauma, as well as foreign bodies, incites an inflammatory response with the production of cytokines, mainly transforming growth factor-β (TGF-β1), a key regulator of tissue fibrosis[27-29]; and (3) trauma also induces tissue hypoxia as a result of interruption of the blood supply to mesothelial cells and submesothelial fibroblasts, leading to increased expression of hypoxia inducible factor-1 α (HIF-1α)[30,31] and vascular endothelial growth factor (VEGF), responsible for collagen formation and angiogenesis[32].

MOLECULAR CROSSTALK

Sticky connected pathways

Molecular pathways involved in fibrinolysis inhibition, inflammation, and tissue hypoxia crosstalk and potentiate the effect of each. The principal molecular aberrations included in this crosstalk are the reduction of tissue plasminogen activator (tPA) and upregulation of TGF-β1 and HIF-1α.

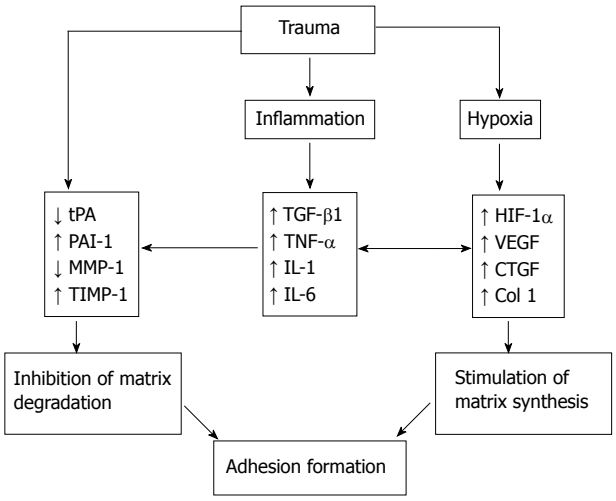

Figure 1 The role of trauma, hypoxia, and inflammation in modulating molecular crosstalk in adhesion formation. tPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitors 1; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitors of MMP; TGF-β1: Transforming growth factor-β; TNF-α: Tumor necrosis factor-α; IL: Interleukin; HIF-1α: Hypoxia inducible factor-1α; VEGF: Vascular endothelial growth factor; CTGF: Connective tissue growth factor.

INHIBITION OF FIBRINOLYSIS AND MATRIX DEGRADATION

The role of fibrinolysis in adhesion formation/reformation is to breakdown the fibrin clots that are formed during the healing process. The inactive proenzyme, plasminogen, is converted to plasmin by the action of tPA. Plasmin degrades fibrin and thus limits adhesion formation. Experimental and clinical studies have identified the presence of PAA in the mesothelium[33,34] and that tPA is the major (95%) physiological mediator of PAA[35,36]. Both mechanical and chemical injury reduce peritoneal PAA, with a progressive reduction in PAA in the first hours following a surgical operation, followed by complete loss of fibrinolytic activity up to 72 h after the operation[37,38]. Laparoscopic surgery also decreases peritoneal tPA[39]. This reduction in PAA is the result of reduced tPA production and increased release of plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2) by mesothelial, endothelial, and inflammatory cells[3,40]. Extensive human and animal studies confirmed the central role of altered tPA/PAI-1 balance in adhesion formation and demonstrated that this imbalance is more exaggerated in severe adhesions[25,26,34,40].

Plasmin also activates latent matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) degradation. The proteolytic activity of MMPs is regulated in part by their physiological inhibitors, tissue inhibitors of MMPs (TIMPs). It has been shown that MMPs and TIMPs are ex-

pressed in the human peritoneum, in adhesion fibroblasts, and in serosal layers of several intraperitoneal organs with and without adhesions[3,41,42]. Chegini *et al*[43] demonstrated that serosal tissue of intraperitoneal organs obtained during open surgery expresses more tissue inhibitor metalloproteinase-1 (TIMP-1) than matrix metalloproteinase-1 (MMP-1), and that adhesions express elevated levels of TIMP-1 and a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum. The association between the imbalance of MMP/TIMP production and adhesion formation has been confirmed in another study in women undergoing laparoscopy[29].

INFLAMMATION AND THE ROLE OF TRANSFORMING GROWTH FACTOR-β-1

Several studies have demonstrated that during the acute phase of the inflammatory response, mesothelial cells and peritoneal macrophages produce a variety of cytokines, including TGF-β1, tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and IL-6. These pro-inflammatory cytokines, individually and synergistically, stimulate the production of PAI-1 and reduce the synthesis of tPA by human mesothelial cells (Figure 1)[3,44-46]. TGF-β not only interacts with the fibrinolytic system and ECM, but also with many other cellular mediators involved in the process of adhesion formation. TGF-β1 overexpression by the peritoneum, as well as increased concentrations of TGF-β in the peritoneal fluid, has been associated with increased incidence of adhesion formation in both humans and animals[3,27,47,48].

Several studies demonstrated that increased TGF-β1 is associated with a reduction of tPA and an increase of PAI-1 release[27,49,50], an excess of TGF-β1 leads to an increase in the severity of adhesions formed[27,51], whereas an inhibitory antibody to TGF-β1 decreased adhesion formation[52]. TGF-β1 contributes to the synthesis of the ECM by stimulating fibroblastic cell production of collagen and fibronectin[53-55]. TGF-β also antagonizes ECM resorption by decreasing the activity of MMPs through decreasing MMP-1 and increasing TIMP-1 expression from mesothelial cells[3,56,57]. This impairment of MMP activity prevents the ECM deposition that occurs early in wound healing from being adequately remodeled and degraded when necessary, as healing progresses.

THE ROLE OF HYPOXIA IN ADHESION FORMATION

Several lines of evidence have demonstrated that peritoneal tissue hypoxia plays a key role in adhesion formation[58-61]. During laparotomy, tissue injury including trauma, desiccation, and vascular disruption (due to ligatures and other vascular hemostatic methods, including cauterization) reduces oxygen supply to the peritoneum. Laparoscopic surgery was shown to be less adhesiogenic, not only because it is less traumatic, but also due to elevated peritoneal tissue oxygen tension levels compared to those during laparotomy[58]. However, adhesions during laparoscopic surgery increase with duration of pneumoperitoneum and with insufflation pressure. These effects were attributed to desiccation and compression of the capillary flow in the superficial peritoneal layers by the pneumoperitoneum. The addition of oxygen to the insufflation gas decreases adhesion formation[59]. Moreover, supplemental perioperative oxygen was found to increase peritoneal tissue oxygen tension and to reduce the severity of adhesions[61].

Hypoxia negatively modulates all pathways involved in adhesion formation. Hypoxia decreases tPA and increases PAI expression in human peritoneal fibroblasts *in vitro*[62] and in peritoneal tissues *in vivo*[61], thereby decreasing plasmin, inhibiting lysis of fibrin, and increasing adhesion formation. The *PAI-1* gene contains oxygen responsive promoter sequences, namely hypoxia response element (HRE-1 and HRE-2), to which HIF-1α binds and induces gene expression[63]. Hypoxia was found to increase expression of TIMP-1, but not MMP-1, in both peritoneal and adhesion fibroblasts[55], thus decreasing matrix degradation. Hypoxic conditions in cultured human mesothelial cells and peritoneal fibroblasts also increased the expression of TGF-β1[55,64,65]. Hypoxia resulted in increased expression of collagen 1 mRNA in both peritoneal and adhesion fibroblasts[55,66], probably through the production of superoxide[66]. Moreover, hypoxia induces proliferation while inhibiting apoptosis in fibroblasts from adhesion, thus favoring adhesion formation[67]. Finally, hypoxia increases VEGF production through activation of HIF-1α in normal and adhesion fibroblasts *in vitro*[68] and *in vivo* in human adhesion mesothelial cells[69] and in animal adhesion tissues[30,70]. VEGF plays a central role in angiogenesis and its role in adhesion blood vessel development has been established[71]. Furthermore, TGF-β1 stimulates VEGF and connective tissue growth factor (CTGF) expression[72]. CTGF stimulates increased expression of ECM fibronectin, collagen 1, and laminin, while CTGF knockdown inhibits ECM production induced by TGF-β1 in human mesothelial cells[73].

CURRENT PREVENTION THERAPIES

Rules of disengagement

Single therapeutic strategies have failed to completely prevent peritoneal adhesions because of the multifactorial nature of adhesion pathogenesis[74]. As these multifactorial etiologies act independently and synergisctically in adhesion formation, it is imperative to simultaneously address the major molecular aberrations, including reduction of tPA and upregulation of TGF-β1 and HIF-1α, for any therapeutic strategy to be successful. The current preventive approaches of reducing surgical trauma, use of physical barriers or administration of single pharma-

Table 1 ***In vivo*** **adhesion prevention gene therapy studies**

Ref.	Vector/dose	Nucleic acid	Adhesion reduction (%)
Atta *et al*[101]	Adenovirus, 5×10^7 pfu	*tPA* gene	34
Guo *et al*[102]	Plasmid, 100 μg, sonoporation	*Smad7* gene	37
Guo *et al*[103]	Adenovirus	*Sphingosine kinase-1* gene	62
Segura *et al*[31]	Polyethylenimine cationic polymer, 2-4 nmol	siRNA HIF-1α siRNA PAI-1	36 to 52
Liu *et al*[104]	Adenovirus, 1×10^9 pfu	*HGF* gene	56

tPA: Tissue plasminogen activator; siRNA: Small interfering RNA; HIF-1α: Hypoxia inducible factor-1α; PAI-1: Plasminogen activator inhibitors 1; HGF: Hepatocyte growth factor.

cological agent or gene therapy have all failed to achieve satisfactory results.

Surgical precautions

The general surgical precautions aiming at minimizing surgical trauma include meticulous surgical techniques, delicate purposeful tissue handling, achieving optimal hemostasis, minimizing the risk of infection, and avoiding contaminants (e.g., fecal matter) and the use of foreign materials (e.g., talcum powder) when possible[74,75]. However, these surgical techniques alone are not effective.

Physical barriers

Physical barriers work by separating surgically injured tissues during the initial postoperative time period while remesothelization is occurring, a process that is usually expected to take three to five days[74]. Currently, three barriers, Interceed® (Johnson and Johnson, Gynecare, Somerville, NJ), Seprafilm® (Genzyme, Cambridge, MA), and ADEPT® (Baxter, Deerfield, IL), are approved by the Food and Drug Administration (FDA) for clinical use in the United States[74]. Although barriers have shown some success[74,76], this experience is not universally confirmed[77]. In fact, the FDA warns surgeons that when Interceed is used laparoscopically, patients have more adhesions than patients in the control group[76]. In the United States, ADEPT is only approved for laparoscopic gynecological surgery, and is contraindicated for patients with infection or allergies to cornstarch, as well as procedures involving laparotomy incision, bowel resection, or appendectomy. If used in these contraindicated procedures, patient may experience dehiscence, cutaneous fistula formation, anastomotic failure, ileus, and/or peritonitis. Thus, application and adoption of this product have been very limited[76]. Furthermore, the surgeon must predict the potential sites of adhesion formation in order to determine the placement site and to optimize barrier function[78].

Molecular therapy

A multitude of pharmacological agents, including recombinant proteins and antibodies, have demonstrated moderate success in reducing adhesion formation in different experimental adhesion models. These agents are applied locally into the peritoneal cavity, and work by correcting aberrant molecular pathways operative during adhesion development. One of the most extensively studied pharmacological agents that has demonstrated consistent success is recombinant tPA (reviewed in Ref 92)[79-92]. Experimental studies have reported reduction in adhesion formation and reformation using intraperitoneal recombinant human tPA in a variety of delivery methods and preparations, without impairing the healing of bowel anastomosis and without reduction in wound strength or causing hemorrhagic complications[79,80,92,93]. The action of tPA is localized to fibrin deposits; therefore, fibrinolytic activity is limited to this site, which prevents indiscriminate fibrinolysis[90]. Similar experiences were obtained in studies using neutralizing antibodies for PAI-1[94], TGFβ-1[45,52,95], TNF-α and IL-1[96], IL-6[97], and for VEGF[98,99]. However, these agents have short half-lives (few minutes) limiting their fibrinolytic effect for a sufficient duration of time (three to five days) until complete healing of peritoneal surfaces[92,100].

Gene therapy

Local molecular therapy is inherently limited; therefore, an alternative strategy using gene therapy has been recently employed to correct molecular aberrations induced by surgical trauma in a regulated manner during the period of remesothelialization. Postoperative peritoneal adhesion is an attractive target for gene therapy because of several inherent biological features. The disease is localized to the site of peritoneal trauma and develops over a short period of time, extending for the first few days following surgical trauma. These characteristics lend themselves perfectly to gene therapy using non-integrating vectors. The vector can be applied locally following completion of the operation, and the short duration of gene expression would cover the period of altered molecular aberrations (e.g., depressed tPA, elevated PAI-1, TGF-β1, HIF-1α, *etc.*) following surgery. Nevertheless, gene therapy for peritoneal adhesions is still in its infancy, with very few *in vivo* studies reported in the literature (Table 1).

Using different vectors, the five gene therapy studies reported in the literature were able to express therapeutic nucleic acids (transgenes or small interfering RNA) in the peritoneal tissues after intra-peritoneal administration in a rat adhesion model for at least seven days post-administration[31,101-104]. This duration of expression is enough

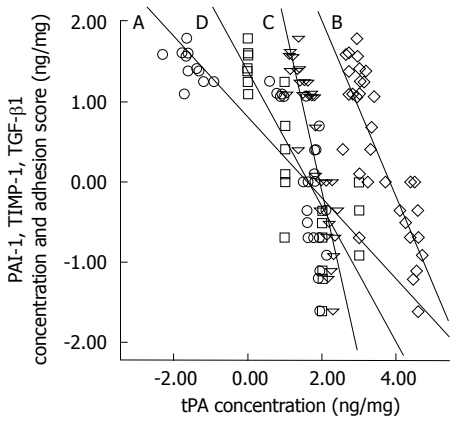

Figure 2 Correlation analysis was performed using two-tailed Spearmen rank correlation test between tPA with respect to PAI-1 (line A, marker ○), TIMP-1 (line B, marker ◇), TGF-β1 (line C, marker ▽) or adhesion score (line D, marker □). Values of tPA, PAI-1, TIMP-1, and TGF-β1 concentration in adhesion tissues were logarithmically transformed to obtain a normal distribution before statistical analysis. There was significant negative correlations ($P < 0.01$) between human tPA and either of PAI-1 (Spearman rank correlation coefficient, $r = -0.89$), TIMP-1 ($r = -0.73$), TGF-β1 ($r = -0.87$), or adhesion score ($r = -0.87$). tPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitors 1; TIMP: Tissue inhibitors of MMP; TGF-β1: Transforming growth factor-β.

to cover the time required for complete healing of the mesothelial cell layer of the peritoneum. The mechanism of adhesion reduction differed among these studies. Two studies showed that an adenovirus encoding the genes of hepatocyte growth factor (HGF) itself or its down-stream signaling molecule sphingosine kinase 1 (SK-1) could achieve adhesion reduction *via* a stimulatory effect on proliferation and migration of mesothelial cells[103,104]. The altered tPA/PAI-1 balance occupies a central role in adhesion formation and two studies tackled this molecular imbalance. Atta *et al*[101] used an adenovirus vector encoding human tPA, while Segura *et al*[31] employed a cationic polymer containing siRNAs to PAI-1 and HIF-1α to downregulate PAI-1, either directly or through its gene inducer, HIF-1α. The expression of TGF-β1 was attenuated by overexpressing its down-stream Smad2/3 natural inhibitor Smad7 using plasmid vector[102].

The moderate success in adhesion reduction in these gene therapy studies supports the concept that the multifactorial nature of molecular aberration during adhesion formation should be collectively and simultaneously addressed for any therapeutic strategy to be effective. This concept does not contradict, but reinforces, the established hypothesis of the central role of tPA reduction following surgical trauma in adhesion formation. In a recent study of adhesion prevention from our laboratory using an adenovirus vector encoding human tPA, we verified that overexpression of human tPA resulted in abrogation of the elevated fibrogenic molecules PAI-1, TIMP-1, and TGF-β1. The study showed that the reduction of these molecules depends on the concentration of the expressed human tPA protein[101]. Further analysis showed that there are significant negative correlations (at 0.01 level, 2-tailed) between human tPA and either of PAI-1 (Spearman's $r = -0.89$), TIMP-1 ($r = -0.73$), TGF-β1 ($r = -0.87$), or adhesion score ($r = -0.87$) (Figure 2).

Prospects

Gene therapy for the prevention of peritoneal adhesions has not been fully explored. Two potential developments for safe and effective gene therapy studies for adhesion prevention include combinatorial gene therapy and vector modifications.

COMBINATORIAL GENE THERAPY

The multifactorial nature of adhesion formation proposes that a combinatorial gene therapy would be more efficacious than a single gene therapy approach. For example, this could include overexpression of the fibrinolytic *tPA* gene together with downregulation of fibrogenic genes, such as TGF-β1 and/or HIF-1α. Overexpression is achieved through delivery of an exogenous gene, while downregulation is accomplished by the delivery of small interfering RNA (siRNA) molecules. Upon delivery, siRNAs complement with specific mRNAs resulting in their degradation, thus enabling the specific silencing of a single gene at the cellular level. As discussed above, single gene overexpression or silencing was moderately successful in reducing experimental adhesions[31,101]. The synergistic effects from the combined fibrinolysis stimulation and fibrogenesis inhibition, however, remain to be confirmed.

SAFE AND EFFICIENT VECTORS

Viral vectors

Delivery of therapeutic nucleic acid molecules to target tissues is accomplished using either viral vectors or non-viral carrier systems. Replication-deficient recombinant adenovirus vectors have become the most widely used viral vectors for *in vivo* gene transfer[101]. Adenovirus vectors have many positive attributes, including their ability to provide efficient *in vivo* gene transfer to both dividing and non-dividing cells, their high *in vivo* stability, and their non integrating nature into the host genome. These merits make adenoviral vectors suitable for proof-of-principle experimental studies. However, the clinical application of virus-mediated gene delivery *in vivo* is hampered by virus-induced acute inflammation, which could be fatal, high immunogenicity, and low tissue specificity. The broad tropism of adenovirus allows the virus to infect many cell types and is responsible for virus dissemination to distant organs. Various modifications of adenoviral vectors are underway to enhance the targeting of adenoviral vectors towards adhesion fibroblasts, which will provide effective and safe methods for localized treatment of postoperative peritoneal adhesions.

Nonviral vectors

Given the unresolved safety limitations of viral vectors, significant research efforts have been directed towards

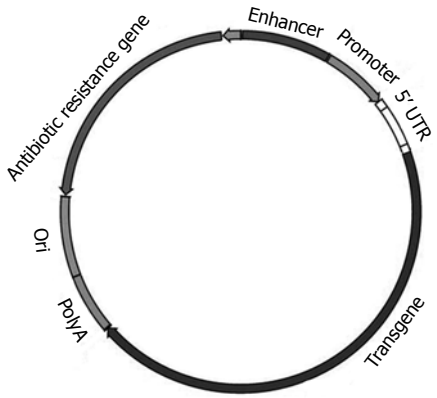

Figure 3 Skeleton of a conventional plasmid. Ori: Origin of replication.

the development of non-viral (plasmid-based) delivery systems. Plasmids are extrachromosomal genetic elements able to replicate autonomously and to be maintained in a host cell. Plasmids, the most basic forms of non-viral gene therapy, carry two main units: a eukaryotic transcription cassette and the bacterial amplification unit (Figure 3). The first bears genetic elements intended for gene expression in mammalian (eukaryotic) cells, such as the enhancer/promoter sequences for gene expression; 5' untranslated region (5' UTR), the gene of interest and polyadenylation (polyA) sequence. The bacterial amplification unit commonly contains an origin for plasmid DNA replication (ori) in bacteria and, generally, an antibiotic resistance selection marker[105]. Plasmids do not enter cells efficiently because of their large size, hydrophilic nature (due to negatively charged phosphate groups), and their susceptibility to nuclease-mediated degradation. Plasmid DNA and siRNA are stable for only 0.5 and 2 h, respectively in human serum[106]. Plasmids can be delivered to cells either naked (carrier-free) by direct injection, electroporation, ultrasound *etc.*, or complexed with cationic lipids (lipoplexes), cationic polymers (polyplexes), peptides or inorganic nanoparticles[107]. Two promising recent modifications of plasmid vectors, minicircles and CpG-depleted vectors, are briefly discussed below. Evaluating the rapid progress in the field of cationic liposomes and polymers is, however, beyond the scope of this brief review, and the reader is referred to excellent recent reviews[107,108].

Minicircle vectors

Limitations of conventional DNA plasmid vectors are related to their size (> 3 kb). Moreover, bacterial sequences contain immunotoxic cytidine-phosphate-guanosine (CpG) dinucleotides motifs, which are approximately four times more prevalent in bacterial than mammalian DNA. Bacterial CpG dinucleotides have been identified to be major contributors to the low and short-lived transgene expression (transgene silencing) in vertebrates after non-viral gene delivery. These bacterial sequences can also interfere with short hairpin RNA (shRNA, precursor of siRNA) expression[106,109]. To overcome these limitations, highly safe and efficient vector systems for gene transfer in eukaryotic cells called minivectors (minicircles) were developed[110]. Minicircles are supercoiled minimal expression cassettes, derived from conventional plasmid DNA by site-specific recombination *in vivo* in Escherichia coli. As a result, two well-defined circular molecules are generated from the parent conventional plasmid, termed minicircle (mammalian expression cassette) and miniplasmid (bacterial backbone elements). Further purification of the minicircle renders it therapeutically applicable[105]. Thus, minicircle DNA lacks the bacterial backbone sequence consisting of an antibiotic resistance gene and an origin of replication. Minicircle DNA is low in immunogenicity due to its lower content of bacterial unmethylated CpG dinucleotides. In addition to their improved safety profile, minicircles have been shown to greatly increase the efficiency of transgene expression in various *in vitro* and *in vivo* studies, compared to the conventional plasmid with the same transgene expression cassette. It has been reported that a minivector incorporating short hairpin RNA efficiently transfected adhesion fibroblasts and was shown to be stable in human serum for > 48 h[107].

CpG-depleted vectors

Bacterial DNA is rich in unmethylated CpG dinucleotides, in contrast to mammalian DNA, which contains a low frequency of CpG dinucleotide, which are mostly methylated. Recognition of unmethylated CpGs present in the bacterial backbone could trigger an innate immune response following detection in the endosome by toll-like receptor 9 (TLR9)[109] and initiate a signaling cascade, leading to the production of proinflammatory cytokines. As plasmids used in *in vivo* gene therapy studies are produced in *Escherichia coli* (*E. coli*), their CpGs are unmethylated and induce immune responses through this host defense mechanism. Recently, plasmids that are completely devoid of CpG dinucleotides have been developed. These plasmids yield high levels of transgene expression both *in vitro* and *in vivo*, and, in contrast to CMV-based plasmids, allow sustained expression *in vivo*[111,112]. In these CpG-free plasmids, all elements required for replication and selection of the plasmid in *E. coli* and for gene expression in mammalian cells (e.g., promoter, polyadenylation signal, reporter gene, *etc.*) either naturally lack CpG dinucleotides, were modified to remove all CpGs, or are entirely synthesized.

CONCLUSION

Gene therapy for the prevention of postoperative peritoneal adhesions is still in its infancy. The potential applications of this strategy have not been fully explored. The recent explosive progress in advanced nonviral gene delivery systems, coupled with the newly developed less immunogenic and more efficient expression plasmids, will undoubtedly accelerate research studies in gene therapy for peritoneal adhesions.

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REVIEW

Role of (^{18}F) 2-fluoro-2-deoxyglucose positron emission tomography in upper gastrointestinal malignancies

Elizabeth C Smyth, Manish A Shah

Elizabeth C Smyth, Manish A Shah, Department of Gastrointestinal Oncology, Weill Cornell Medical College, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States
Manish A Shah, Department of Gastrointestinal Oncology, Weill Cornell Medical College, New York-Presbyterian Hospital, New York, NY 10065, United States
Author contributions: Smyth EC and Shah MA contributed equally to this paper.
Correspondence to: Manish A Shah, MD, Director, Department of Gastrointestinal Oncology, Weill Cornell Medical College, New York-Presbyterian Hospital, 1305 York Avenue, New York, NY 10065, United States. manish.a.shah.md@gmail.com
Telephone: +1-646-9622547 Fax: +1-646-9621607
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Abstract

The role of whole-body FDG [(^{18}F) 2-fluoro-2-deoxyglucose] positron emission tomography (PET) scanning as an imaging modality in the management of patients with malignancy has evolved enormously over the past two decades. FDG-PET has demonstrated significant efficacy in the staging, prognostication and detection of occult metastatic disease in malignancies of the gastrointestinal tract, in addition to assessment of the response to cytotoxic chemotherapy in a more timely manner than has traditionally been possible by more conventional imaging tools. The sensitivity and specificity of FDG-PET for the detection and staging of malignancy depend not only on the site and size of the primary tumor and metastases, but also on histological cell type, reflecting underlying disparities in glucose metabolism. The metabolic response to neo-adjuvant chemotherapy or to chemo-radiotherapy in cancers of the gastro-esophageal junction or stomach has been demonstrated in several prospective studies to correlate significantly with both the histological tumor response to treatment and with consequent improvements in overall survival. This may offer a future paradigm of personalized treatment based on the PET response to chemotherapy. FDG-PET has been less successful in efforts to screen for and detect recurrent upper gastrointestinal malignancies, and in the detection of low volume metastatic peritoneal disease. Efforts to improve the accuracy of PET include the use of novel radiotracers such as (^{18}F) FLT (3-deoxy-3-fluorothymidine) or ^{11}C-choline, or fusion PET-CT with concurrent high-resolution computed tomography. This review focuses on the role of FDG-PET scanning in staging and response assessment in malignancies of the upper gastrointestinal tract, specifically gastric, esophageal and pancreas carcinoma.

Key words: Positron emission tomography; Gastric cancer; Esophageal cancer; Pancreas cancer

Peer reviewer: Dr. Cem Onal, Department of Radiation Oncology, Baskent University Faculty of Medicine, Adana 01120, Turkey

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INTRODUCTION

Whole-body positron emission tomography (PET) scanning after the administration of (^{18}F) 2-fluoro-2-deoxyglucose (FDG) has emerged as a promising new imaging modality in the management of patients with malignancy. The role of FDG-PET scanning in upper gastrointestinal (GI) malignancies has evolved tremendously over the past two decades. Like most imaging modalities, FDG-PET initially made its mark in staging for preoperative risk assessment, prognostication, and in evaluation of

distant metastatic disease. FDG-PET scanning has also improved our ability to identify occult metastatic disease in a number of malignancies, including malignancies of the upper GI tract. When considering glucose uptake as a surrogate for metabolic activity, another important application of FDG-PET scanning is therapeutic response assessment. Traditional computed tomography (CT) scanning has been the mainstay for assessment of the effectiveness of cytotoxic therapy in solid tumor oncology; however with the advent of FDG-PET, it has been increasingly apparent that this new modality may also provide an assessment of the therapeutic effectiveness of cytotoxic therapy, and possibly at an earlier time point.

This review focuses on the role of FDG-PET scanning in staging and therapeutic response assessment in malignancies of the upper GI tract, specifically gastric and esophageal carcinoma.

SCIENCE OF FDG-PET AND CHANGES IN FDG UPTAKE

FDG uptake is considered as a surrogate for the metabolic activity of a malignancy, specifically linked to glucose metabolism in malignant cells[1]. The role of FDG-PET imaging, in fact, may be related to the Warburg effect-the observation made by Otto Warburg in 1924 that suggested that cancer cells metabolize glucose differently from normal non-malignant cells[2]. Specifically, cancer cells tend to grow and metabolize nutrients independent of growth factor stimulus, but not in the most efficient manner for ATP generation, but rather in a manner that would support the acquisition of building blocks for continued, uncontrolled cell division and growth[2]. Central to this hypothesis is dysfunction of the phosphoinositide 3-kinase signaling pathway, commonly identified as pathologic in a majority of malignancies, and which is central to both growth control and glucose metabolism. A change in glucose metabolism, as identified by FDG-PET serial imaging, may therefore uniquely predict subsequent cell death[1].

Glucose uptake by malignant cells is largely mediated by the GLUT-1 transporter[3]. In a study of 60 patients with squamous cell carcinoma of the esophagus, Hiyoshi *et al*[4] demonstrated that GLUT-1 expression was correlated with the depth of tumor, lymph node metastasis and pathological stage, in addition to FDG avidity on PET imaging. Mu *et al*[5] correlated the standardized uptake value (SUV) with the expression of GLUT-1 and the Ki-67 proliferative marker, and found that with increasing clinical stage and pathological dedifferentiation, the expression of both markers increased concurrently, indicating an association with tumor aggressiveness. Tohma *et al*[6] demonstrated that FDG uptake may have a more significant association with the intracellular enzyme hexokinase-2 expression than with GLUT-1 expression. In contrast, FDG uptake is not associated with cyclin D1, p53, epidermal growth factor receptor or vascular endothelial growth factor expression in esophageal tumors[7].

ESOPHAGEAL CARCINOMA

Role of PET in staging the depth of disease-esophageal carcinoma

Clinical significance of T stage: Penetration of the primary tumor through successive layers of the walls of the esophagus is described using the T stage of the tumor. Deeper levels of mucosal involvement are associated with a higher risk of nodal and distant metastasis, and diminishing overall survival. The location of the primary tumor within the esophagus has particular relevance to the draining lymph node stations for that area. Nodal metastasis beyond the locoregional nodes may render the patient unresectable as a result. Early cancers (T2 or less) may undergo primary surgical resection. Those tumors with T3 or greater depth of penetration may undergo preoperative chemotherapy or chemoradiotherapy with a view to future resection, or definitive combined modality therapy.

FDG-PET and T stage

In an initial study of FDG-PET in the assessment of esophageal cancer by Flamen *et al*[8], FDG-PET detected 70 out of 74 esophageal lesions. It failed to detect 4 small (< 8 mm) T1 lesions. This study demonstrated no correlation between the SUV and the T stage. A retrospective series from Japan similarly demonstrated superior sensitivity of PET for the detection of T2 or greater disease; 25/25 patients with T2 or greater tumors had FDG uptake, compared to 0/7 with T1 tumors. Significant correlations with increased SUV uptake were seen with both the size of the primary and with the depth of tumor invasion[9].

In a prospective series of 81 patients who underwent surgery with no preoperative treatment, PET detected the primary lesion in 43% of pT1 tumors. Sensitivity was significantly better for pT1b disease at 61%, compared with 18% for pT1a. PET positivity increased with increasing levels of tumor invasion, being 83% at T2, 97% at T3 and 100% at T4[10]. Importantly, in another study examining patients with early stage tumors who underwent primary surgical treatment, PET-CT could not distinguish between those with carcinoma *in situ* (Tis) *vs* those with T1 disease, with FDG uptake in 5/11 (45%) and 26/47 (55%) respectively. The investigators noted a trend towards both increased frequency of FDG uptake and increased SUV with increasing depth of invasion.

It may be concluded from this data that PET, and indeed PET-CT, is an inadequate modality for assessing depth of tumor penetration within the mucosal wall of the esophagus, and also that it cannot distinguish adequately between carcinoma *in situ* and invasive disease. However, with increasing depth of invasion, an FDG-PET scan is increasingly likely to identify the malignancy.

In addition, FDG avidity on FDG-PET scans should be taken in context due to the small but real rate of false positive scans. Specifically, areas of increased FDG uptake within the esophagus may have an alternate cause such as chemotherapy or radiation-induced esophagitis, candida or other benign causes[11-14]. PET lacks the specificity to differentiate between these conditions, under-

Table 1 Prospective studies comparing the accuracy of positron emission tomography with computed tomography and/or endo-ultrasonography for the detection of lymph-node metastases

Ref.	Yr	Histology	*n*	Imaging	Sensitivity (%)	Specificity (%)
Flamen *et al*[114]	2000	SCC/AC	74	PET	39	97
				CT	63	88
				EUS	22	96
				EUS/CT	54	90
Lerut *et al*[115]	2000	SCC/AC	42	PET	22	91
				CT/EUS	83	45
Yoon *et al*[116]	2003	SCC	81	PET	30	90
				CT	11	95
Sihvo *et al*[18]	2004	AC	55	PET	35	91
				CT	42	45
				EUS	85	60
Lowe *et al*[19]	2005	SCC/AC	75	PET	82	60
				CT	84	67
				EUS	86	67
Shimizu *et al*[20]	2009	SCC	20	PET-CT	11-50	85-100
				Thin slice CT	22-100	69-100

PET: Positron emission tomography; CT: Computed tomography; EUS: Endoscopic ultrasound; SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

scoring the inadequacy of this approach. Due to these factors, endoscopic ultrasound (EUS) is the preferred method for assessment of the depth of invasion of the primary tumor through the wall of the esophagus. This has been demonstrated in a meta-analysis of 49 studies to have a sensitivity of 81%-90% for T staging and a specificity of 99%[15]. EUS is limited by inability to pass through stenotic tumors in these cases, PET or PET-CT based imaging may serve as a useful adjunct.

Role of PET in staging nodal disease-esophageal cancer

Clinical significance of nodal stage: Nodal status in esophageal cancer is determined by the presence or absence of involved locoregional lymph nodes. The regional designation of a lymph node relates to its anatomical relationship to the primary tumor. Tumors of the upper third of the esophagus drain to superior mediastinal and cervical lymph nodes. Tumors of the middle third drain both superiorly and inferiorly to paratracheal, hilar, subcarinal, periesophageal, and pericardial lymph node stations. Tumors of the lower third of the esophagus drain to lymph node basins in the lower mediastinum and celiac areas Patients with non-regional lymph node spread have a worse prognosis than those with locoregional spread only, but better than those with distant metastases.

Initial reports of PET showed promise due to apparent increased sensitivity in the detection of lymph node metastasis when compared to CT[16]. However this may have been due to the use of outdated CT technology and techniques, and this initial promise with respect to increased sensitivity has not been sustained in well designed prospective studies.

In an initial report, Flamen *et al*[8] reported that 74 patients demonstrated a lower sensitivity of PET for the detection of regional lymph node metastasis when compared to EUS (81% *vs* 33%) but with a non-significant trend towards higher specificity (84% *vs* 69%). PET showed a higher specificity than CT and EUS combined when staging both regional and non-regional lymph node metastases for esophageal cancer. In a prospective study of 58 patients comparing CT and PET in the detection of lymph node metastasis within the abdomen by Kneist *et al*[17], the investigators observed a sensitivity of only 24% for PET compared to 73% for CT. Sensitivity of PET was significantly less in the area of the lesser curvature and the celiac trunk. Specificity was 75% and 95%, respectively. Within the thorax, PET demonstrated an improved but still inferior sensitivity (42% *vs* 75%) and again a superior specificity to CT. A prospective evaluation of CT, EUS and PET by Sihvo *et al*[18] demonstrated that EUS had a higher sensitivity for the detection of nodal disease (85%) than CT or PET (42% and 35%). The combination of CT, EUS and PET did not appreciably increase the sensitivity of the assessment. Neither was there any synergy between modalities with respect to specificity. A 2005 study performed by Lowe *et al*[19] comparing CT, PET and EUS for the staging of esophageal cancer showed comparable sensitivities between the three modalities for the detection of nodal disease (82%-86%). Specificity was also not significantly different at 67% for CT and EUS, and 60% for PET.

Progress in the development of both CT and PET imaging may lead to improvements in the diagnostic accuracy of both modalities. A recent study comparing thin slice CT to PET-CT in the detection of subclinical lymph node metastasis in patients with operable squamous cell carcinoma demonstrated the superiority of CT for the detection of disease at all lymph node stations, with the caveat that sensitivity appeared to decrease from the cervical area (100%) to the abdominal area (22%). Specificity was high for both CT and PET in the cervical and abdominal lymph node basins, with superior specificity for PET demonstrated only within the mediastinum[20].

The results of the above studies are described in Table 1. In order to better characterize these heterogenous results, a meta-analysis was performed by van Westreenan *et al*[50]. This included both prospectively and retrospectively obtained data. Pooled sensitivity for the detection of locoregional lymph node metastases was 51% (range, 8%-92%) with pooled specificity of 84% (range, 67%-100%)[21]. The low sensitivity of PET in prospective studies may be due to a selection bias in many cases. These results may be biased by the inclusion only of apparently early stage patients who proceeded immediately to surgery. Those who required preoperative chemotherapy and/or radiation were excluded, leading to an over-representation of solely micrometastatic foci, which are less reliably detected. For reasons of this relatively low sensitivity of PET for locoregional disease, and due to its excellent specificity, FDG-PET is better as an adjunct to conventional

Table 2 Prospective studies comparing the accuracy of positron emission tomography with computed tomography and/or endo-ultrasonography in the detection of distant metastases

Ref.	Yr	Histology	*n*	Imaging	Sensitivity (%)	Specificity (%)
Flamen *et al*[8]	2000	SCC/AC	74	PET	71	90
				CT	41	83
				EUS	42	94
				EUS/CT	47	78
Lerut *et al*[115]	2000	SCC/AC	42	PET	77	90
				CT/EUS	46	69
Sihvo *et al*[18]	2004	SCC	81	PET	35	91
				CT	42	45
Heeren *et al*[25]	2004	SC/AC	74	PET	71	98
				CT	21	98
				CT/EUS	29	96
Lowe *et al*[19]	2005	SCC/AC	75	PET	81	91
				CT	81	82
				EUS	73	76

PET: Positron emission tomography; CT: Computed tomography; EUS: Endoscopic ultrasound; SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

imaging modalities for the detection of lymph node metastases rather than a comprehensive staging investigation in its own right.

Efforts to improve accuracy of PET in the detection of lymph node metastasis

The limited spatial resolution of PET may lead to difficulties due to the fact that uptake within lymph nodes close to the primary tumor may be difficult to distinguish from the tumor itself. Fusion PET-CT and correlation with metabolic and tumor-related parameters may offer superior sensitivity for the detection of nodal disease. A 2009 study by Roedl *et al*[22] compared fusion PET-CT with PET viewed side by side with CT images, in addition to axial tumor area, tumor width diameter and SUV uptake. Fusion PET-CT was more sensitive and more specific for the detection of lymph node metastasis at 70% *vs* 62% and 95% *vs* 91%, respectively. Sensitivity and specificity of 87% and 85% were increased by the addition of tumor diameter measurements. However when qualitative visual analysis was added to quantitative tumor dimension measurement in addition to PET-CT the sensitivity was 96% and the specificity 95%.

Dual time PET may assist in the differentiation between benign and malignant lesions, and may also improve the accuracy of detection of lymph node metastasis in esophageal cancer. Small malignant lesions and malignant lymph nodes show an increase in SUV uptake over time, whereas benign disease does not, and shows an early peak only. An improvement in diagnostic accuracy from 83% to 91% was seen with dual time imaging of squamous cell carcinomas of the thoracic esophagus. In addition, false positive uptake in the lung hilum due to inflammatory processes was distinguished from malignant disease in 19/42 (45%) of patients using this method[23].

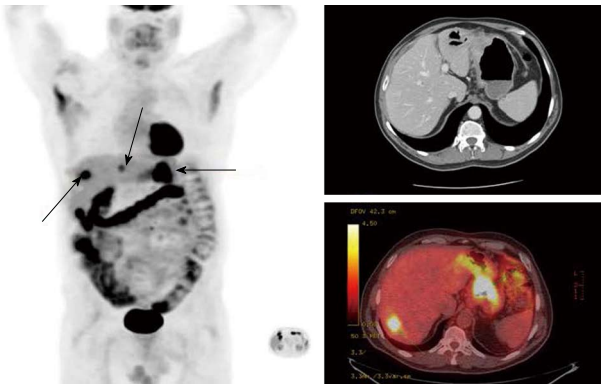

Figure 1 (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography/computed tomography image of a patient with a proximal gastric cancer and occult liver metastasis. The liver lesion was not identified on the corresponding staging computed tomography.

DETECTION OF METASTATIC ESOPHAGEAL CANCER USING FDG-PET

PET finds a niche in the detection of metastatic disease, where its performance is superior than in the detection of the depth of the primary lesion or of locoregional lymph node involvement of esophageal carcinoma (Table 2).

An initial prospective study by Luketich *et al*[24] demonstrated a sensitivity of PET for detection of metastatic disease of 69% with a specificity of 93.4% and an overall accuracy of 84%. Following this, Flamen *et al*[8] demonstrated that FDG-PET had a superior accuracy for the detection of metastatic disease compared to combined CT and EUS (82% *vs* 64%), largely driven by the higher sensitivity of PET (74% *vs* 47%). PET correctly upstaged 15% of patients from M0 to M1 disease. The study by Lowe *et al*[19] demonstrated similar sensitivity of PET and CT at 81%, and superior specificity for PET. This may relate to improvements in CT scanning techniques in recent years.

A 2004 study by Heeren *et al*[25] demonstrated that PET upstaged up to 20% of patient to M1 disease. The accuracy of CT was 86% compared to CT/EUS at 69%. All three modalities combined provided an accuracy of 92%. In this study 13% of patients in whom M1 disease was detected on PET were spared an unnecessary surgical procedure, however 87% did require laparoscopy to confirm PET positive findings underscoring the importance of cytological confirmation of metastatic disease. In a combined analysis of 452 patients from 11 studies the pooled sensitivity and specificity for the detection of metastatic disease by PET was 67% (95% confidence interval (CI): 58%-76%) and 97% (90%-100%) respectively. Figures 1 and 2 demonstrate the detection of occult liver (Figure 1) and bone (Figure 2) metastases by FDG-PET/CT not seen on conventional CT imaging.

IS PET PREDICTIVE OF SURVIVAL IN ESOPHAGEAL CANCER?

Many studies have examined the relationship between

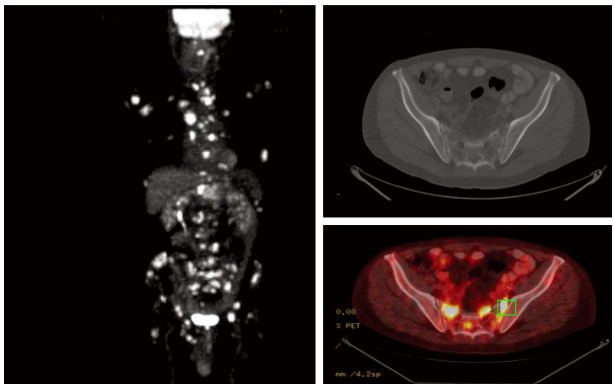

Figure 2 (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography/computed tomography detects diffuse bony metastases not seen on staging computed tomography.

SUVmax and survival. In a recent systematic review, all 12 studies selected for inclusion demonstrated that a higher SUVmax of the primary tumor was associated with inferior survival, however only seven of these reached statistical significance. In a meta-analysis of disease-free and overall survival, the hazard ratios for disease recurrence and death were 2.52 and 1.86, respectively, for those with a higher than median SUV[26]. This correlation with peak SUV and survival may hold true even for those with apparently early stage disease[27].

SUVmax is also often significantly correlated with pathological stage, acting as a potential confounder. On multivariate analysis in several smaller studies, peak SUV was significantly associated with survival in univariate but not multivariate analysis, and thus did not emerge as an independent risk factor[28,29]. However in a large retrospective study of 184 patients with operable esophageal cancer, where SUVmax was significantly correlated with the stage of the primary tumor, lymph node status, and presence of metastasis in univariate analysis, on multivariate analysis SUV remained independently and significantly associated with overall survival when correcting for pathological stage of disease. The 5-year overall survival for those with an SUVmax $\geqslant$ 4.5 was 47% compared to 76% in those with an SUV $\leqslant$ 4.5[30]. It should be noted that the majority (91%) of patients in this study had a diagnosis of squamous cell carcinoma, and that these results contrast sharply with those published by Rizk *et al*[31] in a retrospective series of 189 patients with adenocarcinoma of the distal esophagus or gastro-esophageal (GE) junction who underwent chemoradiation as a primary treatment, in which they failed to show any association between survival for those with a high or a low SUVmax. Those with a high SUVmax did however show a superior response to chemoradiation This led the authors to conclude that although high SUVmax was correlated with inferior survival following resection in their earlier study, because high baseline SUVmax was also associated with a superior response to chemoradiation, this acted as an equalizing factor with respect to survival.

Altogether, these data suggest that high SUVmax is most likely to be associated with increased tumor stage and size of lesion. Whether SUVmax is an independent predictor of patient outcome (specifically independent of tumor stage) is not sufficiently validated.

ROLE OF FDG-PET IN RADIOTHERAPY TREATMENT PLANNING FOR ESOPHAGEAL CANCER

The gross tumor volume (GTV) must be accurately delineated in order to successfully treat the area of malignancy. However, conventional CT scanning has a low discriminatory value for this purpose. FDG-PET has been investigated in order to assess whether this improves the accuracy of this delineation. Excellent correlation has been demonstrated between preoperative FDG-PET and EUS measurements of tumor length and measurements of the same resected surgical specimen[32]. The addition of FDG-PET to conventional CT planning may lead to increases or reductions in the GTV of up to 20%, and changes in the planning target volume in over half of patients[33,34]. Modifications of GTV are most often seen in the longitudinal direction[35], however this may also change based on detection of suspicious lymphadenopathy outside the original planned treatment field[36]. Improved accuracy in GTV delineation may lead to changes in radiation dose intensity to critical structures such as the heart and lungs[33,37], whereas utilization of CT alone may lead to undertreatment of FDG-PET avid disease[34]. However, due to a lack of standardization of FDG-PET assessments of GTV and the presence of significant interobserver variation, the use of FDG-PET is not routine in radiotherapy treatment planning, nor has this been validated in terms of improved outcomes such as survival or locoregional tumor control. A prospective trial is ongoing in this regard (NCT01156831)[38].

DOES SUV PREDICT RESPONSE TO CHEMORADIOTHERAPY?

Several studies have examined whether the change in SUV of the primary tumor with chemotherapy or chemoradiotherapy is useful in determining the response to the intervening therapy. A large proportion of studies have been prospective, but were limited in their scope of analysis to some extent by small numbers. Each study evaluated a different treatment regimen. Most studies used pathological response as the gold standard for evaluation of chemotherapy efficacy. This is commonly measured using the Mandard system[39] or a simple modification of this system, where pathological response is classified according to the percentage of viable tumor cells remaining, with non-responders having > 10% tumor cells remaining, partial response 0%-10%, and complete responders 0% viable tumor cells.

A first prospective study in 2001 by Weber *et al*[40] of

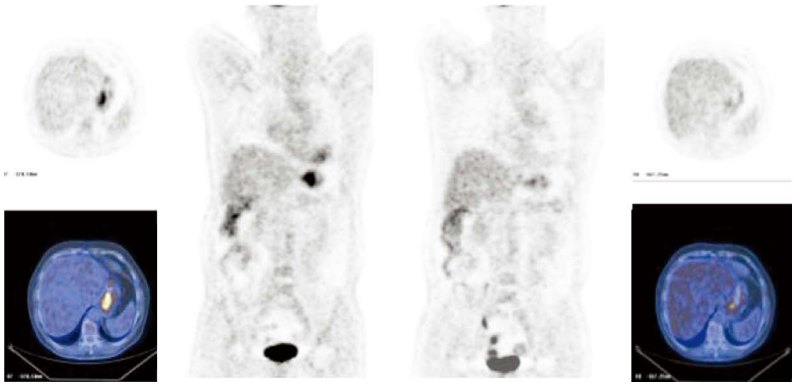

Figure 3 (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography response in a patient with a proximal gastric cancer receiving chemotherapy.

40 patients with adenocarcinoma of the GE junction and gastric cardia demonstrated a median reduction in SUV of responders of more than three times that of non-responders and was significantly correlated with pathological response ($P < 0.001$). Response was also significantly associated with survival. Those with no response had a 2-year survival of 37% *vs* 60% in responders. Figure 3 demonstrates a sample FDG-PET/CT response for a patient with a proximal gastric adenocarcinoma.

A prospective trial by Ott *et al*[41] used a predetermined level of reduction in SUV to determine the cut-off point for metabolic responder *vs* non-responder. This had been previously determined to be a reduction of 35% from baseline, which had been demonstrated to have a sensitivity and specificity of 93% and 95%, respectively, for the detection of a pathological response[40]. Sixty five patients with locally advanced GE junction tumors undergoing preoperative chemotherapy were enrolled. Baseline tumor FDG uptake was 8.1 ± 3.4 SUV for assessable patients. SUV uptake significantly decreased to 5.4 ± 2.0 (approximately 33%) in the follow-up scan. Eighteen patients were classified as metabolic responders and 38 as metabolic nonresponders. The pathological response was highly significantly correlated with the metabolic response ($P < 0.001$); 44% of patients with a metabolic response had a pathological response, compared to 5% of metabolic non-responders. Median overall survival for non-responders was 18 mo, significantly shorter than overall survival for the group as a whole at 32 mo. Median survival for metabolic responders had not yet been reached at the time of publication.

A similar study was performed at Memorial Sloan Kettering Cancer Center as a validation study, and reported in abstract form in 2007[42]. In this study, patients with locally advanced but resectable gastric/GE junction adenocarcinoma received preoperative chemotherapy with irinotecan and cisplatin for two cycles. An FDG-PET CT scan was performed at baseline and again at day 15 and day 35. This study confirmed the results initially reported by Weber *et al*, demonstrating that a significant drop in SUV from baseline was associated with the pathologic response to therapy as well as with patient survival[42].

The primary utility of a change in FDG-PET SUV from baseline as a marker for response to chemotherapy and subsequently survival is that this information is available early in the treatment plan, and thus could potentially be used in order to guide future management. This approach was taken by Lordick *et al*[43] in the MUNICON trial. This study recruited 119 patients with locally advanced tumors of the GE junction undergoing preoperative chemotherapy. Patients who did not meet a predefined metabolic response level on FDG-PET of a 35% reduction from baseline SUVmax 2 wk after commencing treatment did not continue with chemotherapy but proceeded directly to surgery. Metabolic responders completed the course of preoperative chemotherapy and then proceeded to surgery; 49% of patients were metabolic responders and 51% were metabolic non-responders. Of the metabolic responders, 58% achieved a major histological response, with 0% in the non-responders. R0 surgical resection was possible in 96% of metabolic responders and in 74% of metabolic non-responders. On pathologic assessment, metabolic responders demonstrated earlier stage tumors than metabolic non-responders. Metabolic non-responders had a median event-free survival of 14.1 mo compared to 29.7 mo in metabolic responders. It was noted that metabolic responders who did not have a pathological response had survival comparable to those who were metabolic non-responders, implying that a metabolic response was necessary but not sufficient for improved survival[43].

In a cross trial comparison between the original study by Ott *et al*, where chemotherapy was continued despite a metabolic non-response, and MUNICON where non-responders proceeded directly to surgery, amongst those patients that went on to complete surgical resection, survival between non-responders in both groups was similar. This suggests that, amongst metabolic non-responding patients, patient survival was unaffected (either adversely or positively) by continuing with ineffective chemotherapy or by stopping ineffective chemotherapy and proceeding early to surgery. These results have led to an ongoing clinical trial in which failure to respond to initial induction chemotherapy with a reduction in SUV on

PET is followed by introduction of a salvage regimen of non-cross resistant chemotherapy in an effort to improve outcome (NCT00737438 on clinicaltrials.gov; Memorial Sloan Kettering study, IRB 08-081).

In contrast, in a study of 32 patients with esophageal/GE junction adenocarcinoma, a FDG-PET scan performed following a week of chemoradiation failed to detect any significant difference between pathologic responders and non-responders with respect to changes of SUVmax on PET[44]. This may in fact be due to the timing of the PET as radiation is known to have a "stunning" effect with respect to FDG uptake, irrespective of further cell kill, which may cause bias in an interpretation performed at an early interval following radiation.

These studies suggest that the utility of FDG-PET in response assessment in esophageal/GE junction adenocarcinoma remains to be verified at this time, but that it is a potentially promising modality to begin "individualized" care for patients with upper GI malignancies (namely esophageal and gastric adenocarcinoma). It should be noted that the response of PET to chemotherapy when compared with that of CT may lead to clinical confusion, such as when a lesion improves by PET criteria, but fails to shrink or may even enlarge slightly by traditional RESIST criteria[45]. Recently proposed guidelines for response assessment in solid tumors suggest that PET progression may be defined as an SUV increase of $\geqslant$ 20% in a region 1 cm or larger in diameter, whereas a response be defined as a decline in SUV of $\geqslant$ 30% in such a region[46]. Such a guideline would seem to be a good starting point for evaluation of the PET response in many solid tumor malignancies, but will need prospective validation.

FDG-PET FOR THE DETECTION OF ESOPHAGEAL CANCER RECURRENCE

The accuracy of CT and magnetic resonance imaging (MRI) for the detection of recurrent disease, particularly within the area of the initial primary tumor may be decreased by post-surgical or post-chemoradiation related changes such as fibrosis, edema, and inflammation. Guo *et al*[47] followed 112 patients with resected squamous cell carcinoma of the esophagus for recurrence with FDG-PET/CT. PET demonstrated excellent sensitivity at local, regional and distant sites of metastases (96.9%, 85.9% and 90.5%, respectively), but lower specificity for local-regional recurrence (50%, 92.2% and 89.9%, respectively). Of note, five out of nine false positive FDG-PET scans were identified in the area of the surgical anastomosis. A French study examined the routine use of FDG-PET in the prospective follow-up of resected esophageal cancer patients[48]. This study demonstrated that for the detection of locoregional recurrence, PET had a higher sensitivity, slightly lower specificity and a superior accuracy than CT (100% *vs* 65%, 85% *vs* 91%% and 91% *vs* 81%, respectively). PET was also superior to CT in the detection of local metastasis. No patient had a negative PET and a recurrence detected by another modality, i.e., there were no false negative PET scans in this study, leading to a 100% negative predictive value. As this recently published study is the first examining the prospective use of PET to detect recurrence in asymptomatic patient, it is too early to comment on whether changes in management based on this strategy will lead to improvements in patient outcomes.

COMPARISON OF FDG-PET AND OTHER PET TRACERS IN THE DIAGNOSIS AND MANAGEMENT OF ESOPHAGEAL CANCER

FDG is not a tumor specific radiotracer, and this leads to the drawback of false positive uptake in areas of inflammation or infection by neutrophils and macrophages, i.e., when there is contamination of the malignancy with other actively dividing or metabolically active cells. An alternative to FDG-PET is (^{18}F) FLT (3-deoxy-3-fluorothymidine) which is trapped intracellularly following phosphorylation by thymidine kinase 1 into (^{18}F) FLT-monophosphate, forming the rationale for the use of FLT as a proliferation tracer[49]. A study by Westreenan *et al*[50] compared the efficacy of FLT *vs* FDG in the detection of esophageal cancer and demonstrated increased uptake for FDG rather than FLT (FLT-PET missed 20% of primary esophageal tumors in this study). FDG-PET also detected a synchronous primary rectal tumor in one patient, which was not detected by FLT-PET. In addition, there was no correlation between uptake of FLT and Ki-67, a marker of proliferation. For this reason, FDG remains the preferred radiotracer for use in the diagnosis and management of patients with esophageal cancer[50].

^{11}C-choline is a small molecule that is integrated into the cell membrane as phosphatidylcholine and serves as a marker of cell membrane metabolism. Because of late urinary excretion, it has been examined in genitourinary tumors such as prostate cancer[51]. ^{11}C-choline has been investigated in two studies of esophageal cancer. Kobori *et al*[52] studied squamous cell carcinoma of the upper esophagus and claimed a superior sensitivity for choline-PET in the detection of primary tumors and nodal metastases in the mediastinum (94% and 88%, respectively). Specificity was not reported. In this study the sensitivity of FDG-PET was 34% and 38% for the primary tumor and nodal involvement, which is somewhat lower than the literature median. These results contrast with those of Jager *et al*[53], who studied a more diverse group of esophageal and GE junction adenocarcinomas in addition to squamous cell carcinoma of the esophagus and GI stromal tumors. They demonstrated the superiority of FDG-PET, with a sensitivity of 100%, 67%, and 100% for the detection of primary tumor, locoregional and lymph node metastases, respectively, compared to 73%, 60%, and 75%, respectively, for choline-PET. Imaging in the abdominal area

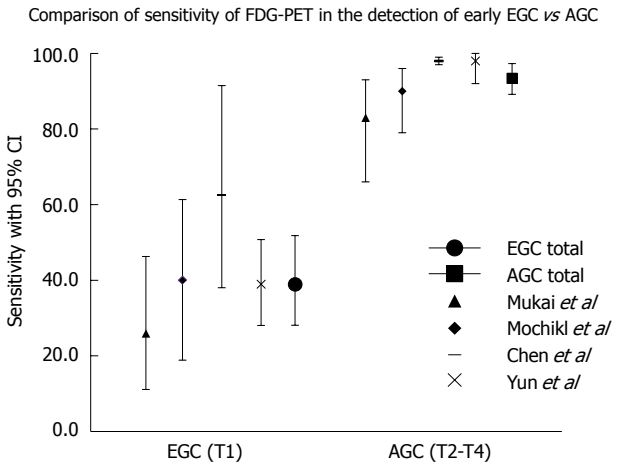

Figure 4 Sensitivity of (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography to identify primary early and advanced gastric carcinoma. FDG-PET: (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography; EGC: Early gastric cancer; AGC: Advanced gastric cancer; CI: Confidence interval.

using choline-PET is limited by the high background uptake of this agent by the liver.

GASTRIC ADENOCARCINOMA

Gastric cancer remains the most common GI malignancy worldwide, responsible for approximately 934 000 new diagnoses annually (8.6% of new cancer cases) and 700 349 deaths worldwide annually[54]. Gastric cancer may be distinguished anatomically such that proximal tumors (associated with chronic reflux and obesity) have worse prognosis than distal tumors which are more commonly associated with chronic infection by *Helicobacter pylori*[55]. Alternatively, gastric cancer may also be distinguished histopathologically as diffuse, intestinal, or mixed histology which describes the pattern of spread of the primary tumor[56]. Based on these distinctions, an emerging concept in understanding the biology and physiology of gastric cancer is that it likely reflects not one disease, but several[55]. How these distinctions impact on FDG-PET imaging is still evolving.

IMAGING PRIMARY GASTRIC CARCINOMA WITH FDG-PET

Unlike esophageal carcinoma, in which the majority of tumors (particularly T2-T4) are identified on FDG-PET imaging, the primary gastric lesion is less well imaged by FDG-PET. This has been demonstrated in several series with sensitivity for detection of gastric lesions ranging from 21% to 100%[57-65]. Specificity ranged from 78% to 100%. There are several factors that affect the sensitivity and specificity to detect a primary gastric carcinoma. Significantly, there is a variable and occasionally intense uptake of FDG of a physiological nature within the gastric wall[61,63,66]. FDG uptake may also correspond to acute inflammation such as superficial or erosive gastritis[67]. This leads to two disadvantages in the detection of gastric cancer. Firstly, an awareness of this phenomenon must exist in order to avoid a high number of false positive diagnoses. Conversely, over-awareness may lead to failure to detect weakly enhancing and diffuse malignant lesions.

TUMOR SIZE AND DEPTH (T STAGE) AND FDG-PET

Tumor size and T stage may influence the sensitivity of PET imaging in the detection of the primary gastric lesion. In one study, sensitivity was as low as 21% for detecting tumors < 30 mm in size, and increased to 76% for lesions over 30 mm[62]. Gastric cancer limited to the mucosa or submucosa (T1 lesions), are less likely to be detected by PET than more advanced T2-T4 lesions. Sensitivity for detection of early gastric cancers (T1) ranges from 26% to 63%, whereas that for more advanced disease (T2-T4) ranges from 83%-98%[57,61,62,65]. Figure 4 graphically depicts the range of sensitivity in diagnosis of early and advanced gastric cancer.

Histological subtype variants also influence glucose uptake and therefore the ability of PET to detect the primary lesion. The ability of FDG-PET to detect non-intestinal gastric primary tumors can range from 0% for T1 non-intestinal primaries to 77% for advanced non-intestinal disease. For intestinal type tumors, sensitivity ranges from 44% for T1 tumors to 92% for T2 or greater disease[62,63,68]. This may relate to the fact that the GLUT-1 transporter has been shown to be preferentially expressed on the intestinal type gastric carcinoma cell subtype, with decreased expression on mucous-secreting and signet ring type cells[69,70]. GLUT-1 expression has been shown in multivariate analysis to be the most influential factor relating to FDG uptake in gastric carcinoma, although the relationship between histological subtype and SUV uptake and sensitivity of FDG-PET has not been consistent across studies[71,59-61].

TECHNIQUES TO IMPROVE DETECTION OF THE PRIMARY GASTRIC LESION

Simple measures such as distention of the stomach by water or, less commonly, food have been shown to improve the accuracy of detection of gastric lesions both pre-operatively and in the post-operative remnant stomach[72-74]. In an effort to improve detection of gastric cancer by PET, the pyrimidine analog FLT has been used as an alternative radiotracer. One study demonstrated increased sensitivity of FLT-PET for detection of gastric tumors, especially if those tumors which were not FDG avid[58]. This may improve detection of previously difficult-to-detect tumor types such as mucin-producing and signet ring cell tumors. A second smaller study showed comparable efficacy between the two moieties[59]. In both studies, mean SUV uptake was lower for FLT-PET than for FDG-PET. Additional improvements may be made

Table 3 Gastric cancer lymph node staging by positron emission tomography

Ref.		*n*	Sensitivity (%) PET	Specificity (%) PET	Sensitivity (%) CT	Specificity (%) CT
Chen *et al*[57]		61	61	92	77	62
Kim *et al*[60]		73	40	95	71	71
Mochiki *et al*[61]		85	23	100	65	77
Mukai *et al*[62]		62	34.50	97	62.10	87.90
Yeung *et al*[64]		23	22	97		
Yoshioka *et al*[75]	Low resolution	42	47	62		
	High resolution	41	73	78		
Yun *et al*[65]		81	35	97	52	94
Tian *et al*[78]		38	60	100		
Yang *et al*[79] (PET-CT)		78	37	97.20	60.50	83.30

PET: Positron emission tomography; CT: Computed tomography.

possible by improving spatial resolution of the imaging equipment[75].

SCREENING FOR GASTRIC CARCINOMA WITH FDG-PET

FDG-PET has not been shown to be an effective screening tool for the diagnosis of gastric cancer. In one study, combined with endoscopy in asymptomatic individuals, PET-CT detected 2/20 cancers from 2861 patients screened giving a sensitivity of only 10% and a positive predictive value of 8.3%; 18/20 cancers were early gastric cancers (T1). There were 22 false positives on this study. There was no significant difference between the SUV values of the false positives and the true positives[76]. A second study of 1336 asymptomatic patients detected two gastric cancers in addition to nine other malignancies. The rate of false positive in this study was three times the rate of true positive findings[77]. Therefore, the screening sensitivity of FDG-PET in an asymptomatic population is less again than that in a diseased population.

FDG-PET AND LYMPH NODE STATUS: GASTRIC ADENOCARCINOMA

Survival in gastric cancer patients decreases with lymph node involvement, and with the number of lymph nodes involved. Knowledge of lymph node status therefore is not only of importance with respect to prognosis, but may also guide surgical treatment planning and which patients may benefit from neoadjuvant chemotherapy.

FDG-PET has been examined both alone, in comparison with CT imaging, and combined as CT-PET, in the preoperative assessment of the nodal status of gastric cancer (see Table 3). The sensitivity of PET is generally low for the detection of lymph node metastases, ranging from 22% to 60% for normal resolution scans[57,60-62,64,65,75,78,79]. It is possible that this may reflect the low spatial resolution of PET at 7 mm-9 mm which leads to difficulty discriminating perigastric lymph nodes from the gastric primary tumor, as sensitivity has been shown to increase to up to 73% with a higher resolution scan[75]. This compares poorly with the sensitivity of CT which ranges from 52% to 77% in the same series. By contrast the specificity of PET is higher than that of CT, ranging from 62%-100%, compared to CT (range, 62%-94%)

The sensitivity and specificity of PET are also influenced by lymph node staging status (i.e., N1, N2, or N3 nodal metastases). In three studies which stratified sensitivity by lymph node status, CT was significantly more sensitive for N1 disease[60,61,65], whereas similar levels of sensitivity and specificity were seen in N3 disease for both imaging modalities; however, this may have reflected the low prevalence of N3 disease in the study groups. Increased SUV of the primary tumor was correlated positively with lymph node metastases in two studies[57,61], possibly indicating increased glucose transport capacity which may in turn correlate with increased aggressiveness of the primary tumor[69].

PERITONEAL DISEASE

A common site of spread for gastric adenocarcinoma is the peritoneum. As many as 25% of patients with locally advanced tumors on EUS will have sub-radiographic occult peritoneal disease that may be identified only at laparoscopy[80]. PET is not a reliable indicator of peritoneal disease, with sensitivity for detection of peritoneal carcinomatosis of between 9% and 30% with normal resolution scans, and increased to 50% sensitivity with the use of a higher resolution 3.9 mm slice. This compares unfavorably with CT which demonstrates a sensitivity of 76%-80% for peritoneal cancer[57,75,81]. Peritoneal lesions are often small and diffuse in nature, which may go some way to explaining the low detection rate. Specificity remains high at 79%-98% in the same series, with less specificity with higher resolution imaging. Due to the need to confirm the absence of metastatic peritoneal spread prior to definitive surgery, staging laparoscopy may still be necessary, as this is the most sensitive modality to evaluate the peritoneum[82,83].

RESPONSE TO TREATMENT

With the introduction of neoadjuvant or perioperative

Table 4 Positron emission tomography computed tomography for the detection of gastric cancer recurrence

Author	Yr	*n*	Discriminating factor	Sensitivity (%) PET	Specificity (%) PET
De Potter *et al*[85]	2002	33		70	69
Jadvar *et al*[90]	2003	16		94	100
Yoshioka *et al*[75]	2003		Liver	78-85	82-74
			Lung	67	88
			Bone	30	82
			Pleural	4	100
			Ascites	24	76
Patriti *et al*[89]	2007	51		100	
Nakamoto *et al*[88]	2009	44	Previous suspicious imaging	80	100
		14	Tumor markers positive	73	83
		26	Routine	50	88
Park *et al*[117]	2009	105		75	77
Sim *et al*[86]	2009	52		68.40	71.40
Sohn *et al*[118]	2009	212	Post ablation	0	

PET: Positron emission tomography.

chemotherapy it is of interest to try to determine those who may respond to such chemotherapy, and those who are likely to fail to respond. This may be crucial in future in order to spare non-responders further potentially toxic chemotherapy, or to switch to another, non cross resistant regimen. The advantage of PET over CT in this regard is that the CT response by RECIST (Response Evaluation Criteria in Solid Tumors) as measured by the change in size may be a late manifestation of a response. PET may demonstrate a decrease in FDG uptake at an earlier stage than could be demonstrated by conventional imaging.

In one study of 44 pure gastric carcinoma patients treated with neoadjuvant cisplatin and 5-fluororuacil, 35 showed FDG uptake at baseline, before the initiation of chemotherapy. The PET response at 14 d post-chemotherapy was correlated with histopathological response at the time of surgery. The PET response was defined as > 35% reduction in the SUV value of the target lesion. A histopathological response was defined as < 10% viable tumor cells remaining in the operative surgical specimen. A metabolic response correctly predicted the histological response after completion of chemotherapy in 10/13 responding and 19/22 non-responding tumors, corresponding with a sensitivity of 77% (95% CI: 46%-95%) and a specificity of 86% (95% CI: 65%-97%)[41]. Metabolic response appeared to correlate significantly with survival. At 2-year follow-up, survival in the metabolic responder group was 90%, compared with 25% in the metabolic non responder group. A second smaller study in the setting of metastatic gastric cancer using chemotherapy and the biologic agent cetuximab demonstrated in this study, PET demonstrated a sensitivity of 83% and a specificity of 75% for the prediction of ultimate best response by RECIST. There was also a significant correlation between metabolic response and progression-free survival in this cohort[84].

FDG-PET AND PREDICTION OF PATIENT SURVIVAL: GASTRIC ADENOCARCINOMA

Data on survival with respect to PET-positive tumors may be confounded by the fact that PET-negative tumors in most studies may represent earlier stage disease. For example, in one study, The 2-year survival rate for patients with PET-positive cancers was 65.9%, and for those with PET-negative cancers was 94.4%, but a significant proportion of PET-negative tumors were T1/T2 *vs* T3/T4 for the tumors visible on PET[61]. One study on recurrent gastric carcinoma with 33 patients showed a higher median survival for those with PET negative recurrence *vs* PET positive recurrence of 18.5 mo *vs* 6.9 mo respectively, however, other studies have failed to corroborate this finding[63,85].

FDG-PET TO DETECT RECURRENCE OF RESECTED DISEASE: GASTRIC ADENOCARCINOMA

When compared to contrast CT, PET showed a non-significant trend towards decreased sensitivity and increased specificity in the detection of recurrent disease. Contrast-enhanced CT was significantly more sensitive for the diagnosis of peritoneal recurrence (87% *vs* 47%)[86]. This concurs with another series demonstrating a high sensitivity of 78% and 67% for liver and lung lesions, respectively, with a lower sensitivity of 30% for bone metastases. Sensitivity for pleural carcinomatosis and ascites were also similarly low[75]. As FDG also demonstrates uptake in acute inflammation and fractures in addition to physiological uptake in the abdomen, this may lead to false positives in the detection of boney disease[87]. Table 4 summarizes these data.

Notably, the utility of FGD-PET in the detection of recurrent gastric cancer is largely dependent on the prevalence of recurrent disease in the screened population. In a population undergoing routine screening examination following definitive primary therapy the sensitivity of screening may be as low as 50%-70%. In contrast, positive predictive value is high in a high prevalence population (i.e., those in whom disease is suspected). This is illustrated when comparing the positive predictive value of 100% in a population with a suspicion of disease based on previous radiological imaging *vs* 25% in a population with no clinical or radiological suspicion of recurrent disease[85,86,88]. If the population undergoing testing has an a priori suspicion of disease based on previous imaging or tumor markers, then sensitivity for detection may reach 94%-100%. Specificity is generally high at 70%-100% for PET in the detection of recurrent disease[89,90].

PANCREAS ADENOCARCINOMA

Pancreatic cancer ranks as one of the most lethal malignancies and only 20% are suitable for resection at presentation. Accurate delineation of tumoral extent and anatomy are crucial prior to surgery in order to avoid potentially futile laparotomy. Conventional work up includes abdominal ultrasound, CT, EUS and MRCP.

PET AND THE DIAGNOSIS AND MANAGEMENT OF PANCREATIC MALIGNANCY

As the normal pancreas exhibits low FDG uptake, and pancreatic tumors have been demonstrated to have high GLUT-1 expression, the expectation is that pancreatic tumors should not be difficult to differentiate from the normal parenchyma by FDG-PET[91]. In an initial study in 1997 by Zimny *et al*[92], 106 patients with pancreatic lesions were examined using FDG-PET; 85% of pancreatic carcinomas were correctly identified, and in 84% of cases of chronic pancreatitis it was possible to exclude malignancy. Ten of 11 false negatives were due to elevated plasma glucose. In patients with normal plasma glucose the sensitivity, specificity, positive and negative predictive values were 98%, 84%, 96% and 93%, respectively. The SUV of carcinoma was significantly higher than that of chronic pancreatitis (6.4 ± 3.6 for pancreatic carcinoma *vs* 3.6 ± 1.7 for chronic pancreatitis ($P < 0.001$). Inokuma *et al*[93] examined the utility of PET in the diagnosis of pancreas cancer in comparison to CT and EUS. In a study of 45 patients PET had a lower sensitivity than EUS, but a higher specificity than all other modalities, and highest positive predictive value and overall accuracy. In a larger study, comparing PET with CT and MRI, the sensitivity of PET was lower than that of CT but higher than that of MRI (91% CT *vs* 82% PET *vs* 78% MRI), and PET had the highest specificity and positive predictive value among the three modalities. There was no correlation between the SUV of the tumor and the degree of differentiation. The ability of PET to detect disease was improved by the correction of SUV for blood glucose[94]. The ability of PET to detect pancreatic cancer may be greater than CT at smaller lesion sizes[95]. In the differentiation of benign *vs* malignant cystic disease of the pancreas, Sperti *et al*[96] showed that PET was superior to CT with respect to sensitivity, specificity, positive and negative predictive value, and diagnostic accuracy at 94%, 94%, 89%, 97%, and 94%, respectively; these figures for CT were 65%, 88%, 73%, 83%, and 80%. A review by Gambhir *et al* suggested a sensitivity of 94% and a specificity of 90% for PET when compared with that of CT (84% and 75%, respectively)[97]

FDG-PET AND STAGING: PANCREAS ADENOCARCINOMA

FDG-PET is not the preferred modality to stage the depth of invasion or invasion of local-regional structures the primary tumor of the pancreas due to its poor spatial resolution. At this time, thin slice CT or EUS are better able to delineate the anatomical boundaries of the primary tumor and thus resectability. Similarly, PET is poorly sensitive for the detection of loco-regional lymph node metastases, which may be due to their proximity to the primary lesion. Sensitivity has ranged from as low as 49% to as high as 76% for the detection of local field lymph node involvement[98,99]. For pancreatic tumors, similar to gastric adenocarcinoma, FDG-PET is sensitive for the detection of metastatic disease to the liver and bone, but less so to the peritoneum. In a series of 168 patients Fröhlich *et al*[100] determined PET had a sensitivity of 97% for hepatic lesions > 1 cm, but only 43% for those < 1 cm, with 95% specificity. Three quarters of false positives were due to intrahepatic cholestasis. A study of 59 patients by Diederichs *et al*[99] confirmed these findings, with an overall sensitivity for the detection of hepatic metastases of 70%, again missing some metastases < 1 cm in diameter. The sensitivity for the detection of peritoneal disease was 25%.

IS SUV UPTAKE PROGNOSTIC IN PANCREATIC CANCER?

An SUV cut-off of ⩾ 4.0 was used by Sperti and colleagues to characterize patients with pancreatic cancer into two groups. Those with an SUV ⩾ 4.0 had an overall survival of only 7 mo, compared to 32 mo in the lower SUV group. This applied also to those who underwent resection. Tumor SUV was confirmed in multivariate analysis to be an independent predictor of survival[96]. This is in agreement with data published by Nakata *et al*[101] for patients with inoperable pancreatic tumors, in which those with a tumor SUV of > 3.0 were shown to have inferior survival to those with SUV uptake of < 3.0. In contrast to many other malignancies, proliferative activity as measured by the Ki67 index did not correlate with FDG uptake in pancreatic tumors[102].

PET AS A PREDICTOR OF RESPONSE TO CHEMOTHERAPY: PANCREAS ADENOCARCINOMA

PET has been used in an attempt to measure the response to neoadjuvant chemoradiotherapy in pancreatic cancer. In a study of 20 patients with locally advanced pancreas adenocarcinoma, of those who had > 50% reduction from the baseline SUV, 10% had a complete surgical resection, compared to 6% of those who had < 50% reduction. Those with a significant response also had a 23.2 mo survival compared to 11.3 mo in those who did not respond[103]. This is in agreement with a study by Bang *et al* which demonstrated the superiority of PET in the detection of a treatment response to chemoradiotherapy, detecting a response in one-third of patients, where conventional

CT failed to detect any response. Those who developed a response on PET also had significantly longer survival than those who did not. The PET and tumor marker response following palliative chemotherapy were also correlated positively with patient survival in a recent Japanese study[104] which contrasts with results of a study by Kobayashi *et al*[105] in which only a fall in tumor markers and not SUV was correlated with survival.

DETECTION OF RECURRENT DISEASE

Ruf *et al*[106], in a study of 31 patients with suspected recurrence after surgery, demonstrated that PET was superior to the combination of CT and MRI in the detection of recurrence (96% *vs* 39%). CT/MRI failed to detect any local recurrence, but did perform well in the detection of small hepatic metastases when compared to PET (92% *vs* 42%). Thus PET may be superior in the detection of recurrence within the tumor bed, but CT/MRI may have better discriminatory power within the hepatic parenchyma. PET may also complement the use of tumor markers or CT for the detection of recurrent disease when CT findings are equivocal, as demonstrated in a small study by Rose *et al*[95], where PET detected 100% of recurrences felt to be equivocal on CT. In a recent study of 45 patients with suspected recurrent disease, PET fused with contrast CT was shown to have a sensitivity of 94.7% for the detection histologically proven metastatic disease. Notably there was also a high sensitivity in this study for the detection of all sites of recurrence, with sensitivity for detection of local recurrence, abdominal lymph node metastasis, and peritoneal dissemination being 83.3%, 87.5%, and 83.3%, respectively[107].

METHODS OF IMPROVING THE ACCURACY OF PET IN PANCREAS CANCER

Although PET is superior to CT for the differentiation of benign *vs* malignant lesions, false positives may occur, most commonly due to pancreatitis, post instrumentation of the biliary tree, due to retroperitoneal fibrosis or hemorrhage or inflammation of a pancreatic pseudocyst. If C-reactive protein serum levels are elevated, the specificity of PET may fall to 50%[108]. Using delayed PET may aid in the differentiation of benign *vs* malignant lesions as evidenced in a prospective series of 47 patients where the diagnostic accuracy for malignant *vs* benign disease was 91.5% using this method[109]. Optimal glycemic control is also an important factor in the accuracy of PET scanning in pancreatic disease as noted in the study by Zimny where 91% of false negative results were due to hyperglycemia reducing the sensitivity of PET from 96% to 63% in those with an abnormally high serum glucose[92].

The fusion of PET-CT may show promise. A retrospective study by Lemke *et al*[110] showed that use of PET-CT improved the sensitivity of either individual imaging modality. Sensitivity was 76% for CT, 84% for PET and 89% for PET-CT but this came at a cost of a loss of specificity. Addition of CT imaging to fusion PET-CT may lead to further gains. In another study the sensitivity for the detection of metastatic disease by PET-CT, CT, and PET-CT plus CT was 61%, 57%, and 87%, respectively[111]. Enhanced PET-CT has also been shown to be superior to PET alone compared to unenhanced PET-CT imaging in two studies[107,112]. Use of the alternative radiotracer FLT has not been shown to be of benefit in pancreas cancer. In a small pilot study, FLT-PET demonstrated low levels of uptake in the primary tumor and detected only 40% of primary pancreatic tumors compared to 100% with FDG-PET[113].

CONCLUSION

FDG-PET imaging is now a standard practice in staging cancers of the esophagus. The role of FDG-PET/CT imaging in staging gastric carcinoma, however, is complicated by the higher rate of FDG-non-avid malignancies and by the false positive rate within the stomach due to inflammatory conditions. For each upper GI malignancy, depth of invasion and nodal status are not well evaluated by FDG-PET scans. However, for locally-advanced malignancies, an FDG-PET scan may be used to identify occult metastatic disease which may then significantly then change the treatment plan. A newer application of this imaging modality is the assessment of metabolic response, which correlates with chemotherapy sensitivity and survival. Preliminary prospective clinical studies suggest FDG-PET scans can predict response to therapy. With these data, the utility of FDG-PET scanning in upper GI malignancies is increasingly commonplace. With the identification of new FDG-PET tracers, we expect a further expansion of the application of PET imaging in upper GI malignancies.

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ORIGINAL ARTICLE

Osteopontin expression is associated with hepatopathologic changes in *Schistosoma japonicum* infected mice

Bo-Lin Chen, Gui-Ying Zhang, Wei-Jian Yuan, Shi-Ping Wang, Yue-Ming Shen, Lu Yan, Huan Gu, Jia Li

Bo-Lin Chen, Gui-Ying Zhang, Wei-Jian Yuan, Lu Yan, Huan Gu, Jia Li, Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China
Shi-Ping Wang, Department of Pathogenic Biology, College of Xiangya Basic Medicine, Central South University, Changsha 410008, Hunan Province, China
Yue-Ming Shen, Department of Gastroenterology, Changsha Central Hospital, Changsha 410004, Hunan Province, China
Author contributions: Chen BL designed the research, performed the majority of the experiments, and wrote the manuscript; Zhang GY guided the research; Wang SP provided many facilities in animal modeling; Yuan WJ was involved in editing the manuscript; Shen YM, Yan L, Gu H, and Li J helped perform the research and participated in data analysis.
Supported by Grants from the National Natural Science Foundation of China, No. 81072038/H1617
Correspondence to: Gui-Ying Zhang, Dr., Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China. guiyingzhang@hotmail.com
Telephone: +86-0731-84327282 Fax: +86-0731-84327321
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Abstract

AIM: To investigate osteopontin expression and its association with hepatopathologic changes in BALB/C mice infected with *Schistosoma japonicum*.

METHODS: The schistosomal hepatopathologic mouse model was established by abdominal infection with schistosomal cercaria. Liver samples were obtained from mice sacrificed at 6, 8, 10, 14, and 18 wk after infection. Liver histopathological changes were observed with hematoxylin-eosin and Masson trichrome staining. The expression of osteopontin was determined with immunohistochemistry, reverse transcription-polymerase chain reaction, and Western blotting. The expression of α-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) were determined by immunohistochemistry. Correlations of osteopontin expression with other variables (α-SMA, TGF-β1, hepatopathologic features including granuloma formation and degree of liver fibrosis) were analyzed.

RESULTS: Typical schistosomal hepatopathologic changes were induced in the animals. Dynamic changes in the expression of osteopontin were observed at week 6. The expression increased, peaked at week 10 ($P < 0.01$), and then gradually decreased. Positive correlations between osteopontin expression and α-SMA ($r = 0.720$, $P < 0.01$), TGF-β1 ($r = 0.905$, $P < 0.01$), granuloma formation ($r = 0.875$, $P < 0.01$), and degree of liver fibrosis ($r = 0.858$, $P < 0.01$) were also observed.

CONCLUSION: Osteopontin may play an important role in schistosomal hepatopathology and may promote granuloma formation and liver fibrosis through an unexplored mechanism.

Key words: *Schistosoma japonicum*; Granuloma; Liver fibrosis; Osteopontin; BALB/C mice

Peer reviewers: Satoshi Mamori, MD, PhD, Department of Gastroenterology and Hepatology, Shinko Hospital, 1-4-47 Wakihama-cho, Chuo-ku, Kobe, Hyogo 651-0072, Japan; Hussein M Atta, MD, PhD, Professor, Department of Surgery, Faculty of Medicine, Minia University, Misr-Aswan Road, El-Minia 61519, Egypt

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INTRODUCTION

Schistosomiasis remains a huge threat to human health in tropical areas. Almost 12% of the population is in danger of this disease, with more than 200 million people infected annually[1]. In the past few decades, this disease has re-emerged and is still endemic in marsh, lake, and even mountainous regions of China, causing social hardship and economic burden[2]. It is now believed that the immune response of humans to schistosome eggs and the granulomatous responses they induce are the major causes of pathology in schistosomiasis[3]. The granulomas that form around the eggs impair blood flow in the liver and consequently induce portal hypertension[4]. On the other hand, the granulomas destroy the eggs and sequester or neutralize pathogenic egg antigens, leading to fibrosis in host tissues[5]. Once the immune response is activated, it is unlikely to be self-limiting, and schistosomiasis liver damage will continue, even after effective insecticide treatment[6]. Increasing reports of praziquantel treatment failures have highlighted the need for advanced knowledge of schistosomal hepatopathologic mechanisms and for new therapeutic strategies.

Osteopontin is granulomatogenic and has chemokine functions (mediating T lymphocyte and macrophage migration), cytokine activity (modulating T-helper 1 and 2 cytokine production), and several inflammatory and anti-inflammatory effects (regulation of nitric oxide generation)[7]. Recent work has demonstrated the important role osteopontin plays in mediating hepatic inflammation[8]. Upregulation of osteopontin expression early in the development of steatohepatitis, and its possible role in signaling the onset of liver injury and fibrosis in experimental nonalcoholic steatohepatitis have been reported[9].

These limited findings led us to hypothesize that osteopontin may be engaged in the immunopathogenesis of schistosomiasis liver damage. In our current study, we investigated the dynamic changes in osteopontin expression in *Schistosoma japonicum* (*S. japonicum*)-infected mouse liver. We also examined the relationship between osteopontin and hepatopathology and potential promoters of fibrosis progression such as hepatic stellate cells (HSCs) and transforming growth factor-β1 (TGF-β1) to obtain possible clues for further studies on the cellular and molecular mechanisms involved in schistosomal hepatopathology.

MATERIALS AND METHODS

Parasite and laboratory animals

Six-week-old BALB/C female mice were purchased from the Experimental Animal Center (Central South University, Changsha, Hunan, China). All animal experiments were performed in accordance with the Chinese Council on the Animal Care Guide for the Care and Use of Laboratory Animals. *Oncomelania hupensis* harboring *S. japonicum* cercariae were obtained from the Center for Schistosomiasis Control and Prevention (Yueyang, Hunan, China).

Animal treatment

One hundred BALB/C mice were randomly divided into the control group and the model group (n = 50 each). Mice in the model group were percutaneously infected with *S. japonicum* by placing a glass slide carrying 15 ± 1 cercariae in non-chlorine water on its abdomen for 20 min. Mice in the control group were treated with non-chlorine water containing no cercariae. All mice were kept at 20-25 ℃ in a 12-h light/12-h dark cycle with free access to food and water. At 6, 8, 10, 14, and 18 wk after infection, 10 mice from each group were randomly selected and sacrificed. Liver tissues were extracted and cut into two parts: the left lobes of the liver were fixed in 4% paraformaldehyde for 12 h; the remaining portion of the liver was preserved at -80 ℃ until use.

Histopathological study

Paraformaldehyde-fixed liver specimens were dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks, cut into 5-μm thick sections, and placed on glass slides. The sections were then stained with hematoxylin and eosin (HE) and Masson trichrome (MT) according to standard procedures. To describe and evaluate liver pathological changes, a pathologist who was blinded to the research design examined 10 different low-power fields of HE- and MT-stained sections (selected fields were in almost the same location) for each mouse. In addition, the percentage of collagen calculated by a multimedia color image analysis system (Image-Pro Plus 6.0) was measured as a relative objective index (because a histological/fibrosis score that is evaluated by pathologists is susceptible to the ability and subjective judgment of the pathologist) to evaluate the degree of liver fibrosis. Each MT-stained section was examined at 100 × magnification. Every field analyzed contained a granuloma, portal area, or a centrilobular vein. Fibrotic areas were scanned and summed by the software. The percentage of collagen was expressed as the ratio of the collagen-containing area to the whole area, and the result was determined as the mean of ten different fields of each section. Furthermore, the granuloma dimension was also measured at a magnification of 100× using an ocular micrometer. Only non-confluent granulomas containing eggs in their centers were measured[10]. Granuloma dimension = maximum width × maximum length. Mean granuloma dimension of each section = sum of all granuloma dimensions in each section/number of granuloma in each section.

Immunohistochemistry

Immunohistochemical staining was performed with the PV-6001/6002 Two-Step IHC Detection Reagent (ZSGB-BIO, China). The sections were dewaxed, dehydrated, immersed in citrate buffer (0.01 mol/L, pH 6.0), heated at 100 ℃ in a microwave oven 6 × 2 min, incubated in 3% H_2O_2 in deionized water for 10 min to block endogenous peroxidase activity, and washed 2 × 3 min with phosphate-buffered saline (PBS). The sections were

then incubated overnight at 4 ℃ with antibodies against osteopontin (mouse monoclonal; 1:300; Santa Cruz Biotechnology, United States), α-SMA (mouse monoclonal; 1:300; Santa Cruz Biotechnology), and TGF-β1 (rabbit polyclonal; 1:300; Santa Cruz Biotechnology). After washing 2 × 3 min with PBS, the appropriate second antibody was added to the sections and incubated at 37 ℃ for 30 min. Then, the sections were washed 2 × 3 min with PBS, and the color was developed with diaminobenzidine (DAB) for about 5 min. Nuclei were lightly counterstained with hematoxylin. Negative controls included incubation with PBS without the primary antibody. The integral optical density (IOD) was measured with Image-Pro Plus 6.0, and the result was determined as the sum of five different fields (one in the center and four in the periphery) of each section. The IOD of the target protein was defined as the sum of the optical densities of all the positive pixels in the image, which represents the quantity of the targeted protein. The IOD is considered to be more accurate than average optical density as it considers both the intensity and area.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from frozen liver tissue with TRIZOL Reagent (Invitrogen, United States). Complementary DNA (cDNA) was synthesized from total RNA using a ReverTra Ace-α-TM First Strand cDNA Synthesis kit (Toyobo, Japan). Relative quantification of target gene expression was performed using the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The primer sequences were osteopontin forward 5'-CCAGGTTTCTGATGAACAGT-3' and reverse 5'-GTGTGTTTCCAGACTTGGTT-3', which yielded a fragment of 193 bp, and GAPDH forward 5'-AACTTTGGCATTGTGGAAGG-3' and reverse 5'-GGATGCAGGGATGATGTTCT-3', which yielded a fragment of 132 bp. For the first step, the following components were mixed to obtain the specified concentrations in a final 20 μL reaction volume: 1 μL denatured total RNA (1 μg/μL), 4 μL 5 × buffer, 2 μL dNTP mixture (10 mmol/L), 1 μL RNase inhibitor (10 U/μL), 10 μL RNase-free H_2O, 1 μL Oligo $(dT)_{20}$ (10 pmol/μL), and 1 μL ReverTra Ace. The reaction was performed at 42 ℃ for 20 min, followed by 99 ℃ for 5 min, and 4 ℃ for 5 min. In the second step, 1 μL cDNA was mixed with 0.5 μL each sense and anti-sense primers (100 μmol/L each), 2 μL dNTP mixture (2 mmol/L), 1.5 μL $MgCl_2$ (25 mmol/L), 2 μL 10 × polymerase chain reaction (PCR) buffer, 0.5 μL Taq DNA Polymerase (500 U), and 12 μL PCR H_2O. PCR was performed as follows: denaturation at 95 ℃ for 5 min; 32 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and elongation at 72 ℃ for 30 s; and final elongation at 72 ℃ for 5 min. The PCR products were separated by electrophoresis on 1.5% agarose gels (sample volume: 10 μL, voltage: 120 V) and visualized with ethidium bromide staining and ultraviolet illumination. We used gel OD analysis software (Gel-Pro 4.0) to scan and calculate the IOD of strips. The relative mRNA expression of osteopontin was represented as the ratio of osteopontin:IOD and GAPDH:IOD.

Western blotting

Frozen tissue specimens (500 mg) were homogenized on ice in 1 mL lysate prepared from a Total Protein Extraction kit (ProMab, United States) and then ultrasonicated for 3 × 3 s. The crude protein fractions were obtained by centrifuging the homogenates at 9000 × *g* for 10 min at 4 ℃. The supernatant was used as the protein fraction. Gel samples were prepared by mixing protein samples with sample buffer and boiling at 100 ℃ for 3 min. Nuclear and cytoplasmic proteins were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis in running buffer. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Pierce, United States) in transfer buffer at 300 mA constant current for 70 min on ice. Non-specific binding sites were blocked by incubating in PBS containing 5% nonfat milk for 2 h at 37 ℃. Membranes were then incubated with primary antibodies (mouse osteopontin monoclonal; 1:500; Santa Cruz Biotechnology and mouse monoclonal GAPDH; 1:1000; ProMab, United States) overnight at 4 ℃. The membranes were then washed 5 × 4 min with PBS-Tween 20 (PBST) and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; 1:50 000; Zymed, United States) for 1 h at 37 ℃. After the membranes were washed 5 × 4 min in PBST, enhanced chemiluminescence detection of the target protein was performed. The film was scanned, and the image was analyzed with Gel-Pro 4.0. The relative levels of osteopontin were represented as the ratio of osteopontin:IOD and GAPDH:IOD.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Data were expressed as mean ± SD. A normality test was performed before statistical analysis. Comparisons between groups and time points were performed using one-way analysis of variation (homogeneity of variance: S-N-K; heterogeneity of variance: Tamhane). Correlation analysis was performed with linear regression. *P* values less than 0.01 (heterogeneity of variance) or 0.05 were considered statistically significant.

RESULTS

Schistosomal hepatopathology

Both HE and MT staining revealed a parallel change over time (Figure 1, left and middle). The control group showed normal hepatocyte morphology (Figure 1A), but the model group showed typical hepatopathological characteristics of schistosomiasis with remarkable acute granuloma formation and subsequent liver fibrosis from week 6 through week 18 (Figure 1B-F). At week 6, inflammatory cells had infiltrated around the schistosome eggs and formed granulomas, which were mainly distributed in portal areas; collagen fibers were only interspersed among the periphery of the granulomas (Figure 1B). The granuloma size and the quantity of inflammatory

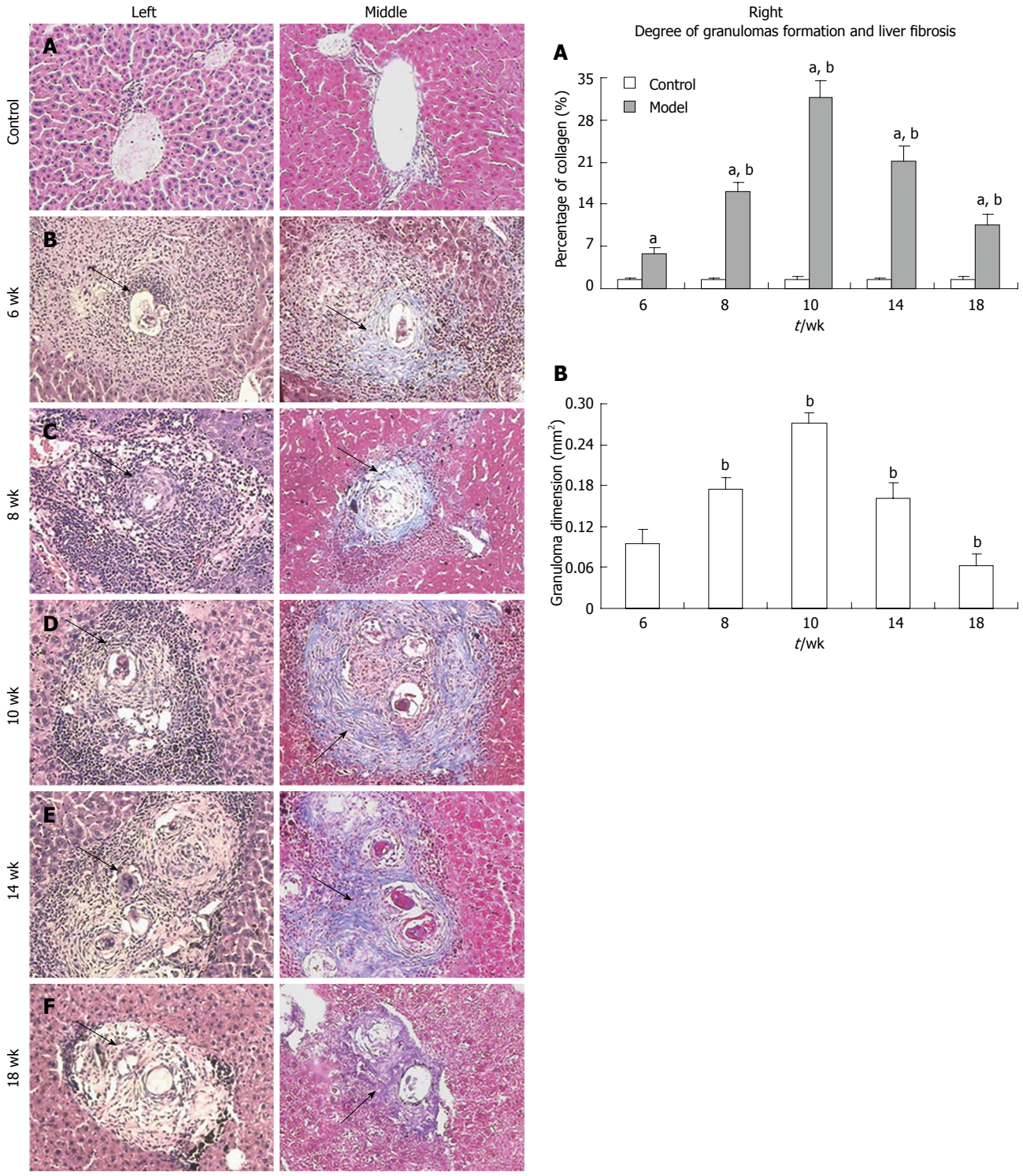

Figure 1 Representative images of hepatopathological changes over time. HE (left) and MT (middle) staining and data showing the degree of granuloma formation and liver fibrosis (right). Arrows show granulomas. Collagen fibers are stained blue (MT staining). 100 × original magnification. HE: Hematoxylin and eosin; MT: Masson trichrome. [a]$P < 0.05$ *vs* control; [b]$P < 0.05$ *vs* previous.

cells increased at week 8. Numerous fibrocytes appeared at the periphery of the lesions, and the collagen fibers became longer and thicker (Figure 1C). The granulomas reached their peak in size and quantity at week 10 (Figure 1D). Numerous inflammatory cells such as neutrophils, lymphocytes, and eosinophils were seen infiltrating in the granulomas, and numerous collagen fibers were wrapped or stretched into the interior of the granulomas. Some fibers extended from portal areas or inflammatory lesions to the lobule, which had been cut apart and re-built. In some serious cases, pseudolobules formed. At week 14, fibrocytes and collagen fibers eventually became the predominant feature of granulomas and formed typical chronic granulomas, whereas other cell types decreased in

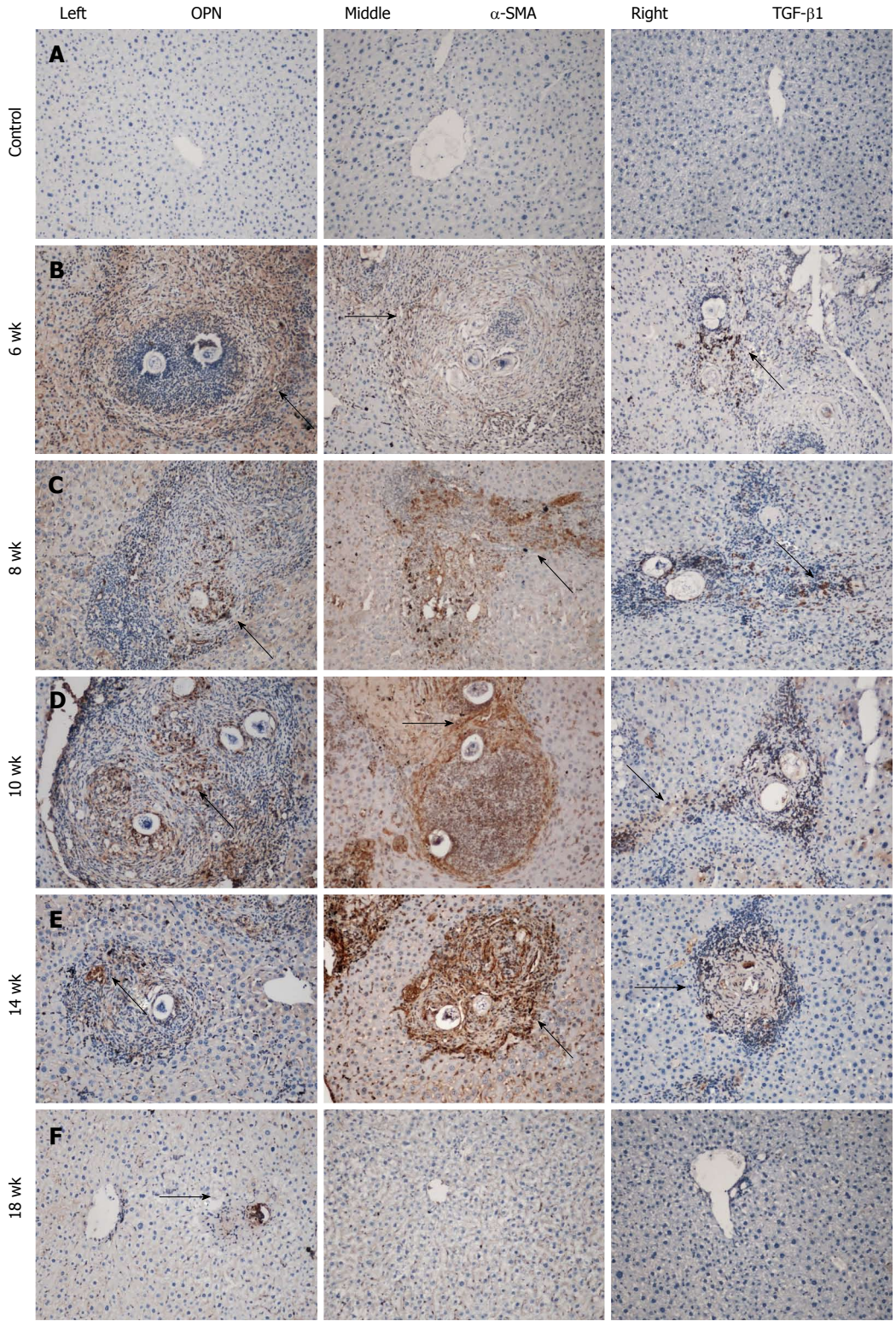

Figure 2 Representative images of immunostaining for osteopontin, α-SMA, and TGF-β1 over time in mouse liver. Positive staining is yellow brown, and arrowheads show positive cells. 100 × original magnification. OPN: Osteopontin; SMA: Smooth muscle actin; TGF: Transforming growth factor.

number (Figure 1E). The schistosome eggs degenerated and disintegrated at week 18, and fibrosis was obviously reduced but more stable (Figure 1F). The collagen percentage and the granuloma dimension in each group also showed a similar change over time (Figure 1, graphs A and B, right).

Expression of osteopontin, α-SMA, and TGF-β1 with immunohistochemistry

Immunohistochemistry for osteopontin, α-SMA, and TGF-β1 demonstrated a similar change that paralleled the development of hepatopathology over time (Figure 2A-F, left to right). Few if any, scarcely distributed cells with

Table 1 Integral optical density of immunostaining in the groups over time

Groups	Staining	Week 6	Week 8	Week 10	Week 14	Week 18
Control group IOD	Osteopontin (× 10^2)	0.52 ± 0.06	0.55 ± 0.07	0.56 ± 0.06	0.50 ± 0.06	0.50 ± 0.06
	α-SMA (× 10^2)[3]	0	0	0	0	0
	TGF-β1 (× 10^3)	0.20 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	0.20 ± 0.02
Model group IOD	Osteopontin (× 10^2)	6.45 ± 0.54[1,2]	10.21 ± 0.80[1,2]	31.20 ± 2.83[1,2]	6.00 ± 0.54[1,2]	2.26 ± 0.28[1,2]
	α-SMA (× 10^2)	0.93 ± 0.09[2]	18.19 ± 1.62[2]	39.13 ± 4.37[2]	30.93 ± 3.87[2]	1.45 ± 0.16[2]
	TGF-β1 (× 10^3)	2.52 ± 0.24[1,2]	4.90 ± 0.50[1,2]	9.20 ± 1.25[1,2]	3.84 ± 0.36[1,2]	0.19 ± 0.02[2]

[1]Compared with control group: $P < 0.01$; [2]Compared with previous time point: $P < 0.01$; [3]The staining of α-SMA in the control group was measured, and the result was zero. Results are expressed as the mean IOD (× 10^2 or × 10^3) ± SD. SMA: Smooth muscle actin; IOD: Integral optical density.

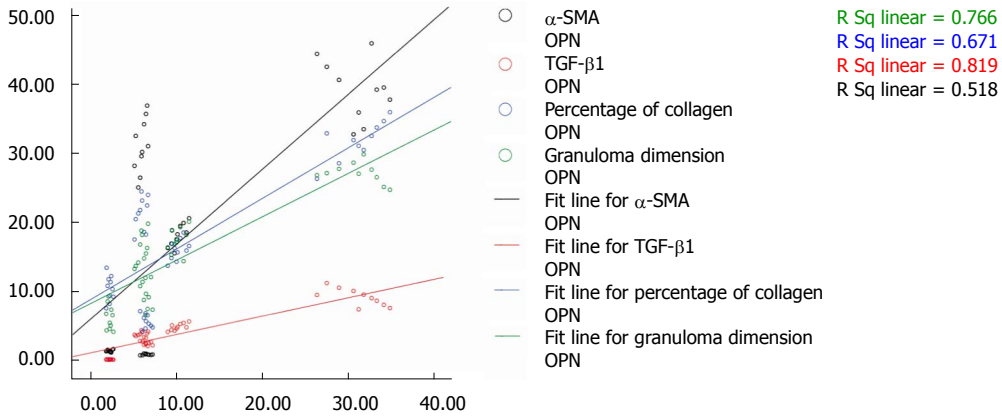

Figure 3 Correlation of osteopontin mRNA expression with α-SMA, TGF-β1, percentage of collagen, and the granuloma dimensions in the model group. x-axis: IOD of osteopontin (× 10^2); y-axis: Black line: IOD of α-SMA (× 10^2); Red line: IOD of TGF-β1 (× 10^3); Blue line: Collagen (%); Green line: Granuloma dimension, mm^2 (× 10^2). OPN: Osteopontin; SMA: Smooth muscle actin; TGF: Transforming growth factor.

faint staining were seen in the normal control group (Figure 2A, left to right) throughout the experiment. At week 6 in the model group, positively stained cells were widely distributed in the area of inflammatory cell infiltration, which formed acute granulomas (Figure 2B, left to right). Strongly upregulated expression of these proteins was seen from week 8 to week 10. Many densely stained positive cells surrounded and infiltrated into the egg granulomas, accumulated in fibrotic areas, and stretched along the fibrous septum (Figure 2C and D, left to right). At week 14, there were still many positive cells distributed in the fibrotic granulomas and dispersed at the periphery of the granulomas. However, the expression weakened and returned to near normal levels at week 18 (Figure 2E and F, left to right). The IODs of immunostaining of individual proteins in the groups over time are shown in Table 1.

Correlation analysis

Correlation analysis revealed that the expression of osteopontin was positively correlated with expression of α-SMA and TGF-β1, and with hepatopathological changes (Figure 3). The R-square values were 0.720, 0.905, 0.815, and 0.875 for osteopontin expression with α-SMA, TGF-β1, percentage of collagen, and granuloma dimension, respectively ($P < 0.01$), implying that the expression of osteopontin may play an important role in the development of liver damage in *S. japonicum*-infected mice in this study.

Expression of osteopontin mRNA (RT-PCR) and protein (Western blotting)

Consistent with the above results, parallel changes were seen in the expression of osteopontin mRNA and protein (Figure 4). The control group showed no changes in expression throughout the experiment, but the expression levels of both osteopontin mRNA and protein in the model group were upregulated at week 6, reached a peak at week 8 ($P < 0.05$), and decreased gradually. Expression levels remained higher than those in the control group ($P < 0.05$).

DISCUSSION

Osteopontin is considered a strong chemoattractant and proinflammatory molecule that is involved in a wide range of physiologic and pathologic events, including angiogenesis, tumor metastasis, wound healing, tissue remodeling, and fibrosis[11-13]. Osteopontin is believed to constitute the central pathway of HSC activation, a key step in the development of hepatic fibrosis[14,15]. However, the role of osteopontin in schistosomal liver damage should be explored further.

In our current study, typical hepatopathological changes were induced in *S. japonicum*-infected animals. Over time during the experiment, the infected liver showed granuloma formation and obvious fibrosis that appeared

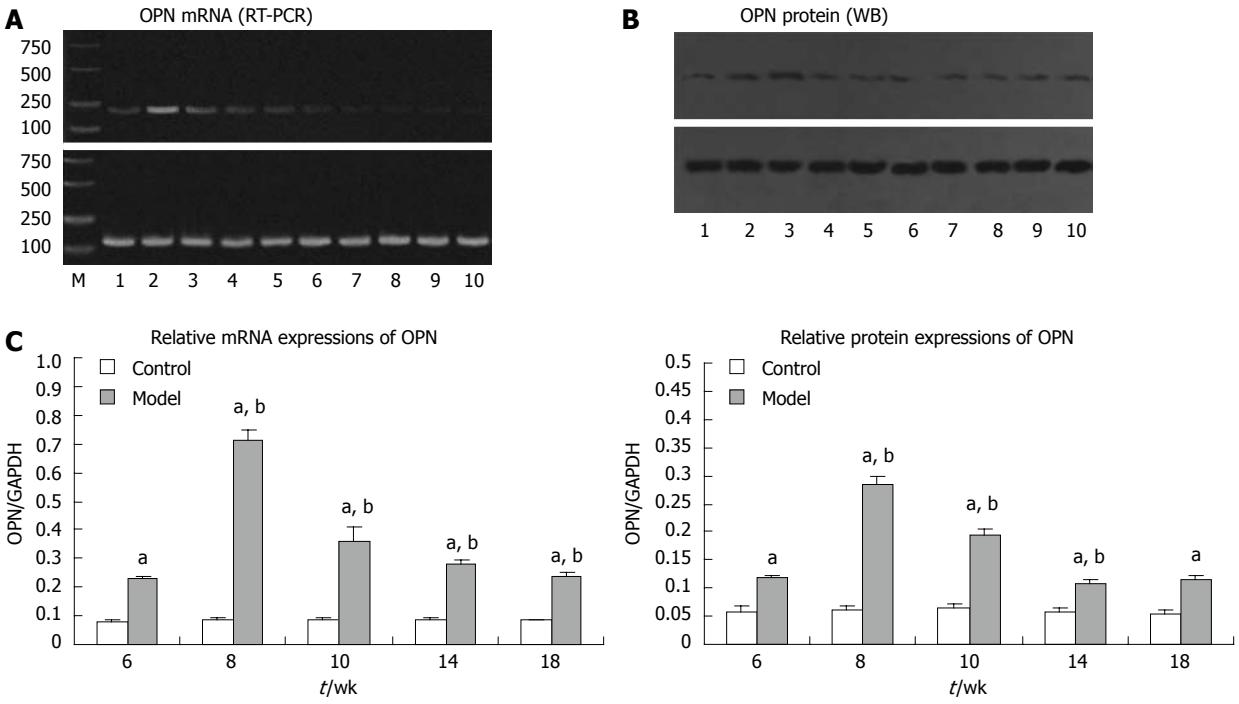

Figure 4 Profiles of osteopontin mRNA (RT-PCR) and protein (Western blotting) expression. No. 1-5 represent the model group at week 6, 8, 10, 14, and 18, respectively; No. 6-10 represent the control group at week 6, 8, 10, 14, and 18, respectively. A: Expression of osteopontin and GAPDH mRNA over time, the 100-bp GAPDH mRNA fragment was used as an internal control; B: Expression of osteopontin and GAPDH protein over time, the 37-kDa GAPDH band was used as an internal control; C: The IOD of osteopontin/GAPDH was expressed as the mean ± SD. M: Marker; n = 10 at each time. OPN: Osteopontin; RT-PCR: Reverse transcription polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IOD: Integral optical density. $^{a}P < 0.05$ *vs* control; $^{b}P < 0.05$ *vs* previous.

at week 6 and peaked from week 8 to 14. Accompanying this pathological change, osteopontin expression (mRNA and protein) was highly upregulated and showed a strong correlation with pathologic changes in the liver and with the immunohistochemistry profiles of α-SMA and TGF-β1. Thus, our data imply involvement of osteopontin in the development of schistosomal hepatopathologic changes.

Determining the mechanisms and the detailed biological role of osteopontin in the process of schistosomal hepatopathology is beyond the scope of our current experiment, but other reports suggest some possibilities. Niki *et al*[16] reported that α-SMA is significantly increased following HSC activation and is considered a marker for HSC activation. TGF-β1 is not only upregulated following HSC activation, but also directly activates HSCs through the TGF-β1/Smad signal transduction pathway, leading to hepatic fibrosis[17]. The upregulated expression of both α-SMA and TGF-β1 and their positive correlation with osteopontin expression observed in this study add more evidence supporting their important role in the process of schistosomal hepatopathology and liver fibrosis.

In summary, typical schistosomal hepatopathological changes occurred during this experiment. The development of hepatopathology, including granuloma formation and liver fibrosis, was accompanied by dynamic expression of osteopontin, which correlated well with the expression of both α-SMA and TGF-β1 over time. Thus, osteopontin may play a key role in schistosomal hepatopathology, the mechanisms of which will require additional studies.

COMMENTS

Background

There are currently few effective therapies for *Schistosoma japonicum* (*S. japonicum*)-infected patients due to a lack of understanding of appropriate intervention targets. Further studies on new cellular and molecular mechanisms are urgently needed as the global *S. japonicum* epidemic is becoming more serious.

Research frontiers

Osteopontin, which is a chemoattractant and proinflammatory molecule, has been shown to be involved in tissue injury and remodeling in other diseases. However, its roles in schistosomal liver damage have yet to be explored.

Innovations and breakthroughs

This study first reports the dynamic changes in osteopontin expression (mRNA and protein) and its associations with pathologic changes in the liver of BALB/C mice infected with *S. japonicum*. This study analyzes the correlations of osteopontin expression with α-SMA and TGF-β1 expression and hepatopathologic features including granuloma formation and liver fibrosis.

Applications

By increasing our understanding of whether and how osteopontin is involved in schistosomal liver damage, this study may provide clues for further studies on the cellular and molecular mechanisms of schistosomal hepatopathology.

Peer review

The study is novel and the methodology is good.

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BRIEF ARTICLE

Hepatocellular carcinoma in cirrhotic patients with portal hypertension: Is liver resection always contraindicated?

Andrea Ruzzenente, Alessandro Valdegamberi, Tommaso Campagnaro, Simone Conci, Silvia Pachera, Calogero Iacono, Alfredo Guglielmi

Andrea Ruzzenente, Alessandro Valdegamberi, Tommaso Campagnaro, Simone Conci, Silvia Pachera, Calogero Iacono, Alfredo Guglielmi, Department of Surgery, Division of General Surgery "A", GB Rossi Hospital, University of Verona, 37134 Verona, Italy

Author contributions: Guglielmi A, Iacono C and Ruzzenente A designed the research and supervised this work; Valdegamberi A and Campagnaro T wrote the paper; Conci S and Pachera S performed the research; Ruzzenente A analyzed the data.

Correspondence to: Andrea Ruzzenente, MD, Department of Surgery, Division of General Surgery "A", GB Rossi Hospital, University of Verona, 37134 Verona, Italy. andrea.ruzzenente@fastwebnet.it

Telephone: +39-45-8124941 Fax: +39-45-585612

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Abstract

AIM: To analyze the outcome of hepatocellular carcinoma (HCC) resection in cirrhosis patients, related to presence of portal hypertension (PH) and extent of hepatectomy.

METHODS: A retrospective analysis of 135 patients with HCC on a background of cirrhosis was submitted to curative liver resection.

RESULTS: PH was present in 44 (32.5%) patients. Overall mortality and morbidity were 2.2% and 33.7%, respectively. Median survival time in patients with or without PH was 31.6 and 65.1 mo, respectively (P = 0.047); in the subgroup with Child-Pugh class A cirrhosis, median survival was 65.1 mo and 60.5 mo, respectively (P = 0.257). Survival for patients submitted to limited liver resection was not significantly different in presence or absence of PH. Conversely, median survival for patients after resection of 2 or more segments with or without PH was 64.4 mo and 163.9 mo, respectively (P = 0.035).

CONCLUSION: PH is not an absolute contraindication to liver resection in Child-Pugh class A cirrhotic patients, but resection of 2 or more segments should not be recommended in patients with PH.

Key words: Liver surgery; Hepatic resection; Hepatocellular carcinoma; Portal hypertension

Peer reviewers: Chun-Qing Zhang, Professor, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China; Faisal M Sanai, Assistant Professor, Hepatobiliary Sciences, King Abdulaziz Medical City, PO Box 22490, Riyadh 11462, Saudi Arabia

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide[1-3]. Surgical treatment is an effective treatment for HCC and the mortality after surgery has decreased in recent years in relation to improved surgical techniques and peri-operative management of patients[4-6]. The overall survival at 5 years after liver resection varies from 33% to 69% according to recent surgical series, although recurrence is still the major issue after surgery[7-13].

The indications for surgical resection depend on the characteristics of the tumor (stage, number of nodules, size, presence of vascular invasion), on the general condition of patient, and on liver functional reserve[14].

The presence of liver cirrhosis is the most important risk factor for the development of HCC, as 85%-95% of HCCs arise in cirrhotic livers[15-17]. HBV and HCV infections and alcohol abuse are the most frequent causes of cirrhosis: about 80%-85% of cases[7]. Portal hypertension (PH) is related to an increase in intrahepatic resistance due to the structural subversion of the liver and loss of vascular bed, and bleeding from gastroesophageal varices is one of the most important complications of cirrhosis[18]. On the basis of several studies[19,20], the American Association for the Study of Liver Diseases (AASLD)[21] defined as clinical PH the presence of esophageal varices or thrombocytopenia (platelet count < 100 000/mm^3) associated with splenomegaly. The European Association for the Study of the Liver (EASL)/AASLD guidelines consider PH as a relative contraindication to liver resection, because of the high risk of postoperative liver failure, as reported in some clinical series[21,22]. These results, however, have not been confirmed in more recent clinical studies[23-25].

The aims of this study are to assess the results of liver resection in patients with HCC and cirrhosis with PH and the relationship in terms of survival between Child-Pugh stage, the extent of hepatic resection and the presence of clinical PH.

MATERIALS AND METHODS

We retrospectively analyzed clinical data of 135 patients with cirrhosis undergoing liver resection with radical intent for HCC from 1995 to 2008 at the single Surgical Division of the Department of Surgery of the University of Verona. The patients' liver function was assessed by Child-Pugh classification. Clinical PH was defined according to AASLD guidelines as the presence of esophageal varices or thrombocytopenia (platelet count < 100 000/mm^3) associated with splenomegaly[21]. The extent of resection was defined according to the classification of Brisbane[26]. After liver resection, patients underwent follow-up with serum α-fetoprotein levels and abdominal ultrasound every 6 mo and computed tomography scan every 12 mo. The mean follow-up after surgery was 38.3 mo.

Statistical analysis

Data were collected and analyzed with SPSS statistical software (SPSS version 16.0 Inc., Chicago Ⅲ). The differences between categorical variables were analyzed with a χ^2 test. The differences between continuous variables were also analyzed with a χ^2 test.

Survival analysis was carried out with the Kaplan-Meier method. Univariate analysis for survival was performed with the Kaplan Meier method, with the Log Rank test to verify significance of differences. The statistical analysis included two different steps; in the first we analyzed the prognostic significance of the PH in all patients and in the second step we analyzed the prognostic significance of PH in different subgroups according to the Child-Pugh class and the extent of liver resection (wedge/segmentectomy or ⩾ 2 segments). Finally, multivariate analysis with Cox's regression model was performed with the following variables: Child-Pugh class, PH and type of hepatectomy. A *P* value lower than 0.05 was considered significant.

RESULTS

The prevalence of PH in all patients was 32.5%. The analysis of our data showed that patients with PH who underwent surgery had worse liver function compared to those without PH (patients in Child-Pugh B class 33% *vs* 11% respectively, $P < 0.01$), with serum bilirubin level > 2 mg/dL in 29% *vs* 3% respectively, $P < 0.01$ and serum transaminases AST > 80 U/L in 52% *vs* 25%, $P = 0.01$ and ALT > 80 U/L in 48% *vs* 19% respectively, $P = 0.01$ (Table 1). The one and 3-mo mortality rates were 4.6% and 13.9% and 1.1% and 3.3% for patients with and without PH, respectively ($P = 0.20$ and $P = 0.05$). The morbidity rate reached no statistical significance for patients with and without PH respectively (37% *vs* 32%, $P = 0.59$). The liver-related morbidity (ascites, encephalopathy, jaundice) was significantly higher in patients with PH than in patients without PH, 32% *vs* 13% respectively ($P = 0.03$) (Table 2).

The 3-year and 5-year survival in patients without PH was higher than in patients with PH (68.4% and 61.2% *vs* 48.7% and 44.9% respectively, $P = 0.047$). These results are reported in Figure 1A.

Survival analysis in patients with Child-Pugh B cirrhosis did not demonstrate significant differences in patients with or without PH (3-year survival of 31.3% *vs* 11.9%, $P = 0.465$) (Table 3).

Also, in Child-Pugh class A patients the survival analysis did not show significant differences in patients with or without PH, with a 3-year and 5-year survival of 63.0% and 57.3% *vs* 72.0% and 63.2% respectively ($P = 0.257$). These results are summarized in Table 3.

Furthermore, we evaluated the relationship between survival, extent of liver resection and PH in Child-Pugh class A patients.

In limited resections (wedge or one segment) we found no statistical differences between patients with or without PH. In these patients the 5-year survival was 72.4% and 61.4% respectively ($P = 0.458$, Figure 1B, Table 3).

When resection of two or more segments was performed, survival was significantly longer in patients without PH with a 5-year survival of 64.5% compared to 25.0% in patients with PH respectively ($P = 0.035$, Figure 1C, Table 3).

Multivariate analysis with Cox's regression model confirmed that Child-Pugh class was related to survival with a HR of 2.57 ($P < 0.01$), whereas PH and type of hepatectomy were not related to survival (Table 4).

DISCUSSION

Liver resection is currently the treatment of choice for

Table 1 Patients characteristics according to the presence/absence of portal hypertension

Variable	Portal hypertension		*P* value
	Yes (%)	No (%)	
n	44	91	
Age (yr)			0.55
< 70	31 (70)	60 (66)	
> 70	13 (30)	31 (34)	
Etiology of liver disease			0.21
Alcohol	7 (16)	24 (26)	
Viral hepatitis	36 (82)	59 (65)	
Other	1 (2)	8 (9)	
Serum ALT level (U/L)			0.01
< 80	23 (52)	74 (81)	
> 80	21 (48)	17 (19)	
Serum AST level (U/L)			0.01
< 80	21 (48)	68 (75)	
> 80	23 (52)	23 (25)	
Child-Pugh class			0.01
Class A	29 (66)	81 (89)	
Class B	15 (33)	10 (11)	
Bilirubin level (mg/dL)			0.01
< 2	31 (71)	88 (97)	
2-3	8 (18)	3 (3)	
> 3	5 (11)	0 (0)	
Albumin level (g/L)			0.01
< 28	7 (16)	8 (9)	
28-35	18 (41)	14 (15)	
> 35	19 (43)	69 (76)	
Platelet count			0.01
⩽ 100 000/mm^3	31 (70)	0	
> 100 000/mm^3	13 (30)	91 (100)	
Esophageal varices			0.01
Yes	17 (39)	0	
No	27 (61)	91 (100)	
α-fetoprotein level (ng/dL)			0.86
< 20	19 (43)	42 (46)	
> 20	25 (57)	49 (54)	
Size (cm)			0.03
< 3	14 (32)	30 (33)	
3-5	21 (48)	23 (25)	
> 5	9 (20)	38 (42)	
Number of nodules			0.51
Single	32 (73)	65 (72)	
2 HCC	8 (18)	12 (13)	
3 HCC or more	4 (9)	14 (15)	
Macrovascular Invasion			0.16
No	42 (95)	73 (80)	
Yes	2 (5)	18 (20)	
Microvascular invasion			0.24
No	29 (66)	43 (47)	
Yes	15 (34)	48 (53)	
Type of hepatectomy			0.58
Wedge resection	10 (23)	16 (18)	
Segmentectomy	26 (59)	52 (57)	
More than 1 segment	8 (18)	23 (25)	

ALT: Alanine transaminase; AST: Aspartate transaminase; HCC: Hepatocellular carcinoma.

single HCC and it is a safe treatment in terms of peri-operative complications, even in patients with liver cirrhosis[6,27]. The outcome of surgical resection is strongly related to hepatic functional reserve. For this reason, the majority of patients with liver cirrhosis cannot undergo surgery because of the high risk of postoperative liver failure.

Table 2 Mortality and morbidity rates according to the presence/absence of portal hypertension

	Portal hypertension		*P* value
	Yes (%)	No (%)	
n	44	91	
1 mo mortality	2 (4.6)	1 (1.1)	0.20
3 mo mortality	6 (13.6)	3 (3.3)	0.05
Overall morbidity	16 (37)	29 (32)	0.59
Cardiac complications	0	3 (3.3)	0.20
Pulmonary complications	10 (23)	18 (20)	0.71
Hepatic complications	14 (32)	12 (13)	0.03

Table 3 Survival analysis according to different groups of patient and presence/absence of portal hypertension

Variable	*n*	Median survival (mo, 95% CI)	Survival		*P* value
			3-yr	5-yr	
Overall					0.047
Without PH	91	65.1 (49.7-80.4)	68.4	61.2	
With PH	44	31.6 (3.4-59.9)	48.7	44.9	
Child-Pugh B					0.465
Without PH	10	27.7 (1.3-65.5)	31.3	31.3	
With PH	15	15.1 (11.7-18.5)	11.9	-	
Child-Pugh A					0.257
Without PH	81	65.1 (47.7-82.4)	72.0	63.2	
With PH	29	60.5 (6.4-114.6)	63.0	57.3	
CP A-limited Hx					0.458
Without PH	58	64.9 (62.9-67.0)	72.4	61.4	
With PH	21	94.0 (54.0-134.0)	72.7	72.7	
CP A-Hx ⩾ 2 segments					0.035
Without PH	23	163.9 (-)	64.5	64.5	
With PH	8	64.4 (54.0-134.0)	50.0	25.0	

CI: Confidence interval; PH: Portal hypertension; CP: Child-Pugh score; Hx: Hepatic resection.

Table 4 Multivariate Cox's regression model of variables related to survival

Variable	HR	95% CI	*P* value
Child-Pugh class (B *vs* A)	2.57	1.31-5.01	0.005
Type of hepatectomy (⩾ 2 segments *vs* limited)	1.05	0.50-2.21	0.880
PH (presence *vs* absence)	1.51	0.84-2.68	0.160

CI: Confidence interval; PH: Portal hypertension.

PH in cirrhotic patients is considered a relative contraindication for surgery in EASL/AASLD guidelines. Bruix *et al*[12] analyzed the outcome of 29 Child-Pugh class A patients with PH [defined as porto-hepatic gradient (HVPG) greater than 10 mm Hg] and observed a higher likelihood of postoperative liver failure in these patients compared to those without PH. The authors justified these results because liver resection in patients with PH can reduce the portal vascular bed without a reduction of portal flow and this condition can lead to a further increase of portal pressure. These hypotheses were not confirmed by a study by Fujisaki *et al*[28] that reported

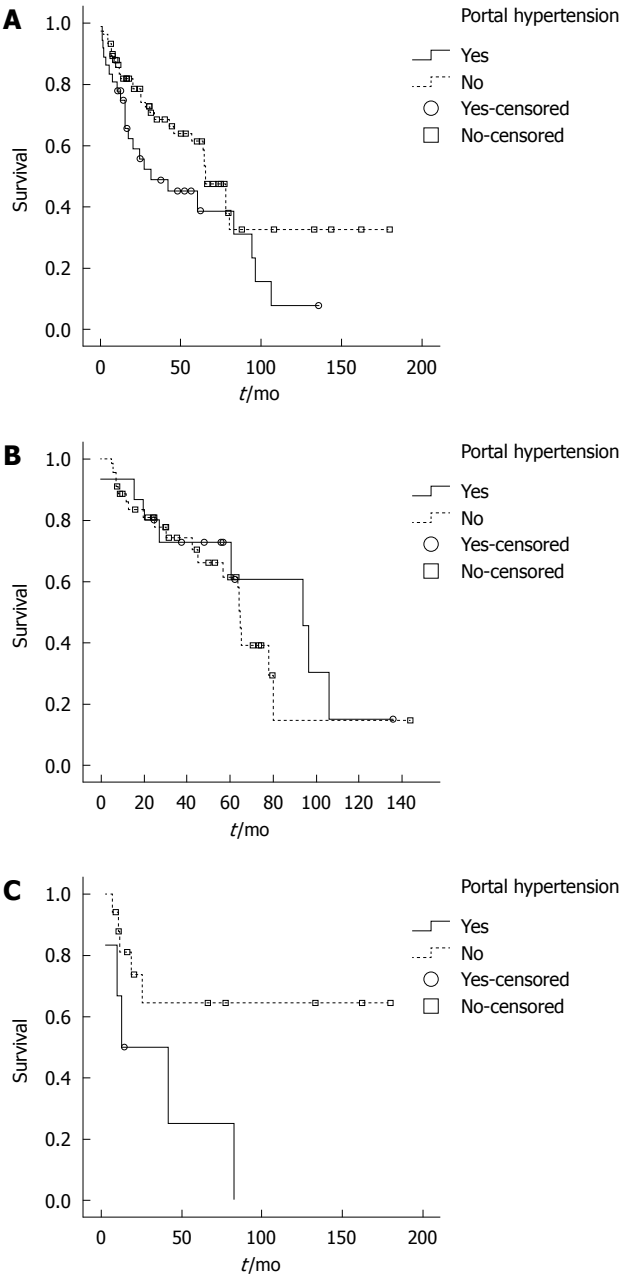

Figure 1 Overall survival analysis in patients. A: Overall survival analysis in patients with or without portal hypertension (PH); the difference between the two groups was statistically significant ($P = 0.04$); B: Overall survival analysis in Child-Pugh score (CP) A patients with or without PH, in the subgroup of patients submitted to limited resection ($P = 0.45$); C: Overall survival analysis in CP A patients with or without PH, in the subgroup of patients submitted to resection of 2 or more segments ($P = 0.03$).

on a group of 54 patients, in whom severity of PH was not worsened by liver resection. Llovet *et al*[29] in another study showed that clinical PH (assessed by the simultaneous presence of gastric-esophageal varices, thrombocytopenia lower than 100 000/mm^3 and splenomegaly, or on a portal-hepatic venous pressure gradient greater than 10 mm Hg) is a predictor of postoperative liver failure and that it is related to long term survival. These authors studied 77 patients divided into 3 groups based on the presence or absence of PH and bilirubin level. The 5-year survival of patients without PH was 74%, while the patients with PH and bilirubin lower than 1 mg/dL had a 5-year survival of 50%, and the patients with PH and bilirubin greater than 1 mg/dL had a 5-year survival of 25%. Other studies confirmed a correlation between PH and increased mortality and complications. Poon *et al*[30], in a study of 1222 patients undergoing liver resection for hepato-biliary cancers, showed that the presence of thrombocytopenia at the time of surgery is a risk factor in multivariate analysis for surgical complications. Similarly, Jarnagin *et al*[31] in a study of 1803 patients showed that preoperative thrombocytopenia is associated with a postoperative increased risk of mortality.

On the contrary, other authors did not detect a significant correlation between PH and liver failure after surgery[32,33]. Ishizawa *et al*[23] analyzed 322 Child-Pugh class A patients and found good long term results in patients with or without PH, (3- and 5-year survival of 71% and 56% *vs* 81% and 71% respectively, $P = 0.008$). Capussotti *et al*[24], in a study of 217 patients including 99 with PH at the time of surgery, showed that the 3- and 5-year survival rates are greater in patients without the presence of PH (62% and 40% *vs* 45% and 29% respectively, $P = 0.020$). However, resection in patients with PH and good hepatic function (Child-Pugh A) had similar results in terms of 3- and 5-year survival (65% and 41% *vs* 60% and 41%, $P = 0.503$). This study also shows that patients with PH have a higher incidence of postoperative complications, particularly those related to the deterioration of liver function (27% *vs* 15%, $P = 0.030$). Kawano *et al*[25] evaluated the results of liver resection in patients with esophageal varices. This study found that patients with esophageal varices had a better 5-year survival (70% *vs* 47%, $P = 0.045$); the authors underlined that patients with PH had a more frequent early diagnosis of HCC due to more careful follow up. More recently, Choi *et al*[34] reported that the 5-year survival rate of patients with clinical PH affected by single nodular HCC without macrovascular invasion was 78.4%, even if in Child-Pugh A cirrhotic patients, the presence of clinically significant PH was significantly associated with postoperative hepatic failure and poor prognosis after resection of HCC.

The data from our study confirm the recent experiences in the literature. Patients with PH at the time of surgery showed worse liver function and this justifies the increased number of complications related to the deterioration of liver function and the increased postoperative 3-mo mortality. Long term survival was significantly related to PH with significantly shorter survival ($P < 0.04$).

Among different Child-Pugh class patients we did not observe statistically significant differences in 3- and 5-year survival between patients with or without PH. In Child-Pugh class A patients submitted to minor resection, survival was not significantly affected by PH. Our study is the first in the literature to demonstrate the relationship between survival, PH and extent of hepatectomy. Surgery can produce good long term survival in patients with PH submitted to limited resection; conversely, PH had strong

adverse prognostic significance in patients who underwent resection of two or more segments. This data can help in the selection of patients and improve safety and long term results of surgery, as well as identify a group of patients who require resection of 2 or more segments, in whom resection is contraindicated and other non-surgical therapies should be applied.

This is a retrospective analysis from a single center. This allowed homogeneous data; however, multi-center studies are needed to confirm these results to reduce technical bias.

Our study confirms that the presence of PH at the time of surgery is not an absolute contraindication to resection in patients with liver cirrhosis. Although the rate of postoperative complications in patients with PH is greater, the results in terms of survival in the group of Child-Pugh class A patients is similar in patients without PH. Also, in patients with PH, limited liver resection can be performed with results comparable to those in patients without PH. Conversely, surgical resection of 2 or more segments in patients with PH results in significantly shorter survival and should not be recommended.

COMMENTS

Background

The presence of liver cirrhosis is the most important risk factor for the development of hepatocellular carcinoma (HCC): 85%-95% of HCCs arise in cirrhotic livers. Portal hypertension (PH) is related to increase in intrahepatic resistance due to the structural subversion of the cirrhotic liver and loss of vascular bed, and bleeding from gastro-esophageal varices is one of the most important complications of cirrhosis. The European Association for the Study of the Liver/American Association for the Study of Liver Diseases (AASLD) guidelines consider PH as a relative contraindication to liver resection, because of the high risk of postoperative liver failure, as reported in some clinical series. On the contrary, other authors have not detected a significant correlation between PH and liver failure after surgery.

Research frontiers

Surgical treatment is the most effective treatment for HCC and the mortality after surgery has decreased in recent years in relation to improved surgical techniques and peri-operative management of patients. Moreover, only about 30% of patients affected by HCC can be submitted to surgery, because of the characteristics of the tumor (stage, number of nodules, size, presence of vascular invasion), the general condition of the patient, or liver functional impairment, such as PH.

Innovations and breakthroughs

Recently, authors have not detected a significant correlation between PH and liver failure after surgery. The data from this study confirm the recent experiences in the literature. Survival analysis in patients with Child-Pugh A and B cirrhosis did not demonstrate significant differences in patients with or without PH. Moreover, limited resections (wedge or one segment) showed no statistical differences between patients with or without PH. When resection of two or more segments was performed, survival was significantly longer in patients without PH. Multivariate analysis with Cox's regression model confirmed that Child-Pugh, but not PH and type of hepatectomy class, was related to survival. This study is the first in the literature to demonstrate the relationship between survival, PH and extent of hepatectomy. Surgery can result in good long term survival in patients with PH submitted to limited resection; conversely, PH had strong adverse prognostic significance in patients who underwent resection of two or more segments.

Applications

This study confirms that the presence of PH at the time of surgery is not an absolute contraindication to resection in patients with liver cirrhosis. Results in terms of survival in the group of Child-Pugh class A patients are similar to patients without PH. Furthermore, in patients with PH, limited liver resection can be performed with results comparable to those in patients without PH. Conversely, surgical resection of 2 or more segments in patients with PH resulted in significantly shorter survival and is not to be recommended.

Terminology

Clinical PH is defined according to AASLD guidelines as the presence of esophageal varices or thrombocytopenia (platelet count < 100 000/mm^3), associated with splenomegaly.

Peer review

In this retrospective study, the authors compare the results of liver resection for HCC in patients with and without PH. The authors show that resection is safe in CTP. A patients regardless of PH, in patients undergoing limited resection. The authors show no difference in survival between those with and without PH in CTP A/B patients.

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BRIEF ARTICLE

Evaluation of latent links between irritable bowel syndrome and sleep quality

Massimo Bellini, Angelo Gemignani, Dario Gambaccini, Simona Toti, Danilo Menicucci, Cristina Stasi, Francesco Costa, Maria Gloria Mumolo, Angelo Ricchiuti, Remo Bedini, Nicola de Bortoli, Santino Marchi

Massimo Bellini, Dario Gambaccini, Cristina Stasi, Francesco Costa, Maria Gloria Mumolo, Angelo Ricchiuti, Nicola de Bortoli, Santino Marchi, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, 56100 Pisa, Italy
Angelo Gemignani, Danilo Menicucci, Remo Bedini, Institute of Clinical Physiology, National Research Council, 56100 Pisa, Italy
Angelo Gemignani, Danilo Menicucci, Remo Bedini, Extreme Centre, Scuola Superiore S Anna, 56100 Pisa, Italy
Angelo Gemignani, Department of Physiological Science, University of Pisa, 56100 Pisa, Italy
Simona Toti, Italian National Institute of Statistics, 00185 Rome, Italy
Author contributions: Bellini M contributed to patient enrollment, diagnosis of IBS, experimental design, and writing the manuscript; Gemignani A contributed to assessment of sleep disturbances, experimental design, and writing the manuscript; Gambaccini D contributed to patient enrollment, diagnosis of IBS, and writing the manuscript; Toti S and Menicucci D contributed to application of the IRT model and statistical analysis; Stasi C, Mumolo MG and de Bortoli N contributed to patient enrollment and diagnosis of IBS; Costa F performed lower digestive endoscopy in patients > 50 years old to exclude non-functional GI disorders; Ricchiuti A performed in all patients abdominal ultrasonography to exclude non-functional GI disorders; Bedini R contributed to statistical analysis and funded the study; Marchi S contributed to patient enrollment, diagnosis of IBS and funded the study.
Correspondence to: Dr. Massimo Bellini, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, Via Paradisa No. 2, 56100 Pisa, Italy. mbellini@med.unipi.it
Telephone: +39-50-997448 Fax: +39-50-997398
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Abstract

AIM: To examine the links between quality of sleep and the severity of intestinal symptoms in irritable bowel syndrome (IBS).

METHODS: One hundred and forty-two outpatients (110 female, 32 male) who met the Rome Ⅲ criteria for IBS with no psychiatric comorbidity were consecutively enrolled in this study. Data on age, body mass index (BMI), and a set of life-habit variables were recorded, and IBS symptoms and sleep quality were evaluated using the questionnaires IBS Symptom Severity Score (IBS-SSS) and Pittsburgh Sleep Quality Index (PSQI). The association between severity of IBS and sleep disturbances was evaluated by comparing the global IBS-SSS and PSQI score (Pearson's correlation and Fisher's exact test) and then analyzing the individual items of the IBS-SSS and PSQI questionnaires by a unitary bowel-sleep model based on item response theory (IRT).

RESULTS: IBS-SSS ranged from mild to severe (120-470). The global PSQI score ranged from 1 to 17 (median 5), and 60 patients were found to be poor sleepers (PSQI > 5). The correlation between the global IBS-SSS and PSQI score indicated a weak association (r = 0.2 and 95% CI: -0.03 to 0.35, $P < 0.05$), which becomes stronger using our unitary model. Indeed, the IBS and sleep disturbances severities, estimated as latent variables, resulted significantly high intra-subject correlation (posterior mean of r = 0.45 and 95% CI: 0.17 to 0.70, $P < 0.05$). Moreover, the correlations between patient features (age, sex, BMI, daily coffee and alcohol intake) and IBS and sleep disturbances were also analyzed through our unitary model. Age was a significant regressor, with patients ⩽ 50 years old showing more severe bowel disturbances (posterior mean = -0.38, $P < 0.05$) and less severe sleep disturbances (posterior mean = 0.49, $P < 0.05$) than older patients. Higher daily coffee intake was correlated with a lower severity of bowel disturbances (posterior mean = -0.31, $P < 0.05$). Sex (female) and daily alcohol intake (modest) were correlated with less severe sleep disturbances.

CONCLUSION: The unitary bowel-sleep model based on IRT revealed a strong positive correlation between the severity of IBS symptoms and sleep disturbances.

Key words: Irritable bowel syndrome; Sleep disorders; Item response theory model; Bayesian model

Peer reviewer: Guang-Yin Xu, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555-0655, United States

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INTRODUCTION

Irritable bowel syndrome (IBS) is quite prevalent in the general population (5%-20%) and is in fact the functional gastrointestinal disorder most frequently encountered in primary and secondary care[1,2]. IBS is characterized by abdominal discomfort, pain and changes in bowel habits (constipation and/or diarrhea)[3]. Visceral hypersensitivity, slowed gastrointestinal transit, and alterations in secretion activity are also often reported[4]. The direct and indirect costs of the syndrome are significant. IBS can have a serious impact on quality of life, and because it is often associated with other disorders, the patient may have to undergo expensive tests and treatments[5-7].

Sleep disturbances connected with gastrointestinal functional disorders, in particular IBS, have been reported[8-10], but no consistent abnormality in sleep patterns has been identified, apart from a significant increase in rapid-eye-movement (REM) sleep[11,12]. Many studies have associated sleep alterations with severity of IBS symptoms[12], but it is not yet known whether there is a direct pathophysiological relationship[13,14]. To date, a fundamental question remains - is sleep disturbance a cause or a consequence of IBS[15]?

Growing awareness of the importance of the "brain-gut axis" has led some gastroenterologists to investigate sleep as an independent factor in the pathogenesis and clinical expression of IBS[11,16].

The aim of the present study was to evaluate the association between the severity of sleep disturbances and intestinal symptoms, measured using two widely accepted questionnaires: the IBS Symptom Severity Score (IBS-SSS)[17] and the Pittsburgh Sleep Quality Index (PSQI)[18]. In addition, individual patient features were also analyzed to investigate their possible interaction with the severity of bowel and sleep disturbances and to exclude their potential confounding role.

MATERIALS AND METHODS

Study patients

Between October 2007 and September 2008, 142 patients who met the Rome Ⅲ criteria for IBS[3] with no confounding psychiatric comorbidity (diagnosed according to DSM Ⅳ axis I criteria) were consecutively enrolled from outpatients attending the Gastrointestinal Unit of the University of Pisa. All patients gave their informed written consent as required by the University Ethics Committee for Clinical Studies. The cohort was comprised of 110 female and 32 male patients (median age: 38 years; range: 18-79 years), of whom, 52 had constipation as the predominant symptom (C-IBS), 48 had diarrhea as the predominant symptom (D-IBS), and 42 had mixed symptoms (M-IBS).

We also evaluated physical and life-habit variables in each patient (Table 1): (1) sex and age; (2) body mass index (BMI)[19]; (3) smoking; (4) alcohol intake[20]; (5) coffee intake; (6) physical activity; and (7) water intake.

Table 1 Clinical subtypes, physical and life-habit variables of irritable bowel syndrome patients

	Item categories	Patients (%)
IBS subtype	M-IBS	29.6
	D-IBS	33.8
	C-IBS	36.6
Age (yr)	≤ 50	78.9
	> 50	21.1
Sex	Male	22.5
	Female	77.5
BMI (kg/m^2)	≤ 25	76.1
	> 25	23.9
Smoking	No	74.6
	Yes	25.4
Alcohol consumption	Non-/occasional drinkers	82.4
	Light drinkers (< 30 g ethanol/d)	17.6
Coffee consumption	< 2 cups/d	55.6
	≥ 2 cups/d	44.4
Physical activity	No	66.2
	Yes	33.8
Water consumption	< 1.5 L/d	61.3
	≥ 1.5 L/d	38.7

IBS: Irritable bowel syndrome; BMI: Body mass index.

IBS and sleep questionnaires

IBS symptoms were evaluated using the IBS-SSS questionnaire[17], which measured five separate items (Table 2): (1) presence and severity of abdominal pain or discomfort; (2) frequency of abdominal pain or discomfort; (3) presence and severity of abdominal distension; (4) degree of satisfaction with defecatory function; and (5) degree of interference of IBS symptoms with daily lifestyle. For each of the five items, the questions generated a score ranging from 0 to 100 that, for the purposes of analysis, was divided into three consecutive intervals: low, medium, and high (Table 2). The scores for the five items were summed to arrive at a global IBS-SSS (range: 0-500) and the IBS severity was then classified as mild (75-175), moderate (175-300) or severe (≥ 300).

Sleep quality was evaluated using the PSQI[18], a self-administered questionnaire based on seven items: subjective sleep quality, sleep latency, sleep duration, habitual

Table 2 Distribution of responses to the items in the irritable bowel syndrome Symptom Severity Score across categories (low, medium and high)

IBS-SSS items	Category (score interval)	Patients (%)
Pain severity	Low: ≤ 40	31.0
	Medium: 40-70	43.7
	High: > 70	25.4
Pain frequency	Low: ≤ 20	19.7
	Medium: 20-70	33.8
	High: > 70	46.5
Distension	Low: ≤ 40	30.3
	Medium: 40-70	28.2
	High: > 70	41.5
Dissatisfaction with defecatory function	Low: ≤ 40	12.7
	Medium: 40-70	26.8
	High: > 70	60.6
Interference with daily life style	Low: ≤ 40	28.9
	Medium: 40-70	32.4
	High: > 70	38.7

IBS-SSS: Irritable bowel syndrome Symptom Severity Score.

sleep efficiency, sleep disturbances, use of sleep inducers, and daytime dysfunction. Each item was scored from 0 to 3, and the PSQI global index was calculated by summing these scores (range: 0-21); a global score > 5 identified poor sleepers.

In both questionnaires, lower values indicated lower severity, and higher values a more severe condition.

Statistical analysis

The association between the severity of IBS symptoms and sleep disturbances was first verified by directly comparing the global IBS-SSS and PSQI score, and then by evaluating the links between individual items in the two questionnaires.

For the first approach, we used both the Pearson correlation coefficient and the generalized Fisher exact test[21]. The Pearson correlation measured the linear dependence between global scores, whereas the Fisher exact test verified the association between IBS-SSS and PSQI through a contingency table.

We adopted a unitary bowel-sleep model based on item response theory (IRT) that enabled us to investigate directly the dependences between IBS-SSS and PSQI single items. Furthermore, we also evaluated the weight of patient features and life habits in affecting the severity of IBS and sleep disturbances.

By focusing on individual items as the unit of analysis rather than the global score, the IRT model could circumvent the problem of different patterns of responses generating identical global scores. To this end, the values of both IBS-SSS single items and patient features were transformed in categories as indicated in the Tables 1 and 2. At variance, the PSQI items were not transformed since each item could have only four values (0-3), thus PSQI single item categories corresponded to the item values.

The output of our IRT model was the estimate of two latent variables: one related to the severity of IBS, and the other to sleep disturbances. In order to evaluate the possible intra-subject associations between IBS and sleep disturbances, the latent variables for the two were jointly modeled using a bivariate normal distribution. The strength of this association was quantified by the covariance parameter. Specifically, we adopted the partial credit model[22], which could be used for the polytomous ordered categories for each item. The partial credit model postulated that the probability of a given response with respect to the next response was a function (logistic curve) of the severity of the subject's symptoms and of structural parameters. The severity of symptoms, considered as a latent variable, was assumed to have a normal distribution[23]. Finally, we used a regression model to remove the effects of the patient features from the latent severities, and to identify the features with a significant effect on each latent severity variable.

In the partial credit model analysis, a sum-to-zero constraint was imposed on the structural parameters[24]. The model was estimated within a Bayesian framework by means of a Markov-chain Monte Carlo algorithm with Gibbs sampling (using WinBUGS 1.4)[25]. This estimation required that one specified the prior distributions of all parameters, but in the present study, no such a priori information was available; thus non-informative priors (i.e., parameter-flat a priori distributions) were used.

The Bayesian method enabled us to estimate the distribution of each parameter (posterior distribution), from which we could derive the central tendency (posterior mean) along with the Bayesian confidence (or Credibility) interval (CI).

In summary, the unitary bowel-sleep model yielded estimates for each patient of the latent severity of their IBS and sleep disturbances, with their covariance parameters.

Finally, the mean latent severity of IBS and sleep disturbances were correlated with patient features (including IBS subtypes). One category was maintained as the reference and we verified whether the regression coefficient (posterior mean), describing the change from the reference to every other category, was significantly different from zero.

RESULTS

Analysis of the global scores

Patients had IBS-SSS ranging from 120 to 470 (median 280): 10 subjects were classified as mild, 69 as moderate, and 63 as severe. In Table 2, the distribution of responses to the items in the IBS-SSS across categories (low, medium and high) is shown.

The sleep quality in our patients ranged from 1 to 17, with a median PSQI score of 5. Sixty patients were poor sleepers (global PSQI > 5). The distribution of the scores for the items in the PSQI is shown in Table 3.

The correlation between the severity of IBS and sleep disturbances was evaluated by comparing the global IBS-SSS and PSQI scores. Pearson's *r* was found to be 0.2

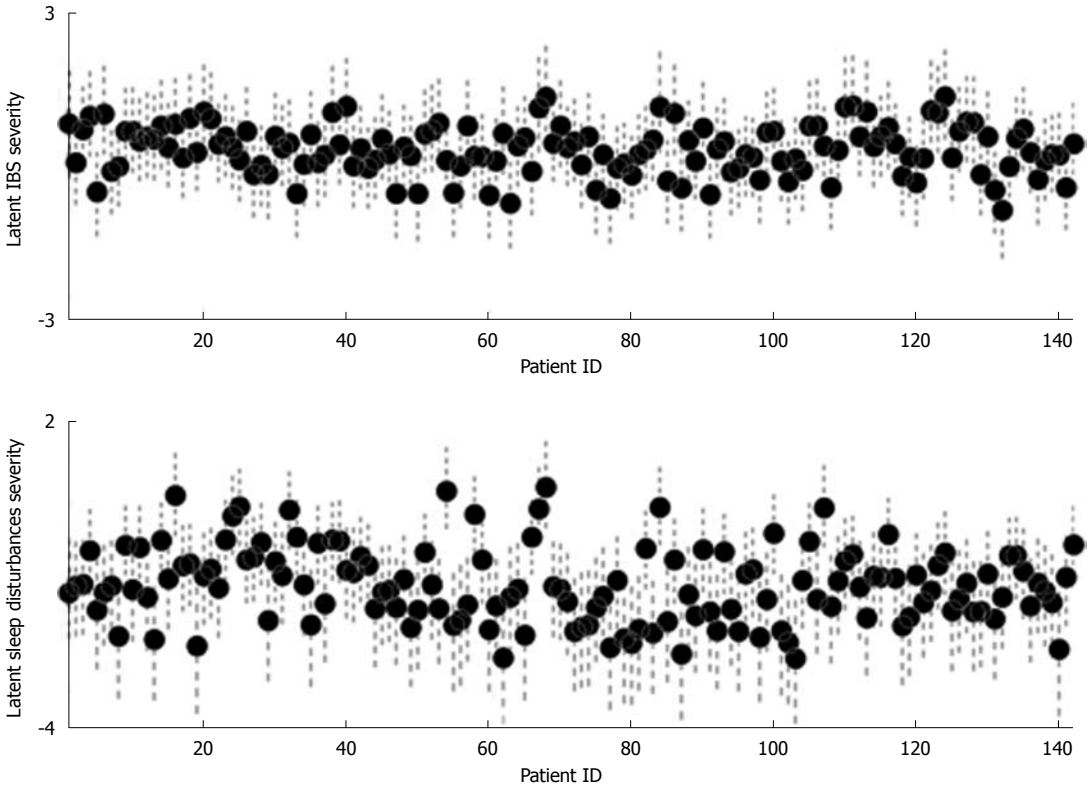

Figure 1 Irritable bowel syndrome latent severity in each patient, with 95% CI (top) and sleep disturbances latent severity in each patient, with 95% CI (bottom). Error bars indicate CI. ID indicates the label of each patient. IBS: Irritable bowel syndrome. CI: Confidence Interval.

Table 3 Distribution of responses to Pittsburgh Sleep Quality Index items

PSQI items	Item scores	Patients (%)
Subjective sleep quality	0	14.1
	1	62.7
	2	20.4
	3	2.8
Sleep latency	0	52.8
	1	28.2
	2	14.1
	3	4.9
Sleep duration	0	43.7
	1	35.9
	2	14.8
	3	5.6
Habitual sleep efficiency	0	54.9
	1	33.8
	2	7.7
	3	3.5
Sleep disturbances	0	8.5
	1	81.0
	2	9.9
	3	0.7
Use of sleeping medication	0	81.0
	1	3.5
	2	2.1
	3	13.4
Daytime dysfunction	0	31.0
	1	50.7
	2	13.4
	3	4.9

PSQI: Pittsburgh Sleep Quality Index.

Table 4 Contingency table for the global irritable bowel syndrome Symptom Severity Score and global Pittsburgh Sleep Quality Index score

	Global PSQI score	
	Good	Poor
Global IBS-SSS		
Mild	7	3
Moderate	44	25
Severe	31	32

IBS-SSS: Irritable bowel syndrome Symptom Severity Score; PSQI: Pittsburgh Sleep Quality Index.

(95% CI: 0.03 to 0.35), indicating a weak significant linear relationship between the two scores. Analysis of a contingency table for IBS-SSS *vs* PSQI categories using Fisher's exact test failed to detect any association between the two indices ($P = 0.18$) (Table 4).

Analysis of the latent severities

To complete the study of the questionnaires, the analysis was extended by modeling the responses to each single item with the partial credit models, and the regression analysis to determine the effect of the individual features on the estimated severity of IBS and sleep disturbances.

In Figure 1, the posterior means of the severity of IBS and sleep disturbances (expressed as latent variables) for the 142 patients are plotted (with their 95% CIs). The

Table 5 For each patient, feature regression coefficients (posterior means) and 95% confidence interval for the latent severities of both irritable bowel syndrome and sleep disturbances are shown

Patient features	Categories	IBS latent severity, coef (95% CI)	Sleep disturbances latent severity, coef (95% CI)
IBS subtype	Mixed	0	0
	Diarrhoea	-0.10 (-0.45 to 0.24)	0.02 (-0.42 to 0.46)
	Constipation	0.32 (-0.05 to 0.69)	0.16 (-0.30 to 0.65)
Age (yr)	≤ 50	0	0
	> 50	-0.38 (-0.75 to -0.02)[1]	0.49 (0.02 to 0.96)[1]
Sex	Male	0	0
	Female	0.10 (-0.24 to 0.46)	-0.53 (-0.95 to -0.07)[1]
BMI (kg/m^2)	≤ 25	0	0
	> 25	0.04 (-0.32 to 0.38)	0.09 (-0.33 to 0.52)
Smoking	No	0	0
	Yes	0.07 (-0.27 to 0.41)	-0.04 (0.45 to 0.38)
Alcohol consumption	Non-/occasional drinkers	0	0
	Light drinkers	-0.22 (-0.60 to 0.16)	-0.60 (-1.08 to -0.12)[1]
Coffee consumption	< 2 cups/d	0	0
	≥ 2 cups/d	-0.31 (-0.60 to -0.01)[1]	-0.08 (-0.44 to 0.26)
Physical activity	No	0	0
	Yes	-0.18 (-0.48 to 0.11)	-0.29 (-0.68 to 0.08)
Water consumption	< 1.5 L/d	0	0
	≥ 1.5 L/d	0.27 (-0.03 to 0.56)	0.07 (-0.29 to 0.42)

[1]Significant differences between covariables. IBS: Irritable bowel syndrome. CI: Confidence interval; BMI: Body mass index.

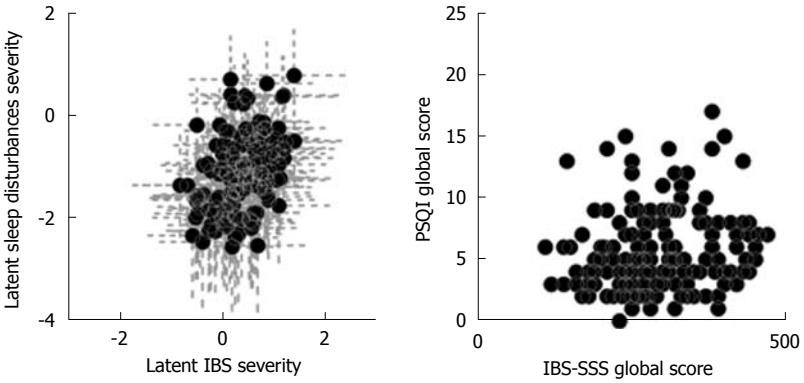

Figure 2 Scatter plot of the global Pittsburgh Sleep Quality Index score *vs* the global irritable bowel syndrome-Symptom Severity Score (left panel), and of sleep disturbances and irritable bowel syndrome latent severities (dots) (right panel). The grey lines indicate the 95% CI. CI: Confidence interval.

latent severity of sleep disturbances was a better discriminant between subjects than IBS, since it showed a more marked between-subject variability: all of the patients had a priori IBS symptoms, whereas the quality of their sleep was measured for the first time in this study.

Our unitary bowel-sleep model demonstrated a significant association between the severity of bowel symptoms and sleep disturbances. In contrast to the results of the global score comparison, a significant intra-subject correlation was found (posterior mean $r = 0.45$ and 95% CI: 0.17 to 0.70).

Figure 2 shows two scatter plots, on the left the IBS-SSS and PSQI global scores, whereas on the right, the two latent severities. The usage of the unitary bowel-sleep model provided a relationship between latent sleep disturbances severity and latent IBS severity. At variance, the usage of global scores did not provide any information about sleep and bowel dysfunction interactions. In the right panel of Figure 2, each black dot corresponds to a single patient and the dashed grey cross indicates the respective CI. Despite the variability between patients, the correlation between the two latent severities was evident, while the scatter plot on the left panel did not confirm any association between the global scores.

Furthermore, the unitary bowel-sleep model allowed us to identify a significant regression between IBS and sleep disturbances latent severities and patient features (Table 5).

The significance of a feature effect corresponded to a significant regression coefficient, and the sign of this coefficient indicated the direction of the relationship: a positive sign signified that the change of category (from the reference one to the other) came with a severity increase and *vice versa*; the 95% CI indicated the significance of the coefficient.

Among the individual features, age was a significant

regressor for both latent severities; young patients (≤ 50 years old) showed more severe bowel disturbances (posterior mean = -0.38) and less severe sleep disturbances (posterior mean = 0.49) than older patients. Daily coffee intake was significantly correlated with severity of bowel disturbances (posterior mean = -0.31); subjects who consumed ≥ 2 cups per day showed less severe bowel disturbances. Sex and daily alcohol intake were found to be significantly correlated with severity of sleep disturbances; female patients exhibited less severe sleep disturbances than male patients, while non-drinkers showed more severe sleep disturbances than those with a moderate alcohol intake. No other life-habit variables were found to be significantly correlated with latent severity of IBS or sleep disturbances.

DISCUSSION

The aim of this study was to evaluate the mutual influences between sleep disturbances and IBS in a large sample of IBS patients. The study provides a detailed statistical analysis of the possible association between bowel disorders, sleep disturbances and life habits.

Our sample may be considered as representative of the IBS population in Italy (this was confirmed by the distribution of IBS subtypes, age and BMI in the cohort). Therefore, our results can be compared and discussed in the light of the results of other studies on IBS patients.

Despite the fact that 42% of the participants had a global PSQI score > 5, only a weak and ambiguous association between IBS-SSS categories and PSQI categories was evident by using standard statistical analysis. Indeed, the correlation coefficient between the global scores was weakly significant and the Fischer exact test did not yield any association.

Conversely a strong link was highlighted by applying the IRT model[26,27] and linear regression.

IRT provides a framework to detect the effect of a series of variables (such as the responses to items on psychological tests) on latent traits of interest (IBS and sleep disturbances). In standard data analyses, the patient's raw score is calculated by summing the scores for the different items, without taking into account differences in the responses to specific items by different patients. It may arise that two patients have the same raw global score but different patterns of symptoms, or different raw global scores but similar symptom patterns. The inability to discriminate between the two possibilities can be resolved by using IRT model, which focuses on individual items rather than the global score. In addition, IRT allows one to introduce covariates (in our case age, BMI, and life habits) and analyze their effect on the parameters of interest.

The IRT model identified a latent link between sleep disturbances and IBS symptoms. These results showed that IBS patients suffered from a considerable degree of sleep impairment, and are in line with those of other studies[8,28,29]. In particular, a recent survey found that sleep disturbances were an independent predictor of IBS in nurses[8].

Through IRT analysis, we also determined that older age, male sex, and no alcohol intake were significant predictors of more severe sleep disturbances.

Our study found that female IBS patients complained with fewer sleep disturbances than males. This would appear to be inconsistent with studies in the general population, which show that females are more likely to suffer from sleep disorders[30], more susceptible to the effects of stress on sleep[31], and after menopause, run a higher risk of developing insomnia[30]. The present findings could be explained by the demographics of our sample, which were characterized by a relatively low mean age and hence a higher percentage of fertile women; indeed, IBS patients tend to be relatively young (< 50 years old)[3].

Evaluation of the predictive parameters for the latent severity of sleep disorders showed that age was positively correlated with sleep disturbances. These data come as no surprise, because aging is widely considered to be a triggering factor for insomnia[32-34].

Finally, this study showed that light alcohol intake was correlated with a lower latent severity of sleep disturbances. It has long been known that a little amount of alcohol consumed by healthy individuals before going to sleep shortens sleep latency, reduces REM sleep, and increases non-REM sleep[35-39]. Alcohol is frequently used as a form of self-medication by patients suffering from sleep disorders, especially insomnia[40].

More unexpectedly, we found that younger age and higher coffee intake were associated with less severe IBS symptoms. The first-line treatment for IBS usually consists of a change in lifestyle and diet, and drinking less coffee is often recommended, even though there are no studies demonstrating a link between the consumption of coffee and the severity of IBS symptoms. Sloots *et al*[41] have reported that the intake of 280 mL of coffee caused no change in rectal compliance or visceral sensibility. However, in our study, coffee drinkers actually consumed no more than two cups per day.

In conclusion, using IRT analysis we found that: (1) there was a positive association between the severity of IBS symptoms and sleep disturbances, and (2) some patient features were significant predictors of severity of IBS and sleep disturbances. These results are consistent with those of other studies[10,14]. The pathophysiological mechanism underlying this association is only partially understood, however. One possibility is that sleep disorders can induce visceral hyperalgesia, which increases the patient perception of gastrointestinal symptoms[8,15]. Several other factors could play a role, including endophenotypic traits such as those associated with stress susceptibility[42].

Further research is needed to clarify whether the association of IBS and sleep disturbances represents comorbidity or the expression of a single disturbance in the brain-gut axis.

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COMMENTS

Background

Sleep disturbances connected with gastrointestinal functional disorders, in particular irritable bowel syndrome (IBS), have been reported and some studies have associated sleep alterations with the severity of IBS symptoms. It is not yet known whether it is a simple comorbidity or if there is a direct pathophysiological relationship. To date, a fundamental question has arisen: is sleep disturbance a cause or a consequence of IBS?

Research frontiers

Sleep disturbances represent a negative factor affecting the outcome of several medical and psychiatric conditions, therefore, growing awareness has led some gastroenterologists to investigate sleep as an independent factor also in the pathogenesis and clinical expression of IBS.

Innovations and breakthroughs

Item response theory (IRT) for the first time has been used to identify latent links between digestive symptoms and sleep quality. Furthermore, this unitary bowel-sleep model enabled us to evaluate better the weight of patient features and life habits in the severity of IBS symptoms and sleep disturbances.

Applications

The results of this study suggest: (1) the use of IRT models for uncovering latent links in clinical comorbidity; (2) the importance of studying sleep quality in functional digestive disorders; and (3) further surveys on the clinical mutual interaction between sleep disturbances and functional digestive disorders (i.e., if the treatment of sleep disturbances improves digestive symptoms and/or *vice versa*)

Terminology

IRT is a paradigm for the analysis of tests and questionnaires. IRT models are often referred to as "latent trait models". The term latent is used to emphasize discrete item responses not directly observed but inferred from the manifest responses. IRT brings greater flexibility and provides more sophisticated information and is generally considered an improvement of classical test theory. The Bayesian method enables one to estimate the distribution of each parameter from which one could derive the central tendency.

Peer review

The authors have looked at sleep quality in patients with IBS to evaluate the relationship between digestive symptoms and sleep quality by using IRT analysis. The results are analyzed in detail and the statistical tool of IRT is used to obtain significant associations. The data are solid, the paper is interesting, has clinical significance and is well written.

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BRIEF ARTICLE

Serum leptin and ghrelin in chronic hepatitis C patients with steatosis

Christos Pavlidis, Georgios I Panoutsopoulos, Dina Tiniakos, Sotirios Koutsounas, John Vlachogiannakos, Irini Zouboulis-Vafiadis

Christos Pavlidis, John Vlachogiannakos, Irini Zouboulis-Vafiadis, First Department of Propaedeutic Medicine "LAIKO" General Hospital, Agiou Thoma 17, Athens 11527, Greece
Georgios I Panoutsopoulos, Department of Nursing, University of Peloponnese, Orthias Artemidos and Plateon, Sparta, Lakonia 23100, Greece
Dina Tiniakos, Laboratory of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Mikras Asias 75, Athens 11527, Greece
Sotirios Koutsounas, Reference Center for Viral Hepatitis, IKA, Athens 10436, Greece
Author contributions: Pavlidis C and Panoutsopoulos GI contributed equally to this work; Pavlidis C and Zoumboulis-Vafiadis I designed the study; Pavlidis C, Zoumboulis-Vafiadis I, Tiniakos D and Koutsounas S performed the study; Panoutsopoulos GI analyzed the data; Pavlidis C, Panoutsopoulos GI, Zoumboulis-Vafiadis I, Tiniakos D and Vlachogiannakos J wrote the manuscript.
Correspondence to: Dr. Christos Pavlidis, First Department of Propaedeutic Medicine "LAIKO" General Hospital, Agiou Thoma 17, Athens 11527, Greece. chrispavlidis@hotmail.com
Telephone: +30-210-6002252 Fax: +30-266-1046106
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Abstract

AIM: To determine the associations between leptin and ghrelin concentrations and sustained virological response (SVR) in chronic hepatitis C patients with steatosis.

METHODS: We retrospectively assessed 56 patients infected with hepatitis C virus (HCV) genotype-1 and 40 with HCV genotype-3. Patients with decompensated cirrhosis, and those with other causes of chronic liver disease, were excluded. Serum HCV-RNA concentrations were measured before the initiation of treatment; at weeks 12 (for genotype 1 patients), 24 and 48 during treatment; and 24 wk after the end of treatment. Genotype was determined using INNO-LIPA HCV assays, and serum leptin and ghrelin concentrations were measured using enzyme-linked immunosorbent assay. Biopsy specimens were scored according to the Ishak system and steatosis was graded as mild, moderate, or severe, according to the Brunt classification.

RESULTS: Overall, SVR was positively related to the presence of genotype-3, to biopsy-determined lower histological stage of liver disease, and lower grade of steatosis. Patients $\geqslant$ 40 years old tended to be less responsive to therapy. In genotype-1 infected patients, SVR was associated with a lower grade of liver steatosis, milder fibrosis, and an absence of insulin resistance. Genotype-1 infected patients who did not achieve SVR had significantly higher leptin concentrations at baseline, with significant increases as the severity of steatosis worsened, whereas those who achieved SVR had higher ghrelin concentrations. In genotype-3 infected patients, SVR was associated only with fibrosis stage and lower homeostasis model assessment insulin resistance at baseline, but not with the degree of steatosis or leptin concentrations. Genotype-3 infected patients who achieved SVR showed significant decreases in ghrelin concentration at end of treatment. Baseline ghrelin concentrations were elevated in responders of both genotypes who had moderate and severe steatosis.

CONCLUSION: Increased serum leptin before treatment may predict non-SVR, especially in HCV genotype-1 infected patients, whereas increased ghrelin may predict SVR in genotype-1.

Key words: Hepatitis C virus; Steatosis; Leptin; Ghrelin; Sustained virological response

Peer reviewer: Rashmi, Kaul, PhD, Assistant Professor, Department of Biochemistry and Microbiology, Oklahoma State

University-Center for Health Sciences, 1111 W 17th St, Tulsa, OK 74107, United States

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INTRODUCTION

Hepatic steatosis is a histopathological feature observed in > 50% of patients with chronic hepatitis C (CHC)[1,2], but occurs less frequently in patients with chronic hepatitis B (27%-51%) and autoimmune hepatitis (16%-19%)[3,4]. Steatosis in CHC patients has been attributed to a combination of mechanisms involved in the pathogenesis of non-alcoholic fatty liver disease, as well as to the direct effect of hepatitis C virus (HCV) on hepatic lipid metabolism leading to triglyceride accumulation[5,6]. In contrast, steatosis in patients chronically infected with hepatitis B virus (HBV) is associated with host metabolic factors[7].

Leptin is an adipokine that contributes to the pathogenesis of liver steatosis[8,9]. In patients with CHC, higher serum leptin concentrations have been associated with the presence of steatosis[10]. Although no clear correlation has been observed between leptin concentrations and the extent of steatosis[11], a recent study reported that high serum leptin concentrations correlated with more severe steatosis, lower viremia, and a lower antiviral response, mainly in patients infected with HCV genotype-1, which constituted 71% of the study population[12].

Leptin, the product of the obese (ob) gene, is mainly expressed by adipose tissue, although it is expressed in other organs, including the liver[13]. Leptin plays an important role in the regulation and metabolism of body fat and may induce insulin resistance, increase fatty acid concentrations in the liver, and enhance lipid peroxidation[5,8,9]. Leptin may act as an immunomodulator, inducing the release of cytokines, such as tumor necrosis factor (TNF)-α, interferon (INF)-γ, interleukin (IL)-18, and tumor growth factor (TGF)-β1, thus promoting liver steatosis and fibrosis[8].

Ghrelin is a peptide that acts as an endogenous ligand of the growth hormone secretatog receptor[14]. Ghrelin is involved in energy metabolism, food intake, and glucose homeostasis[14,15]. Recent studies have assessed whether ghrelin acts as an independent signal of adiposity or as a downstream mediator of leptin, affecting energy balance[16].

Little is known about serum ghrelin concentrations in patients with CHC and steatosis, or on the effects of ghrelin concentration on treatment response. We therefore assessed whether pretreatment serum leptin and ghrelin concentrations differ in steatotic patients infected with HCV genotypes-1 and -3, and whether these concentrations are associated with response to antiviral treatment. We also evaluated the correlations between pretreatment serum leptin and ghrelin concentrations and liver histology and metabolic factors, as well as determining whether the impact of antiviral treatment on leptin and ghrelin concentrations differed by HCV genotype.

MATERIALS AND METHODS

Patient population

We retrospectively assessed patients with serologically, virologically, and histologically confirmed CHC, recruited between 2005 and 2008. Patients were included if they had detectable anti-HCV antibody by enzyme-linked immunosorbent assay (ELISA) Ⅲ at least twice a year, detectable serum HCV-RNA by a sensitive PCR assay within 1 mo prior to the start of treatment, liver biopsy showing chronic hepatitis with steatosis within 6 mo before treatment, and elevated alanine aminotransferase (ALT) activity (> 40 IU/L and < 400 IU/L) at entry and at least once during the 6 mo before the first screening.

Patients with decompensated cirrhosis; other causes of chronic liver disease; a history of intravenous drug abuse or alcohol consumption; use of hepatotoxic drugs, herbal medications or immunosuppressive agents; diabetes; thyroid disorders; chronic renal failure; serious psychiatric disorders; HIV or HBV co-infection; or hepatocellular carcinoma, were excluded.

None of these patients had previously received antiviral treatment or steatosis-inducing therapy. The duration of HCV treatment with PEGylated INF-α and ribavirin was genotype-based. Genotype 1 patients who did not achieve undetectable HCV-RNA or a decrease in 2 logs of HCV-RNA at week 12 (early virological response or EVR), were considered non-responders but included in the study. All patients were clinically, hematologically and biochemically evaluated at weeks 2, 4, 8, 12, 24, 48, and 72 after the start of treatment.

The study protocol was approved by our institutional review board, and all patients provided written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Body composition measurements

Height and weight were determined at baseline, and body mass index (BMI), calculated as weight (in kg)/height (in m)^2, was determined at baseline and at the end of treatment. Waist circumference indicative of visceral obesity was defined as > 102 cm in men and > 88 cm in women.

Laboratory and virology assays

Blood samples were obtained from all subjects after overnight fasting, and serum was obtained after centrifugation and stored in aliquots at -70℃ until assayed. Routine biochemical (aspartate aminotransferase, ALT, γ-glutamyl transpeptidase, alkaline phosphatase, glucose, urea, creatinine, cholesterol, triglycerides, albumin) and

hematological (hemoglobin, white blood cells, platelets) were performed using automated techniques.

Serological markers (hepatitis B surface antigen, anti-HBs, hepatitis B e antigen, anti-HBe, anti-HBc total, anti-HCV, anti-HDV, anti-HIV1,2, and anti-HAV IgM and IgG) were assayed using commercially available enzyme immunoassays (Abbott Laboratories, United States). Serum HCV-RNA levels were measured with a PCR assay (Cobas Amplicor HCV version 2, Roche Diagnostics, United States), both qualitatively (lower detection limit of 50 IU/mL) and quantitatively (range of detection 600-10^6 IU/mL). HCV genotype was determined using an INNO-LIPA HCV assay (Innogenetics, Belgium). Serum HCV-RNA concentrations were measured before the initiation of treatment, at weeks 12 (for genotype 1 patients), 24, and 48 during treatment, and 24 wk after the end of treatment. Serum leptin and ghrelin concentrations were measured using ELISA kits (BioVendor Laboratory, United States), at screening and at the end of treatment and expressed as ng/mL. Insulin resistance index was calculated as: insulin resistance [homeostasis model assessment insulin resistance (HOMA-IR)] = fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5.

Liver histology

Liver biopsies were obtained at baseline using the Menghini technique (mean length of biopsy cores, 1.7 cm). Liver tissue was fixed in 10% neutral formalin and paraffin-embedded sections were stained with hematoxylin-eosin and Masson trichrome stains. Liver biopsies were scored by an experienced liver pathologist using the Ishak scoring system. Steatosis was quantified as the percentage of hepatocytes that contained fat droplets and was graded using a three-tier scale: grade 1/mild (1%-33%), 2/moderate (33%-66%), and 3/severe (> 66%). Patients with histopathological findings of steatohepatitis, including hepatocellular ballooning or perisinusoidal fibrosis in zone 3, were excluded.

Statistical analysis

All continuous variables are presented as mean ± SD or medians ± interquartile ranges (75th-25th percentiles) if they deviated from normality. The association between each genotype and continuous variables was determined by ANOVA (using post-hoc Scheffe's test when the omnibus test was significant) or Student's *t*-test. The association between each genotype and categorical variables was determined using the χ^2 test. Multiple logistic regression analyses were used to determine the association between response to therapy and leptin concentrations, ghrelin concentrations, hepatic steatosis, fibrosis, and HOMA-IR, with results presented as odds ratios and 95% CI. These analyses were performed separately for each genotype and for measurements at baseline and at the end of follow up. For reasons of multicollinearity, it was not possible to include steatosis and fibrosis in the same model. Therefore, fibrosis and steatosis were alternatively introduced (one at a time) into the core model. Mixed effect models were used to examine the relationship between baseline and end of follow-up leptin and ghrelin concentrations among responders and non-responders. There were no significant changes in weight or fat composition in the study population during treatment; therefore, there was no need to adjust leptin and ghrelin concentrations for BMI after treatment. All reported probability values (*P*-values) were based on two-sided tests, with significance set at 0.05. All statistical analyses were performed using the SAS statistical package (Version 9.1, SAS Institute Inc., NC).

Table 1 Clinical, virological, and histological data of the study population (mean ± SD) *n* (%)

	SVR (*n* = 62)	Non-SVR (*n* = 34)	*P*-value
Age	35.01 ± 1.57	39.21 ± 2.07	0.050
Sex (male/female)	36/26	21/13	
BMI	24.700 ± 0.179	25.530 ± 0.637	0.060
Genotype-1	32 (57.1)	24 (42.9)	
Genotype-3	30 (75)	10 (25)	
Stage (0-6)	1.661 ± 0.095	2.412 ± 0.141	< 0.001
Grade (0-18)	5.371 ± 0.136	5.265 ± 0.204	NS
Steatosis (1-3)	1.565 ± 0.082	2.060 ± 0.126	0.001

NS: Non-significant; SVR: Sustained virological response; BMI: Body mass index.

RESULTS

Of the 154 treatment-naive patients with CHC screened, 96 fulfilled our enrollment criteria and completed treatment. The study population consisted of 60 men and 36 women, all of Caucasian origin; of these, 56 were infected with HCV genotype-1 and 40 with HCV genotype-3.

HCV genotypes and response to treatment

All patients received combination therapy with PEGylated INF a-2b or a-2a, plus weight-adjusted ribavirin, for 24 (genotype-3) or 48 (genotype-1) weeks. All tolerated treatment well and completed treatment without any major side effects, significant reductions in drug dose, and significant changes in BMI (reduction more than 2 kg/m^2). Patients infected with HCV genotype-1 who did not achieve sustained virological response (SVR) stopped therapy, whereas those infected with HCV genotype-3 were treated for 24 wk without measurements of HCV-RNA at week 12, according to currently approved treatment guidelines. Of the 96 patients, 62 (64.6%) achieved SVR, whereas the remaining 34 (35.4%) did not.

Clinical, virological, and histological data of patients of both genotypes who did and did not attain SVR are presented in Table 1. Of the 56 patients with genotype 1, 32 (57.1%) achieved SVR, compared with 30 of 40 patients (75%) with genotype-3; thus SVR was significantly related to infection with genotype-3 ($P < 0.05$). In addition, SVR was significantly associated with lower histological stage of liver disease ($P < 0.001$) and lower grade of steatosis in liver biopsy ($P = 0.001$). Patients ⩾

Table 2 Distribution of 56 genotype-1 and 40 genotype-3-infected patients by demographic, host and viral factors with respect to response to hepatitis C virus therapy (mean ± SD) *n* (%)

Variable	Genotype 1 (*n* = 56)			Genotype 3 (*n* = 40)		
	Responders (*n* = 32)	Non-responders (*n* = 24)	*P* value	Responders (*n* = 30)	Non-responders (*n* = 10)	*P*-value
Gender			0.70			0.47
Male	15 (46.9)	10 (41.7)		13 (43.3)	6 (60.0)	
Female	17 (53.1)	14 (58.3)		17 (56.7)	4 (40.0)	
Age (yr)			0.17			0.15
≤ 40	23 (71.9)	13 (54.2)		29 (96.7)	8 (80.0)	
> 40	9 (28.1)	11 (45.8)		1 (3.3)	2 (20.0)	
Body mass index (kg/m^2)			0.28			0.42
< 25	18 (56.3)	10 (41.7)		23 (76.7)	6 (60.0)	
25-28	14 (43.7)	14 (58.3)		7 (23.3)	4 (40.0)	
Grade of hepatic steatosis			0.0001			0.72
Mild	22 (68.7)	5 (20.8)		12 (40.0)	3 (30.0)	
Moderate	10 (31.3)	12 (50.0)		13 (43.3)	4 (40.0)	
Severe	0 (0.0)	7 (29.2)		5 (16.7)	3 (30.0)	
Fibrosis score			0.001			0.04
1	17 (53.1)	3 (12.5)		16 (53.3)	1 (10.0)	
2	11 (34.4)	8 (33.3)		11 (36.7)	7 (70.0)	
3-4	4 (12.5)	13 (54.2)		3 (10.0)	2 (20.0)	
5-6	0	0		0	0	
HOMA-IR			0.01			0.01
< 2	11 (34.4)	4 (16.7)		19 (63.3)	1 (10.0)	
2-3	10 (31.2)	2 (8.3)		10 (33.3)	7 (70.0)	
> 3	11 (34.4)	18 (75.0)		1 (3.3)	2 (20.0)	
Leptin_baseline (ng/mL)	37.66 ± 10.39	49.67 ± 13.44	0.001	24.33 ± 7.98	28.20 ± 9.72	0.22
Ghrelin_baseline (ng/mL)	0.286 ± 0.167	0.190 ± 0.119	0.02	0.778 ± 0.654	0.564 ± 0.324	0.18
Leptin end of follow-up (ng/mL)	26.69 ± 11.77	41.50 ± 16.24	< 0.0001	23.27 ± 9.54	26.70 ± 6.82	0.30
Ghrelin end of follow-up (ng/mL)	0.456 ± 0.254	0.239 ± 0.213	0.001	0.420 ± 0.321	0.450 ± 0.261	0.79

HOMA-IR: Homeostasis model assessment insulin resistance.

Table 3 Leptin and ghrelin concentrations at baseline and end of follow-up in patients infected with hepatitis C virus genotypes-1 and -3 by response to therapy, as well as mixed effect model derived estimates of differences in mean scores (mean ± SD)

Variable	Responders	Non-responders	*P*-value for the adjusted difference[1]
Leptin			
Baseline (ng/mL)	30.99 (9.185)	38.43 (11.58)	NS
End of Follow-up (ng/mL)	23.76 (8.055)	34.10 (11.53)	0.01
Ghrelin			
Baseline (ng/mL)	0.532 (0.410)	0.377 (0.223)	0.01
End of Follow-up (ng/mL)	0.438 (0.288)	0.345 (0.237)	0.05

[1]Adjusted for both leptin and ghrelin concentrations. NS: Non-significant.

40 years old tended to be less responsive to therapy (P = 0.05) (Table 1). Further analysis by genotype showed that, in genotype-1 infected patients, SVR was associated with a lower grade of liver steatosis (P = 0.0001), mild fibrosis (P = 0.001), and absence of insulin resistance (P = 0.01) (Table 2). In genotype-3 infected patients, SVR was associated with stage of fibrosis (P = 0.04) and lower HOMA-IR at baseline (P = 0.01), but not to degree of steatosis (Table 2).

Leptin, ghrelin and response to treatment

Baseline leptin concentrations did not differ between patients who did and did not attain SVR, but were significantly lower after successful treatment in patients who attained SVR (P = 0.01) (Table 3). Among patients infected with HCV genotype-1, non-responders had significantly higher serum leptin concentrations, both at baseline (P = 0.001) and at the end of follow-up (P < 0.0001) than those who attained SVR (Table 2). Using mixed effect model analysis, we observed a statistically significant difference between baseline and follow-up leptin concentrations among genotype-1 infected patients who achieved SVR (P = 0.001), as well as a borderline significant difference among non-responders (P = 0.06) (Table 4).

Among patients infected with HCV genotype-3, however, there were no significant differences in leptin concentrations at baseline and at end of follow-up between those who did and did not achieve SVR (Table 2). Using mixed effect model analysis, leptin remained unchanged, both in responders (P = 0.51) and non-responders (P = 0.61) (Table 4).

Overall, patients who achieved SVR had higher serum ghrelin concentrations, both at baseline (P = 0.01) and at the end of follow up (P = 0.05), than patients who did not achieve SVR (Table 3). Genotype-1 infected patients who achieved SVR had statistically significant higher ghrelin concentrations at baseline (P = 0.02) and at the end

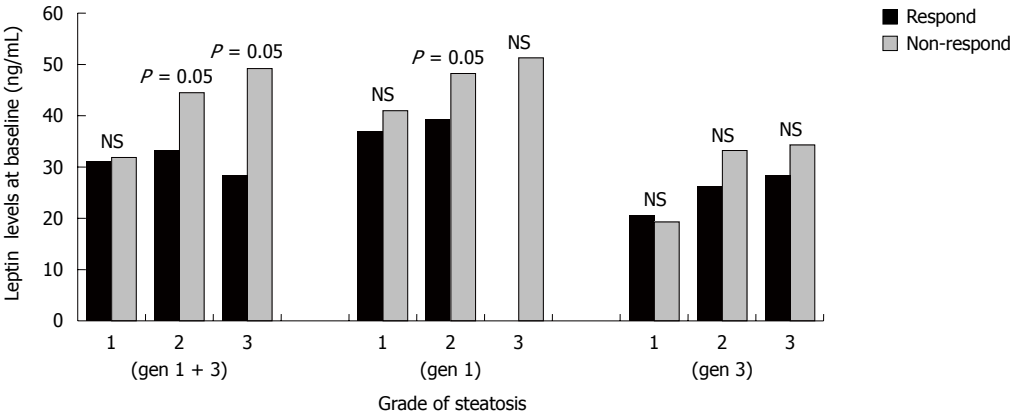

Figure 1 Correlation between leptin concentrations and steatosis in patients infected with hepatitis C virus genotypes-1 and -3 who did and did not achieve sustained virological response following treatment with PEGylated interferon a-2b or a-2a plus ribavirin. NS: Not significant.

Table 4 Leptin and ghrelin concentrations at baseline and at end of follow-up by response to treatment in patients with hepatitis C virus genotypes-1 and -3, as well as mixed effect model derived estimates of the differences in mean scores (mean ± SD)

Variable	Baseline	End of follow-up	*P*-value for the adjusted difference[1]
Genotype 1 SVRs			
Leptin (ng/mL)	37.66 (10.39)	26.69 (11.77)	0.001
Ghrelin (ng/mL)	0.286 (0.167)	0.456 (0.254)	0.001
Genotype 1 non-SVRs			
Leptin (ng/mL)	49.67 (13.44)	43.58 (16.17)	0.060
Ghrelin (ng/mL)	0.190 (0.119)	0.239 (0.213)	0.320
Genotype 3 SVRs			
Leptin (ng/mL)	24.33 (7.98)	23.27 (9.54)	0.510
Ghrelin (ng/mL)	0.778 (0.654)	0.420 (0.321)	0.001
Genotype 3 non-SVRs			
Leptin (ng/mL)	28.20 (9.72)	26.70 (6.82)	0.610
Ghrelin (ng/mL)	0.564 (0.324)	0.450 (0.261)	0.470

[1]Adjusted for both leptin and ghrelin concentrations.

of treatment ($P = 0.001$) than nonresponders (Table 2), with responders showing significantly higher ghrelin concentrations at end of treatment than at baseline ($P = 0.001$) (Table 4). In contrast, ghrelin concentrations in genotype-3 infected patients did not differ between responders and nonresponders, both at baseline and at the end of treatment (Table 2). Mixed effect model analysis showed that ghrelin concentrations were significantly lower at the end of treatment than at baseline in patients who achieved SVR ($P = 0.001$), but not in non-responders ($P = 0.47$) (Table 4).

Leptin, ghrelin and steatosis

Overall, steatosis grade at baseline was higher in non-responders than in patients who achieved SVR, with steatosis grade at baseline being significantly greater as leptin concentrations increased, a difference more obvious in patients with moderate and severe steatosis ($P = 0.05$). This correlation was also observed in genotype-1 non-responders, but not in genotype-3 non-responders or in patients of either genotype who achieved SVR (Figure 1).

Ghrelin concentration at baseline was higher in responders of both genotypes with moderate ($P = 0.05$) and severe ($P = 0.001$) steatosis. In non-responders, however, there was no significant correlation between the grade of steatosis and ghrelin concentrations. In genotype-1 infected patients, both in responders and non-responders, ghrelin concentrations decreased significantly as the grade of steatosis increased ($P = 0.01$), and responders with genotype-3 and moderate or severe steatosis had significantly higher serum concentrations of ghrelin ($P = 0.01$) (Figure 2). A strong correlation between the severity of steatosis and higher viral load at baseline was observed in patients infected with HCV genotype-3 ($P = 0.01$), but not in those infected with HCV genotype-1 (P = NS) (Figure 3).

Multivariate analysis

Using multivariate logistic regression analysis, we found that higher leptin concentrations at baseline were significantly associated with non-response to therapy in patients infected with HCV genotype-1, but not HCV genotype-3. There were no significant associations between response to treatment and ghrelin concentrations in patients of either genotype. Furthermore, HCV genotype-1 infected with moderate or severe steatosis, as well as those with more severe fibrosis, were less likely to respond to therapy (Table 5). In patients with genotype-3, however, there were no significant associations between response to treatment and steatosis, fibrosis, or leptin or ghrelin concentrations, both at baseline and at end of treatment. Only patients with higher levels of HOMA (IR) seemed less likely to respond to therapy (Table 6).

DISCUSSION

The mechanism by which HCV induces steatosis remains unclear. Steatosis in patients infected with the non-3 genotype has been associated with increased BMI, visceral obesity, increased cholesterol and triglyceride concentra-

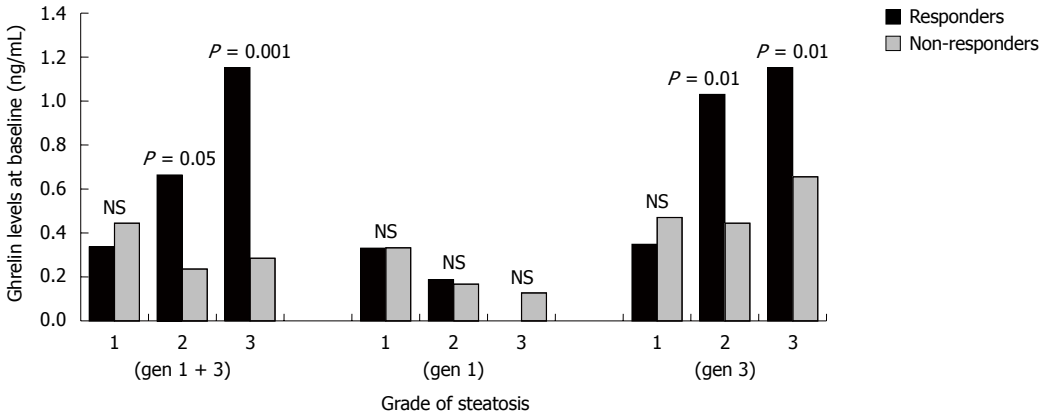

Figure 2 Correlation between ghrelin concentrations levels and steatosis in patients infected with hepatitis C virus genotypes11 and -3 who did and did not achieve sustained virological response following treatment with PEGylated interferon a-2b or a-2a plus ribavirin. NS: Not significant.

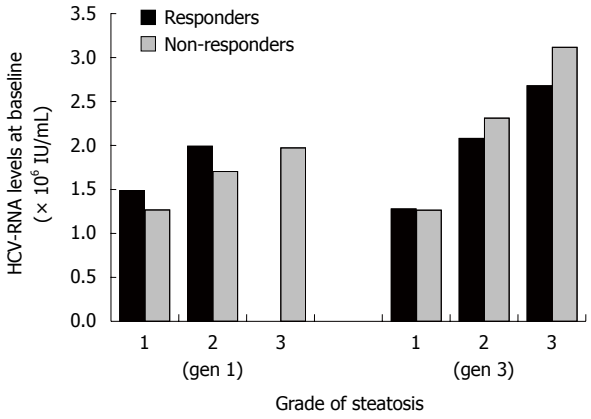

Figure 3 Correlation between viral load and steatosis in patients infected with hepatitis C virus genotypes-1 and -3 who did and did not achieve sustained virological response following treatment with PEGylated interferon a-2b or a-2a plus ribavirin. HCV: Hepatitis C virus.

tions, insulin resistance, metabolic syndrome, diabetes, alcohol consumption, and increased sensitivity of the liver to oxidative stress or cytokine-mediated injury[17,18].

Leptin is a putative link between HCV infection and steatosis[19]. Although a high incidence of hyperleptinemia has been observed in HCV infected patients with liver steatosis[10,20], the underlying mechanism promoting this effect remains undefined. Leptin may increase insulin resistance and fatty acid concentrations in the liver, leading to enhanced lipid peroxidation and promoting steatosis[5]. Leptin may also induce the release of cytokines, such as TNF-α, INF-γ, IL-18, and TGF-β1, which are involved in the pathogenesis of both liver steatosis and fibrosis[8]. In steatosis, activated hepatic stellate cells, but not quiescent cells, can express leptin[21].

Our results clearly show that, in HCV infected patients with liver steatosis, serum leptin levels tend to increase as the grade of steatosis worsens. This finding is significant, especially in genotype-1 patients, suggesting that leptin increases during infection as a part of the host immune response, and may contribute to the development of steatosis. Although steatosis is more common

Table 5 Multiple logistic regression derived odds ratios and 95% confidence intervals for response to hepatitis C virus therapy among patients infected with hepatitis C virus genotype-1 with respect to hepatic steatosis, and leptin and ghrelin concentrations at baseline and at end of follow-up

Variable	Category or increment	ORs	95% CIs	*P*-value
At baseline				
Hepatic steatosis	1 level more	0.12	0.02-0.66	0.01
Leptin	10 ng/mL more	0.43	0.22-0.83	0.01
Ghrelin	0.1 ng/mL more	1.11	0.67-2.01	0.63
HOMA-IR	1 level more	1.34	0.42-4.31	0.63
Alternatively introduced variables				
Fibrosis	1 level more	0.36	0.13 -0.96	0.04
Leptin	10 ng/mL more	0.45	0.24-0.86	0.02
Ghrelin	0.1 ng/mL more	1.35	0.82-2.45	0.26
HOMA-IR	1 level more	0.96	0.33-2.79	0.94
At end of follow-up				
Hepatic steatosis	1 level more	0.13	0.02-0.89	0.04
Leptin	10 ng/mL more	0.38	0.18-0.81	0.01
Ghrelin	0.1 ng/mL more	1.22	0.74-2.01	0.41
HOMA-IR	1 level more	1.98	0.54-7.30	0.30
Alternatively introduced variables				
Fibrosis	1 level more	0.30	0.10-0.93	0.04
Leptin	10 ng/mL more	0.34	0.16-0.72	0.01
Ghrelin	0.1 ng/mL more	1.22	0.74-1.82	0.47
HOMA-IR	1 level more	1.28	0.43-3.79	0.66

CI: Confidence interval; HOMA-IR: Homeostasis model assessment insulin resistance; ORs: Odds ratios.

and more severe in patients infected with HCV genotype-3[17,18], we found that leptin concentrations were not correlated with either the grade of steatosis or response to treatment. Structural and nonstructural proteins of HCV genotype-3 may directly cause steatosis by provoking oxidative stress[6,22,23]. Alternatively, the viral core protein may target microsomal triglyceride transfer protein activity, modifying very low density lipoprotein assembly in, and secretion by hepatocytes[24,25]. The core protein may also affect the cytoplasmic domain of members of the TNF receptor family or act directly on the mitochondria, leading to increased oxidative stress and lipid peroxidation[26]. In patients infected with HCV genotype-3,

Table 6 Multiple logistic regression derived odds ratios and 95% CIs for response to hepatitis C virus therapy among patients infected with hepatitis C virus genotype-3 with respect to hepatic steatosis, and leptin and ghrelin concentrations at baseline and at end of follow-up

Variable	Category or increment	ORs	95% CIs	*P*-value
At baseline				
Hepatic steatosis	1 level more	1.04	0.19-5.55	0.97
Leptin	10 ng/mL more	0.64	0.18-2.24	0.49
Ghrelin	0.1 ng/mL more	1.11	0.90-1.49	0.32
HOMA-IR	1 level more	0.13	0.02-0.68	0.02
Alternatively introduced variables				
Fibrosis	1 level more	0.16	0.02-0.11	0.06
Leptin	10 ng/mL more	1.22	0.37-4.08	0.74
Ghrelin	0.1 ng/mL more	1.22	0.90-1.65	0.16
HOMA-IR	1 level more	0.16	0.03-0.83	0.03
At end of follow-up				
Hepatic steatosis	1 level more	1.77	0.39-7.95	0.46
Leptin	10 ng/mL more	0.40	0.10-1.55	0.18
Ghrelin	0.1 ng/mL more	1.00	0.74-1.22	0.76
HOMA-IR	1 level more	0.10	0.02-0.59	0.01
Alternatively introduced variables				
Fibrosis	1 level more	0.40	0.08-1.52	0.16
Leptin	10 ng/mL more	0.70	0.22-2.18	0.54
Ghrelin	0.1 ng/mL more	1.00	0.74-1.35	0.85
HOMA-IR	1 level more	0.14	0.03-0.71	0.02

CI: Confidence interval; HOMA-IR: Homeostasis model assessment insulin resistance; ORs: Odds ratios.

we found that the grade of steatosis was correlated with higher viral load at baseline, in agreement with the direct "steatogenic" effect of this genotype[6].

Although ghrelin is important in food intake, energy balance, and the regulation of the growth hormone releasing mechanism[15], its role in hepatic disease has not been extensively evaluated to date. Increased serum ghrelin concentrations have been reported in patients with cirrhosis and hepatocellular carcinoma, suggesting that this adipokine may be involved in the anorexia-cachexia syndrome during the terminal stages of liver diseases[27]. Data on ghrelin concentrations in patients with CHC are limited[28].

We found that genotype-1 responders had higher serum ghrelin concentrations at baseline than non-responders, and that its concentration increased significantly in the former at the end of treatment, indicating that ghrelin may prevent or reduce steatosis by negatively regulating leptin. This may enhance the likelihood of SVR, since responders also have lower baseline leptin concentrations. In genotype-3 infected patients, however, ghrelin may be considered an independently acting factor, based on our finding that responders with moderate and severe steatosis had high ghrelin concentrations at baseline and that these concentrations were reduced significantly after treatment. In contrast, no significant differences were observed in non-responders and there were no correlations with leptin concentrations.

Our findings are in accordance with previous reports, which found that steatosis was an independent negative predictor of response to antiviral therapy[29-32]. We also found that genotype 1 patients with elevated leptin concentrations before treatment had a lower likelihood of achieving SVR, irrespective of their viral load. This observation is in keeping with the role of leptin as a suppressor of cytokine signaling 3 in the liver[33], or as a factor that inhibits INF signaling.

In conclusion, our results suggest that the extent of hepatic steatosis, in addition to the stage of fibrosis and the viral genotype, may affect the likelihood of SVR in CHC patients. Leptin appears to contribute to the pathogenesis of steatosis, and we found that elevated serum leptin concentration may be an independent predictor of SVR in HCV genotype-1 infected patients. Increase ghrelin concentrations after successful treatment in genotype 1 patients indicate that this peptide plays a role in the achievement of SVR. Further investigations are needed to determine whether ghrelin acts as a downstream mediator of leptin and to assess the influence of ghrelin on liver steatosis and HCV infection.

COMMENTS

Background

Steatosis is a frequent histopathological feature in patients with chronic hepatitis C (CHC). Leptin and ghrelin are involved in body fat regulation and metabolism. Higher serum leptin concentrations have been associated with steatosis, but less is known about leptin and ghrelin concentrations in patients with CHC and steatosis or the effect of these peptides on response to treatment.

Research frontiers

Leptin is a putative link between hepatitis C virus (HCV) infection and steatosis in HCV genotype-1 infected patients; however, the underlying mechanism remains undefined. Increased ghrelin concentrations have been reported in patients with cirrhosis and hepatocellular carcinoma, but its role in hepatic steatosis has not been extensively evaluated. Steatosis is an independent negative predictor of response to antiviral therapy. Our results clearly show that, in HCV infected patients with steatosis, serum leptin levels tend to increase as the grade of steatosis worsens. Non-responding genotype-1 infected patients have elevated leptin at baseline and genotype-1 and -3 responders have higher ghrelin concentrations at baseline. In genotype-3 infected patients, neither the degree of steatosis nor leptin concentration had any effect on response to treatment.

Innovations and breakthroughs

Several studies have highlighted the importance of steatosis and hyperleptinemia in the achievement of sustained virological response (SVR), but this study is the first to find genotype-dependent associations between the degree of steatosis and leptin and ghrelin concentrations. These findings show the significance of baseline leptin and ghrelin concentrations in the achievement of SVR, as well as the impact of antiviral treatment on serum leptin and ghrelin levels.

Applications

These findings suggest that serum leptin concentrations may be an independent negative predictor of SVR in HCV genotype-1 infected patients with steatosis; the role of ghrelin requires be further investigation.

Terminology

Leptin, the ob gene product, is expressed mainly by adipose tissue, although it is expressed in other organs, including the liver. Leptin is important for body fat regulation and metabolism. Ghrelin, a peptide that acts as an endogenous ligand for the growth hormone secretatog receptor, is involved in energy metabolism, food intake and glucose homeostasis.

Peer review

The research study has an important outcome and could be further strengthened by exploring the existing data for an effect of sex on these parameters, if any.

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BRIEF ARTICLE

In vitro effect of pantoprazole on lower esophageal sphincter tone in rats

Mustafa Duman, Mahmut Özer, Enver Reyhan, Yeliz Demirci, Ali E Atıcı, Tahsin Dalgıç, Erdal B Bostancı, Ece Genç

Mustafa Duman, Enver Reyhan, Ali E Atıcı, Tahsin Dalgıç, Erdal B Bostancı, Department of Gastrointestinal Surgery, Kartal Kosuyolu Education and Research Hospital, Cevizli, Kartal, 34846 Istanbul, Turkey
Mahmut Özer, Yeliz Demirci, Ece Genç, Department of Pharmacology, Medical School, Yeditepe University, Atasehir, 34755 Istanbul, Turkey
Author contributions: Duman M, Özer M and Demirci Y performed the majority of the experiments; Reyhan E, Atıcı AE and Dalgıç T conducted the statistical analysis and interpreted the results; Bostancı EB provided the necessary chemicals and contributed to the design of the experiment; Genç E contributed to the design of the study and writing of the manuscript.
Correspondence to: Ece Genç, Professor, Department of Pharmacology, Medical School, Yeditepe University, Kayışdağı, Ataşehir, 34755 Istanbul, Turkey. egenc@yeditepe.edu.tr
Telephone: +90-216-5780528 Fax: +90-216-5780575
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Abstract

AIM: To investigate the *in vitro* effects of pantoprazole on rat lower esophageal sphincter (LES) tone.

METHODS: Rats weighing 250-300 g, provided by the Yeditepe University Experimental Research Center (YÜDETAM), were used throughout the study. They were anesthetized before decapitation. LES tissues whose mucosal lining were removed were placed in a standard 30-mL organ bath with a modified Krebs solution and continuously aerated with 95% oxygen-5% carbon dioxide gas mixture and kept at room temperature. The tissues were allowed to stabilize for 60 min. Subsequently, the contractile response to 10^{-6} mol/L carbachol was obtained. Different concentrations of freshly prepared pantoprazole were added directly to the tissue bath to generate cumulative concentrations of 5×10^{-6} mol/L, 5×10^{-5} mol/L, and 1.5×10^{-4} mol/L. Activities were recorded on an online computer *via* a 4-channel transducer data acquisition system using the software BSL PRO v 3.7, which also analyzed the data.

RESULTS: Pantoprazole at 5×10^{-6} mol/L caused a small, but statistically insignificant, relaxation in the carbachol-contracted LES (2.23% *vs* 3.95%). The 5×10^{-5} mol/L concentration, however, caused a significant relaxation of 10.47% compared with the control. 1.5×10^{-4} mol/L concentration of pantoprazol caused a 19.89% relaxation in the carbachol contracted LES ($P < 0.001$).

CONCLUSION: This is the first study to demonstrate that pantoprazole has a relaxing effect in isolated LESs. These results might have significant clinical implications for the subset of patients using proton pump inhibitors who do not receive full symptomatic alleviation from gastroesophageal reflux disease.

Key words: Pantoprazole; Lower esophageal sphincter, Gastroesophageal reflux disease

Peer reviewer: Lygia Stewart, MD, Professor of Clinical Surgery, University of California San Francisco, 4150 Clement Street, San Francisco, CA 94121, United States

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INTRODUCTION

The esophagogastric junction is located between the esophagus and the stomach. The high-pressure zone at the junction between the esophagus and the stomach is composed of the lower esophageal sphincter (LES) and

the crural diaphragm[1,2]. Circular smooth muscle from the esophageal body generates little if any tone at rest, whereas the circular smooth muscle of LES is characterized by a spontaneously generated basal tone that prevents the reflux of gastric contents into the esophagus[3,4]. The basal tone of the LES is primarily myogenic in origin, but can be modulated by both neural and hormonal factors[5]. In response to esophageal distension and swallowing, the LES relaxes[6]. The abnormal dynamics of LES function are considered to be the most important factors in the pathogenesis of gastroesophageal reflux disease (GERD)[7-10]. GERD is described as the reflux of gastric contents into the esophagus leading to reflux symptoms and esophagitis sufficient to affect patient wellbeing and/or induce complications. These complications range from esophagitis to adenocarcinoma of the distal esophagus. Furthermore it may cause extra esophageal symptoms, such as cough, laryngitis and asthma[11,12]. GERD is a highly prevalent in the general population, affecting up to 10%-30% of the adult population in western countries[13].

Pharmacological therapy is necessary in the majority of patients. GERD is currently treated with acid suppressing drugs, such as proton pump inhibitors (PPIs); however, for those refractory to pharmacological treatment, surgery is often recommended[13,14]. PPIs are the mainstay of medical management for GERD[11]. They have been widely used since the 1980s and have been considered as ideal drugs because of their highly specific pharmacologic actions[15,16]. Although PPIs have been used as a common treatment modality in GERD, there is a lack of experimental studies of their effects on isolated LES preparations.

The aim of this study was to investigate the effect of a PPI, pantoprazole, on the tone of the isolated rat LES preparations contracted by carbachol. This study provides a significant contribution to this somewhat ignored area of research.

MATERIALS AND METHODS

The experimental protocol was approved by the Ethical Committee of Yeditepe University Experimental Medicine Research Institute and the use of animals was in compliance with US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Sixteen rats weighing 250-300 g, provided by the Yeditepe University Experimental Research Center (YÜDETAM), were used throughout the study. They were kept in plexiglass cages in a room whose temperature and humidity were controlled with 12-h light/dark cycle, and had free excess to food and water.

Rats were anesthetized with a combination of 10 mg/kg xylazine HCl (Rompun® 2%, Bayer HealthCare AG, Leverkusen-Germany) and 100 mg/kg ketamine HCl (Ketasol® 10%, Richter Pharma AG, Weis-Austria) before decapitation.

A midline incision was performed to open up the abdominal cavity and the LES was carefully dissected out and placed in a petri dish containing Krebs solution at room temperature. Thereafter, the mucosal lining was removed and the sphincteric muscle was set up, as a ring segment 2 mm in width, in Krebs solution contained in a standard 30-mL organ bath. The modified Krebs solution comprised NaCl, 118.07 mmol/L; KCl, 4.69 mmol/L; $CaCl_2$, 2.52 mmol/L; $MgSO_4$, 1.16 mmol/L; KH_2PO4, 1.2 mmol/L; $NaHCO_3$, 25 mmol/L, and glucose, 11.10 mmol/L. Krebs solution was continuously aerated with 95% oxygen-5% carbon dioxide gas mixture and kept at 37 ± 0.5 ℃ throughout the experimental period. The tissues were tied to stainless steel hooks at one end of the organ bath; the other end was connected to a force transducer (FDT 05, May, COMMAT Iletisim Co, Ankara-Turkey) under a resting tension of around 1 g. LES ring activities were recorded on an online computer *via* a 4-channel transducer data acquisition system (MP35, BIOPAC Systems Inc. Goleta, CA, United States) using the software BSL PRO v 3.7 (BIOPAC Systems Inc. Goleta, CA, United States), which also analyzed the data.

The following compounds were used: carbachol chloride (Carbamylcholine chloride, Sigma-Aldrich Chemical Co. St. Louis, MO, United States) and pantoprazole (Pantoprazole sodium, Dr. Reddy's Laboratories Ltd. Hyderabad-India). Solutions were prepared daily in distilled water and kept at 4 ℃ during the experiments. Pantoprazole was treated with 1 mol/L HCl and its pH was adjusted to 4.0 before application to the organ bath. Following a 60-min equilibration period for stabilization, the contractile response to carbachol was obtained by application of a single dose of charbachol to a final concentration of 10^{-6} mol/L in the organ bath. After the contractions reached a plateau, concentration-response relationships for pantoprazole (final organ bath concentrations of 5×10^{-6} mol/L, 5×10^{-5} mol/L and 1.5×10^{-4} mol/L, with 15 min allotted between each dose) were obtained in a cumulative manner. (These doses were calculated to be the equivalent of Human doses for the rats). Control experiments were also run with only acidified distilled water added to the organ bath. The relaxations were quantified by integrating the area under the curve for each concentration and control group. At the end of the each experiment, tissues were weighed and the final pH of the Krebs solution was measured.

Statistical analysis

For statistical evaluation, analysis of variance (One way ANOVA) was performed with the program SPSS for windows version 18 (SPSS Inc. Chicago, Illinois). Values of $P < 0.05$ were considered as statistically significant.

RESULTS

The experiment design is outlined in Figure 1. Pantoprazole caused dose dependent relaxation of the carbachol-contracted LES preparations. No such effect was observed in the control group (Figure 1B). The relaxations were quantified by integrating the area under the curve

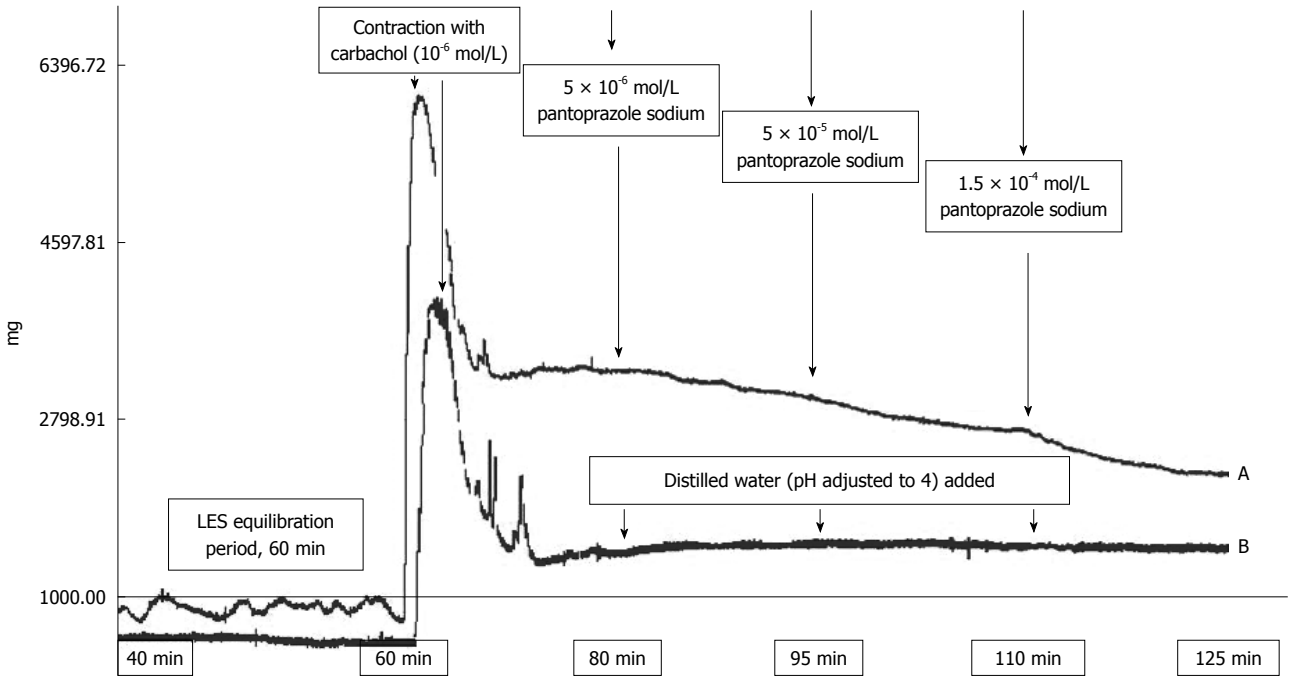

Figure 1 Outline of the experimental procedure. A: The tissues were allowed to stabilize for 60 min in Krebs-containing organ baths. Following that period, their contractile response to 10^{-6} mol/L carbachol was obtained. Pantoprazole was treated with 0.1 mol/L HCl and the pH of the drug solution was adjusted to 4.0. Different concentrations of pantoprazole were added directly to the tissue bath to generate cumulative concentrations of 5×10^{-6} mol/L, 5×10^{-5} mol/L and 1.5×10^{-4} mol/L. The relaxations were quantified by integrating area under the curve for each concentration; B: For the control experiments, acidified distilled water was added at the same time points. LES: Lower esophageal sphincter.

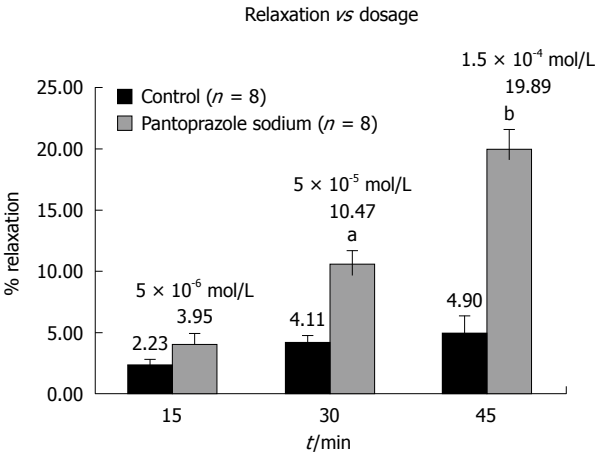

Figure 2 Relaxation *vs* Dosage. 5×10^{-5} mol/L and 1.5×10^{-4} mol/L pantoprazole sodium induced significant relaxation in lower esophageal sphincter preparations *in vitro* ($^{a}P < 0.05$ and $^{b}P < 0.001$). Each bar represents percent relaxation ± SEM for both control and experiment groups. Numbers in parentheses indicate the number of preparations used from different animals.

for each concentration.

The mean of integral values and percent relaxations of eight preparations were compared for statistical evaluation. As shown in Figure 2, application of pantoprazole sodium in a cumulative manner resulted in significant relaxations of LES preparations at 5×10^{-5} mol/L and 1.5×10^{-4} mol/L concentrations.

In the carbachol-contracted LES preparations 5×10^{-6} mol/L pantoprazole caused a 4% relaxation, while higher doses caused significant relaxations. Mean integral relaxation values were 4.11% ± 0.58% (SE) and 10.47% ± 1.2% (SE) for control and 5×10^{-5} mol/L pantoprazole, respectively ($P < 0.05$). Moreover, these values were 4.90% ± 1.4% (SE) and 19.89% ± 1.7% (SE) for control and 1.5×10^{-4} mol/L concentrations, respectively ($P < 0.001$) (Figure 2).

DISCUSSION

The aim of the present work was to assess the *in vitro* effects of pantoprazole on LES tone in rats. The reason why pantoprazole was chosen was the drug's frequent use in our Clinic. The major finding of our study was that pantoprazole caused a dose-dependent decrease in LES tone. This is the first study to demonstrate that pantoprazole has such an effect on isolated LES.

LES is an important specialized smooth muscle in the gastrointestinal tract and has been the subject of investigation by many authors[17-20]. GERD is a highly prevalent condition and is a major burden to society as well as the afflicted individual. Although numerous clinical studies have been conducted to clarify the mechanism of GERD, a clear consensus has not been reached. Regarding the pathophysiologies of GERD, decrease of LES basal tone and transient relaxations of the LES (TLOSRs) as a response to gastric distension[21], and excessive exposure of the esophagus to gastric acid, have been reported to be important[21-24].

GERD is, in most cases, successfully treated with PPIs, which have largely replaced Histamine H_2 receptor blockers because of their well documented efficacy

and because they are well tolerated, with relatively few serious adverse effects. However, a significant number of patients do not receive full symptomatic relief[25,26]. Thus, a significant question that has to be addressed is why some GERD patients are resistant to the effects of PPIs? In addition to neonates and infants who respond poorly to PPIs[27], some adults do not benefit from them either. In a study conducted by Hemmink *et al*[28] in 2008, there were fewer acid reflux episodes in patients on PPI therapy; however, weak acidic reflux episodes increased under the influence of PPIs. The total number of reflux episodes, on the other hand, was not affected. In addition to these, there have been recent papers regarding the adverse effects of PPIs[29,30]. Corley *et al*[31] showed that PPIs are associated with hip fractures among at-risk patients. They can also cause neutropenia in some patients[32]. Acid suppression also causes nosocomial *Clostridium* difficile infections in a dose-dependent manner[33].

These results point out the necessity of developing novel approaches for GERD. Coman *et al*[34] demonstrated the significance of adding prokinetic drugs to the treatment of GERD, in a study conducted on 1118 patients. The effects of specific GABA B receptor agonists have also been studied[35]. Drugs that reduce TLOSRs have also been suggested as pharmacological agents for GERD[36].

At present, the mechanism of the pantoprazole-induced relaxation of LESs can only be speculated. However, there are 2 types of muscles in the LES, circular muscle and sling muscle. Circular smooth muscle is tonically contracted with cholinergic stimulation. In response to swallowing, a peristaltic contraction travels down the length of the esophagus and the LES relaxes.

Nitric oxide (NO)[37,38] and vasoactive intestinal polypeptide (VIP)[39,40] are proposed as neurotransmitters that control relaxation. Both VIP and NO can be released from esophageal nerves with an appropriate stimulus, and NO synthase and VIP are found in myenteric neurons that innervate the circular smooth muscle of the esophagus. Sarioglu *et al*[41], showed the relaxant effect of omeprazole in rabbit corpus cavernosum *in vitro*. They concluded that the relaxant effect is probably due to the L-type Ca^{2+} channel blockage by omeprazole. We can speculate that a similar mechanism is responsible for the effect of pantoprazole on LESs.

The present study is the first to demonstrate a dose-dependent decrease in the carbachol-induced contraction of the LES by pantoprazole. Although this finding has been observed in an isolated tissue, it might have some clinical correlates and might help to understand why the treatment of GERD requires additional pharmacological interventions.

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COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a highly prevalent condition in the general population, affecting up to 10%-30% of the adult population in Western countries[13]. The incidence of GERD is rising very rapidly due to the stressful lives. New approaches are necessary for its treatment.

Research frontiers

Not all patients benefit from the proton pump inhibitors (PPIs) that are frequently used for the treatment of GERD. The authors conducted an experiment to investigate the effects of these drugs on isolated rat lower esophageal sphincters (LESs). There was a dose dependent decrease in LES tone.

Innovations and breakthroughs

The study conducted is the first to demonstrate the effects of pantoprazole on the isolated LESs of rat, including the dose dependent decrease in the tone of LESs under the effect of the drug.

Applications

The study suggests that doctors should be cautious about long-term use of PPIs for the treatment of GERD.

Peer review

This paper should be of interest to a broad readership including gastroenterologists, pharmacologists, and physicians of internal medicine. It is also of interest to gastrointestinal surgeons. This paper is very interesting and is an important study to publish.

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BRIEF ARTICLE

Autofluorescence imaging endoscopy for identification and assessment of inflammatory ulcerative colitis

Taro Osada, Atsushi Arakawa, Naoto Sakamoto, Hiroya Ueyama, Tomoyoshi Shibuya, Tatsuo Ogihara, Takashi Yao, Sumio Watanabe

Taro Osada, Naoto Sakamoto, Tomoyoshi Shibuya, Tatsuo Ogihara, Sumio Watanabe, Department of Gastroenterology, Juntendo University School of Medicine, 113-8421 Tokyo, Japan
Atsushi Arakawa, Hiroya Ueyama, Takashi Yao, Department of Human Pathology, Juntendo University School of Medicine, 113-8421 Tokyo, Japan
Author contributions: Osada T conceived and designed the study; Osada T, Arakawa A, Sakamoto N, Ueyama H, Shibuya T and Ogihara T collected the data; Osada T, Yao T and Watanabe S interpreted the data; Osada T wrote the manuscript; all authors discussed the results and commented on the manuscript; Watanabe S gave final approval of this article.
Correspondence to: Taro Osada, MD, PhD, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, 113-8421 Tokyo, Japan. otaro@juntendo.ac.jp
Telephone: +81-3-58021060 Fax: +81-3-38138862
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Abstract

AIM: To validate the clinical relevance of autofluorescence imaging (AFI) endoscopy for the assessment of inflammatory ulcerative colitis (UC).

METHODS: A total of 572 endoscopic images were selected from 42 UC patients: 286 taken with white light imaging (WLI) and 286 with AFI from the same sites. WLI images were assessed for overall mucosal inflammation according to Mayo endoscopic subscore (MES), and for seven characteristic endoscopic features. Likewise, AFI photographs were scored according to relative abundance of red, green and blue color components within each image based on an RGB additive color model. WLI and AFI endoscopic scores from the same sites were compared. Histological evaluation of biopsies was according to the Riley Index.

RESULTS: Relative to red (r = 0.52, P < 0.01) or blue (r = 0.56, P < 0.01) color component, the green color component of AFI (r = -0.62, P < 0.01) corresponded more closely with mucosal inflammation sites. There were significant differences in green color components between MES-0 (0.396 ± 0.043) and MES-1 (0.340 ± 0.035) (P < 0.01), and between MES-1 and ⩾ MES-2 (0.318 ± 0.037) (P < 0.01). The WLI scores for "vascular patterns" (r = -0.65, P < 0.01), "edema" (r = -0.62, P < 0.01), histology scores for "polymorphonuclear cells in the lamina propria" (r = -0.51, P < 0.01) and "crypt architectural irregularities" (r = -0.51, P < 0.01) showed correlation with the green color component of AFI. There were significant differences in green color components between limited (0.399 ± 0.042) and extensive (0.375 ± 0.044) (P = 0.014) polymorphonuclear cell infiltration within MES-0. As the severity of the mucosal inflammation increased, the green color component of AFI decreased. The AFI green color component was well correlated with the characteristic endoscopic and histological inflammatory features of UC.

CONCLUSION: AFI has application in detecting inflammatory lesions, including microscopic activity in the colonic mucosa of UC patients, based on the green color component of images.

Key words: Autofluorescence imaging endoscopy; Endoscopic activity; Histological activity; Microscopic inflammation; Green color component

Peer reviewers: Arjuna De Silva, Professor in Medicine, Department of Medicine, Faculty of Medicine, University of Kelaniya, PO Box 6, Tallagolla Road, 11010 Ragama, Sri Lanka; Jukka-Pekka Mecklin, MD, PhD, Professor, Surgeon-in-Chief, Department of Surgery, Jyvaskyla Central Hospital, 40620 Jyvaskyla, Finland

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INTRODUCTION

Ulcerative colitis (UC) activity is routinely assessed by a combination of clinical, endoscopic, and histological findings. However, systematic measurement of disease activity in UC patients is essential for determining the efficacy of treatment interventions. Endoscopy in particular is the most immediate and objective method for evaluating colonic mucosal damage. Furthermore, the severity of mucosal inflammation in UC is evaluated based on several disease activity indices, but there is no validated gold standard. To standardize the assessment of disease activity for clinical trials, in 1987, Schroeder *et al*[1] described the Mayo Score, which includes endoscopic findings. This index includes the factors "erythema", "vascular pattern", "friability", "erosion" and "spontaneous bleeding". "Edema" and "granularity" are also factored in other endoscopic indices, such as Matts' endoscopic activity index. These features are considered to be characteristic endoscopic findings in UC. However, inter- and intra-observer variations, and variations depending on the observer's experience in the endoscopic assessment of UC based on conventional endoscopy, have been reported[2].

Histological activity generally shows strong correlation with the activity evaluated by endoscopy. However, microscopic evaluations reflect clinical symptoms more accurately than endoscopic evaluations[3]. Endoscopic appearance alone tends to underestimate the severity of disease activity as compared with histological evaluation, and is not able to detect microscopic activity[4-6]. The features of mucosal lesions in UC are considered to include the presence of polymorphonuclear leukocytes in the lamina propria, the formation of crypt abscesses, ulcers and mononuclear cell infiltrate in the lamina propria together with crypt architectural irregularities[7]. In the Riley Index, 6 histological features of UC are factored and each feature is graded on a four-point scale[8]; this index has been applied in some clinical trials.

Autofluorescence imaging (AFI) videoendoscopy produces real-time pseudocolor images based on tissue autofluorescence emitted by excitation of endogenous tissue fluorophores, which mainly consist of collagen type- I [9]. If the change of hue on AFI indicated the extent and severity of UC activity, the analysis would be an objective and reproducible method regardless of the observer's experience. With this in mind, the aim of this study was to evaluate the correlations between the results of analysis of the change in hue on AFI with the results of white light imaging (WLI), together with histological findings on UC activity, to better understand the clinical relevance of AFI.

MATERIALS AND METHODS

Selection of images

This study included 42 patients with a diagnosis of UC, 31 male and 11 female, aged 36.2 ± 11.0 (mean ± SD) years who underwent colonoscopic examinations with a CF-FH260AZL/I colonovideoscope (Olympus Inc., Tokyo Japan). This endoscopic system comprised a high resolution white-light endoscope with optical zoom (magnification 75 ×) equipped with an AFI and narrow band imaging (NBI). The same sites were observed by WLI and AFI. Stored images were retrieved from the computerized database of the Endoscopy Centre at Juntendo University Hospital (Tokyo, Japan) by using an endoscopic filing system, Scope Reader M1 (AZ Co., Ltd., Sendai, Japan). A total of 572 endoscopic images, 286 with WLI and 286 with AFI, from the same sites where tissue biopsy specimens were subsequently taken were selected. The diagnosis of UC was based on the following criteria: history of recurrent bloody and mucous stools, endoscopic findings of ulceration, mucosal friability, loss of vascular architecture or presence of diffuse lesions increasing in severity towards the rectum, and no evidence of pathogenic micro-organisms in stool cultures. The images could be downloaded from the server in JPEG (Joint Photographic Experts Group) format without loss of quality. The file size of each downloaded image was about 100 kilobytes, with a pixel array of 640 × 480, and in 24-bit color.

Examination of images

Initially, all endoscopic examinations were done by one expert endoscopist. Subsequently, the images of WLI were provided for examination by two expert endoscopists (NS and TO), who discussed their assessments and reached a consensus view on the endoscopic activity. Each of these expert endoscopists had performed more than 5000 colonoscopy procedures, and was familiar with UC disease activity *via* endoscopy. Each WLI image was examined and graded for overall mucosal inflammation according to the Mayo endoscopic subscore (MES), scored on a scale of 0 to 3 (Table 1), and for seven endoscopic characteristic features of UC including vascular patterns, erythema, edema, granularity, erosions, ulcers and friability, scored on a scale of 0 to 10 (Table 2).

The optical findings with AFI colonoscopy were displayed as pseudocolored images, and laid over a composite image on the video display. All computer and video displays used the RGB additive color model in which red, green and blue lights are added together in various ways to produce a broad array of colors (256 × 256 × 256 = 16 million). AFI images were evaluated by analyzing the hue, and scored for the relative abundance of red [R/(R + G + B)], green [G/(R + G + B)] and blue

Table 1 Endoscopic scoring system scored from 0 to 3 (Mayo endoscopic subscore)

Score	
0	Normal or inactive disease
1	Mild disease (erythema, decreased vascular pattern, mild friability)
2	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	Severe disease (spontaneous bleeding, ulceration)

Table 2 Seven endoscopic features scored from 0 to 10 points

Vascular pattern	0 (normal) to 10 (absent)
Erythema	0 (absent) to 10 (marked)
Edema	0 (absent) to 10 (marked)
Granularity	0 (absent) to 10 (marked)
Erosions	0 (absent) to 10 (multiple)
Ulcerations	0 (absent) to 10 (multiple)
Friability	0 (absent) to 10 (marked)

Table 3 Histological scoring system (Riley index)

1	Round cells in the lamina propria
2	Polymorphonuclear cells in the lamina propria
3	Crypt abscesses
4	Mucin depletion
5	Surface epithelial integrity
6	Crypt architectural irregularities

Each item is scored from 0 (absent) to 3 points (severe).

(B/[R+G+B]) color components based on the RGB color model which involves the use of Adobe® Photoshop® Elements® 5.0. The color of the region on AFI, where tissue biopsy specimens were subsequently taken, was divided into red, green and blue color components. Although the actual count of each color varied as its brightness varied, the abundance of the actual count was fairly constant (Figure 1). All AFI and WLI images were displayed anonymously to the observers and revealed neither clinical data nor the date on which the images were taken.

Histological evaluations were done by two expert histopathologists (AA and HU) who were familiar with the histological activity in UC and had no knowledge of the clinical findings. They discussed their evaluation and determined the histological activity by using the Riley Index. Six histological features of UC were factored: acute inflammatory cell infiltrate (polymorphonuclear cells in the lamina propria), crypt abscesses, mucin depletion, surface epithelial integrity, chronic inflammatory cell infiltrate (round cells in the lamina propria), and crypt architectural irregularities. Each feature was graded on a four-point scale from 0 to 3 (Table 3).

The outcomes of the AFI analysis using the RGB color model were compared with the endoscopic activity, endoscopic and histological features at the same sites. In addition, the ability of AFI to detect the microscopic inflammatory lesions which were extensively infiltrated by polymorphonuclear cells within MES-0 was evaluated.

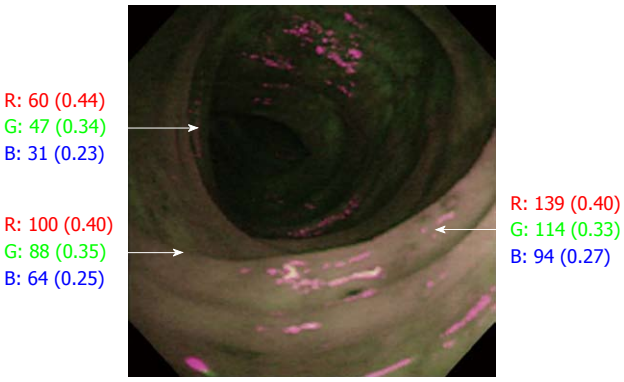

Figure 1 Endoscopic photograph by autofluorescence imaging showing the actual counts of red, green and blue colors based on the RGB additive model in different mucosal areas. The actual counts of red, green and blue colors varied depending on the brightness of the examined area, but the abundance of the actual counts, in parentheses, was approximately constant in the same picture.

Statistical analyses

The relative abundance of color components was expressed as the mean ± SD values. The relationships between endoscopic findings, histological evaluations, and the relative abundance of red, green and blue color components based on the RGB color model were determined by using the Spearman rank correlation coefficient (r). The Tukey-Kramer multiple comparison test was used for statistical analysis of the rate of color components based on the RGB color model among MES from 0 to 3. Before the Tukey-Kramer multiple comparison test, one-way analysis of variance (ANOVA) was performed. The Student's t-test (unpaired) was applied to compare the green color components based on the RGB color model between limited (combined grades 0 and 1) and extensive (combined grades 2 and 3) polymorphonuclear cell infiltration. Differences with P values < 0.05 were considered to be statistically significant.

RESULTS

The relationships between the RGB color components of AFI and WLI findings were evaluated in 572 endoscopic images from a total of 286 sites (Figure 2). Relative to the red (r = 0.52, P < 0.01) or blue (r = 0.56, P < 0.01) color component, the green color component (r = -0.62, P < 0.01) corresponded most often with sites of mucosal inflammation in different colonoscopy images (Figure 3A). However, there was an inverse relationship between the green color components and mucosal inflammation, so that the green color components of AFI diminished as the mucosal inflammation became more severe.

The average number of green color components for images classified into each MES subscore was 0.396 ± 0.043 for MES-0, 0.340 ± 0.035 for MES-1, 0.324 ± 0.034

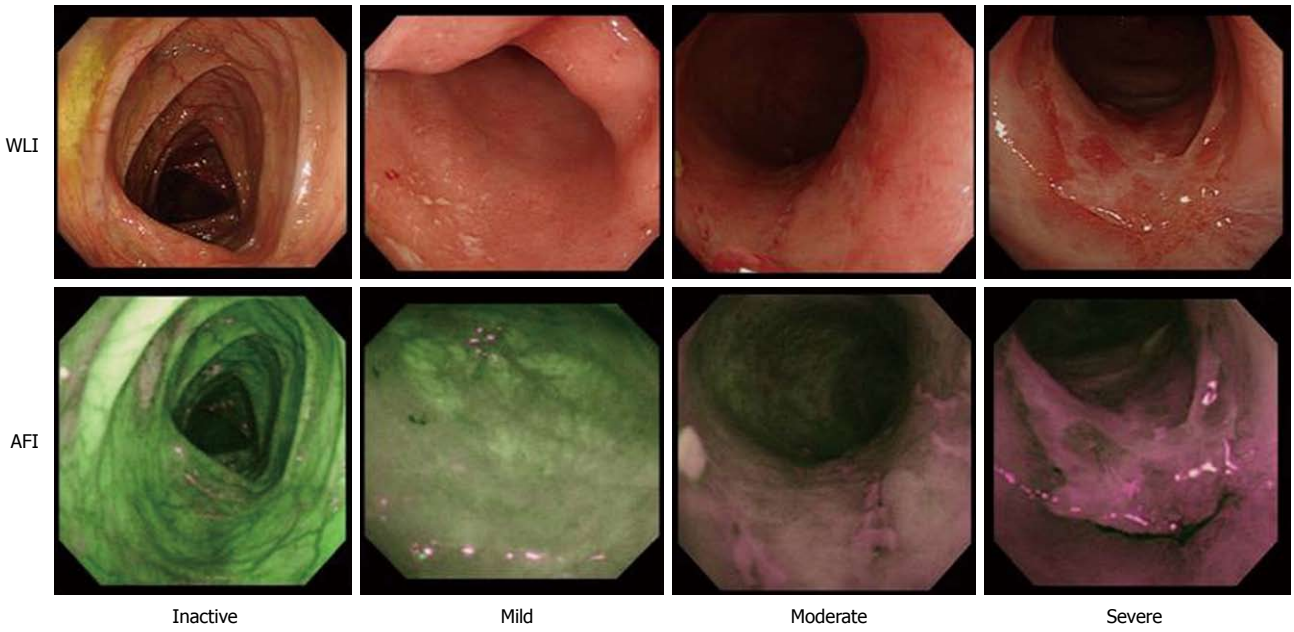

Figure 2 Representative endoscopic photographs of ulcerative colitis using white light imaging (upper row) and autofluorescence imaging (lower row) at the same sites according to the level of endoscopic ulcerative colitis activity (inactive, mild, moderate and severe). The color of the large intestinal mucosa on the autofluorescence imaging (AFI) changes by degrees, from green to grayish and magenta color. WLI: White light imaging.

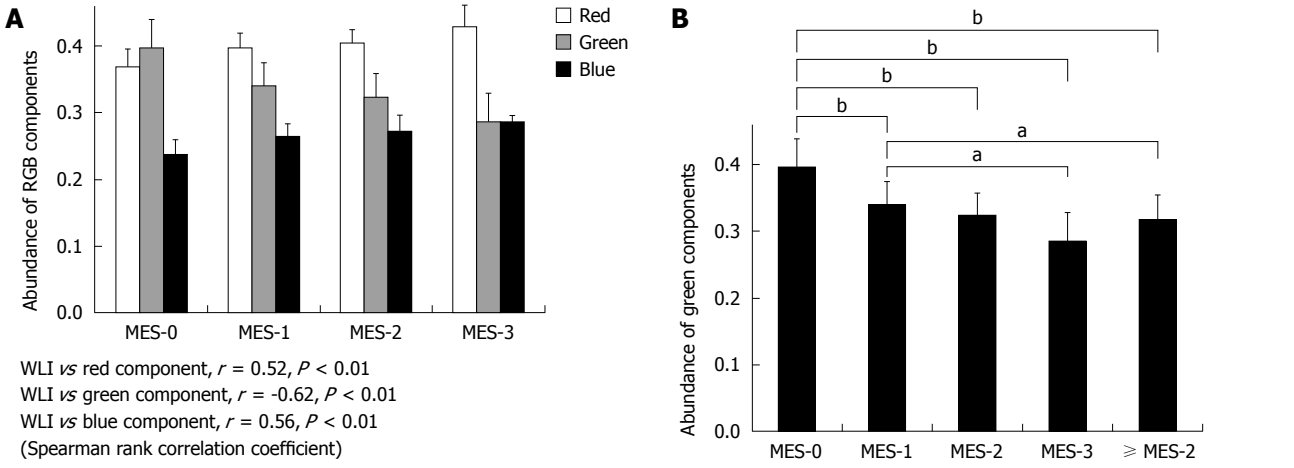

Figure 3 Comparison of overall mucosal inflammation on white light imaging and the abundance of RGB color components on autofluorescence imaging. A: The green color component corresponds most often with sites of mucosal inflammation in different colonoscopy images (according to Spearman rank correlation coefficient); B: There are significant differences in the green color component values between Mayo endoscopic subscore (MES)-0 and MES-1 ($^{b}P < 0.01$), between MES-0 and MES-2 ($^{b}P < 0.01$), between MES-0 and MES-3 ($^{b}P < 0.01$), between MES-1 and MES-3 ($^{a}P < 0.05$), between MES-0 and ⩾ MES-2 ($^{b}P < 0.01$) and between MES-1 and ⩾ MES-2 ($^{a}P < 0.05$), but not between MES-1 and MES-2 or between MES-2 and MES-3 (Tukey-Kramer multiple comparison test). WLI: White light imaging.

for MES-2, and 0.286 ± 0.043 for MES-3. There were significant differences in green color component values between MES-0 and MES-1 ($P < 0.01$), MES-0 and MES-2 ($P < 0.01$), MES-0 and MES-3 ($P < 0.01$), and MES-1 and MES-3 ($P < 0.05$), but not between MES-1 and MES-2, nor between MES-2 and MES-3. Likewise, there was a significant difference in green color component values between MES-1 and scores of ⩾ MES-2 (0.318 ± 0.037) as a combined group of MES-2 and MES-3 ($P < 0.01$) (Figure 3B). As the severity of the mucosal inflammation increased, the green color component of AFI decreased. However, the value of the color did not alter much between moderate and severe inflammation.

The relationships between the AFI green color component and WLI scores for the seven characteristic endoscopic features of UC are presented in Figure 4. The correlation coefficients for the AFI green color component with the scores of the seven characteristics were as follows: vascular pattern $r = -0.65$, $P < 0.01$; erythema $r = -0.55$, $P < 0.01$; edema $r = -0.62$, $P < 0.01$; granularity $r = -0.53$, $P < 0.01$; erosions $r = -0.45$, $P < 0.01$; ulcers $r = -0.23$, $P < 0.01$; and friability $r = -0.52$, $P < 0.01$. The AFI green color component was well correlated with the scores for vascular pattern and edema, but not with the

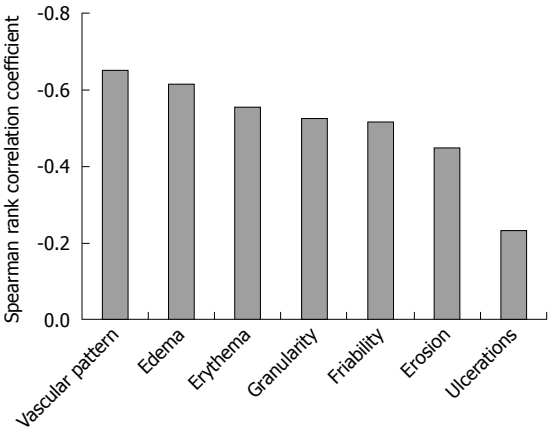

Figure 4 Comparison of seven endoscopic ulcerative colitis features on white light imaging and the green color component on autofluorescence imaging. The green color component on autofluorescence imaging is well correlated with vascular pattern (r = -0.65, P < 0.01) and edema (r = -0.62, P < 0.01) scores on WLI, but not with the ulcer score (r = -0.23, P < 0.01) (Spearman rank correlation coefficient). WLI: White light imaging.

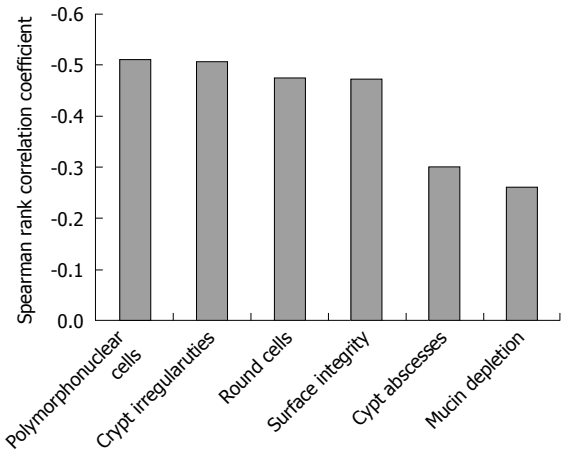

Figure 5 Comparison of histology scores, six histological characteristic ulcerative colitis features and the green color component on autofluorescence imaging. The autofluorescence imaging green color component is relatively well correlated with the scores for polymorphonuclear cells in the lamina propria (r = -0.51, P < 0.01) and crypt architectural irregularities (r = -0.51, P < 0.01), but not with the scores for crypt abscesses (r = -0.30, P < 0.01), and mucin depletion (r = -0.26, P < 0.01) based on Spearman rank correlation coefficient.

score for ulcers. It also showed relatively good correlation with the other endoscopic features of UC. The relationships between the AFI green color component and histology scores of six histological characteristic features of UC are shown in Figure 5. The correlation coefficients for the AFI green color component with the scores for the histological features were as follows: polymorphonuclear cells in the lamina propria r = -0.51, P < 0.01; crypt abscesses r = -0.30, P < 0.01; mucin depletion r = -0.26, P < 0.01; surface epithelial integrity r = -0.47, P < 0.01; round cells in the lamina propria r = -0.48, P < 0.01; and crypt architectural irregularities r = -0.51, P < 0.01. The AFI green color component was relatively well correlated with the scores for polymorphonuclear cells in the lamina propria

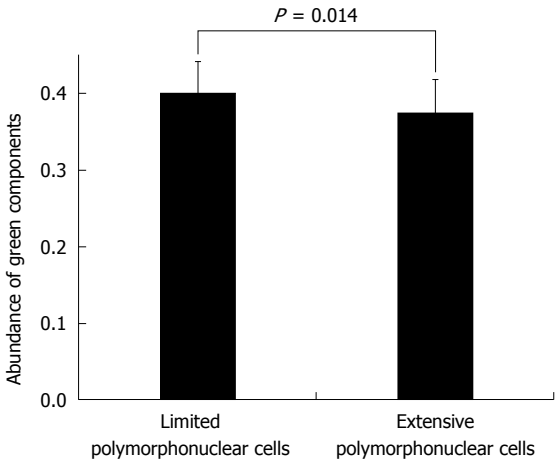

Figure 6 Comparison of limited (combined grades 0 and 1) and extensive (combined grades 2 and 3) polymorphonuclear cell infiltration of the green color components in Mayo endoscopic subscore-0. The mean number of green color components for images classified into the amount of polymorphonuclear cell infiltration in Mayo endoscopic subscore (MES)-0 is 0.399 ± 0.042 for limited and 0.375 ± 0.044 for extensive infiltration. There are significant differences in green color component values between limited and extensive polymorphonuclear cell infiltration in MES-0 (P = 0.014, by Student's t-test).

and with the crypt architectural irregularities, but not with the scores for crypt abscesses and mucin depletion.

The relationship between limited (combined grades 0 and 1) and extensive (combined grades 2 and 3) polymorphonuclear cell infiltration based on the green color components within MES-0 based on WLI are shown in Figure 6. The mean numbers of green color components for images classified according to the level of polymorphonuclear cell infiltration within MES-0 were 0.399 ± 0.042 and 0.375 ± 0.044 for limited and extensive infiltration, respectively. There were significant differences in green color component values between limited and extensive polymorphonuclear cell infiltration within MES-0 (P = 0.014). The abundance of green color components of AFI was also significantly different between microscopic inflammatory sites and histologically-quiescent UC lesions.

DISCUSSION

AFI has been used to highlight neoplastic tissue[10], minimal change in reflux esophagitis[11], the extent of chronic atrophic fundal gastritis[12], and Barrett's esophagus[13]. In all of these reports the lesions on the AFI are divided into two colors, such as green and magenta, green and pink, green and gray, green and purple, because investigators were concerned with the presence and the extent of the lesions, rather than with determining the severity of inflammation. In the present study, AFI pictures were scored according to the relative abundance of red, green and blue color components within each image based on an RGB additive model. Therefore, each component was expressed as a sequential variable number, and this facilitated evaluation of the severity of mucosal damage.

Further, the analysis was convenient to perform by using the Adobe® Photoshop® Elements® 5.0 software. Among the three color components, the green color component corresponded most often (and inversely) with sites of mucosal inflammation. Although fluorophores exist in both the mucosa and the submucosa, collagen in the submucosa emits strong green autofluorescence[14]. Our impression is that the presence of mucosal inflammation caused a high cell density and thickened the mucosal layer, making it difficult for the autofluorescence from the submucosa to penetrate the inflamed mucosa. Therefore, the green color component was reduced. The degree of the green color component might be valuable for assessing the extent of inflammation in the large intestine.

The overall mucosal inflammation in WLI, as assessed by MES, and the green color component in the AFI were well correlated. Specifically, there were significant differences in green color component values between MES-0 and MES-1, and between MES-1 and ⩾ MES-2, but not between MES-2 and MES-3. The degree of mucosal structural change around erosions and ulcers due to inflammation may differ only slightly between MES-2 and MES-3. Accordingly, the green color component of the AFI may not have been able to clearly distinguish moderate from severe inflammation.

Regarding the relationships between each of the seven characteristic endoscopic UC features on the WLI and the green color component of the AFI, vascular pattern and edema were well correlated with the green color component. These features are present from the early phase of UC, and decreased vascular pattern was particularly associated with MES-1, Baron score 1[15], modified Baron score 1[16], and Rachmilewitz index score 1[17]. This suggests that the green color component of the AFI is appropriate for detecting mucosal inflammation. In contrast, ulceration was not well correlated with the green color component of the AFI. The level of penetration of the green color component of the AFI from the submucosa through more than moderately inflamed mucosa might be constant regardless of the presence of ulcers. However, it was relatively simple to distinguish moderate from severe inflammation by using only WLI, based on the findings from spontaneous bleeding and multiple ulcers.

It has been reported that two measures of histological findings, such as architectural abnormalities including crypt architectural distortion and inflammatory features including large numbers of neutrophils, allow a distinction between inflammatory bowel disease and other causes of colorectal inflammation[18]. In our study, among six histological characteristic features of UC, polymorphonuclear cells in the lamina propria and crypt architectural irregularities were relatively well correlated with the green color component of the AFI. These two features are common in UC[19]. Polymorphonuclear cells in the lamina propria and crypt architectural irregularities are recognized in the active disease phase. The green color component of the AFI might reflect active mucosal inflammation. Other histological features of UC, especially mucin depletion and crypt abscesses, were not correlated with the green color component of the AFI. Although mucin depletion is a characteristic feature of UC, the degree of mucin depletion is not associated with the degree of inflammation. Crypt abscesses were observed in only about 7% of the biopsy specimens (26.2% of patients).

Although endoscopic remission was obtained after treatment, histological evidence of acute inflammatory cells often remained. The presence of microscopic inflammation contributed a 2- to 3-fold increase in the relapse rate[8]. The microscopic findings on inflammation were helpful in predicting relapse and for deciding on medical intervention. In this study, although the difference between microscopic inflammation and histological quiescence and the intensity of the green color components was relatively small, nevertheless the difference was significant. It is likely that microscopic inflammation was determined based on an evaluation of the green color component of the AFI.

In conclusion, the outcomes of the present investigation indicate that AFI is an appropriate new approach for detecting inflammatory regions and approximately estimating the severity of inflammation in UC patients, regardless of endoscopic experience. In particular, microscopic to moderate inflammation can be detected based on the green color component of the RGB additive color model. Further studies should strengthen our findings on the clinical relevance of AFI in patients with UC.

COMMENTS

Background

Endoscopic and histological findings define the degree of inflammatory activity in the clinical diagnosis of ulcerative colitis (UC). Although endoscopy is the most immediate method for assessing intestinal mucosal damage, the consistency of inter- and intra-observer findings and diagnoses have been reported to vary considerably. Substantial experience is necessary to accurately assess the disease activity in UC, particularly as microscopic inflammation cannot be detected by using conventional endoscopy.

Research frontiers

Autofluorescence imaging (AFI) videoendoscopy produces real-time pseudocolor images based on tissue autofluorescence emitted by excitation of endogenous tissue fluorophores. AFI has been used to highlight various lesions. There is no report on the change of hue related to AFI indicating extent and severity of UC activity.

Innovations and breakthroughs

AFI images were evaluated by analyzing the hue, and were scored relative to the abundance of red, green and blue color components of the RGB color model. As the green color components of AFI diminished, the mucosal inflammation was indicated to be more severe.

Applications

AFI appears to be an appropriate new approach and should be valuable for detecting inflammatory regions and approximately estimating the severity of inflammation in UC patients regardless of endoscopic experience, but based on the green color components of the RGB additive color model.

Terminology

AFI is a novel technique for detecting the autofluorescence emitted by the gastrointestinal tissues, mainly from collagen type-I in the submucosal layer, in real time. In an AFI mode, excitation light (395-475 nm) for inducing autofluorescence, together with green light (550 nm) and red light (610 nm) for obtaining reflection images, are generated by a light source equipped with a 300W xenon

arc lamp through a rotating filter. An excitation light cut filter is incorporated in the CCD for the AFI mode to allow only 490-625 nm light to reach the CCD.

Peer review

The present study demonstrates some technical progress when the AFI pictures were scored in a new way and the green color component correlated to the inflammatory changes on a significant level. However, the difference between mild inflammation and severe inflammation was small (about 10% in the abundance of green light components) and therefore our attitude to the results and conclusion should be cautious. As a preliminary and pioneering work this could be accepted for publication.

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BRIEF ARTICLE

Aspirin-induced small bowel injuries and the preventive effect of rebamipide

Kazuhiro Mizukami, Kazunari Murakami, Takashi Abe, Kunimitsu Inoue, Masahiro Uchida, Tadayoshi Okimoto, Masaaki Kodama, Toshio Fujioka

Kazuhiro Mizukami, Kazunari Murakami, Takashi Abe, Kunimitsu Inoue, Masahiro Uchida, Tadayoshi Okimoto, Masaaki Kodama, Toshio Fujioka, Department of General Medicine and Gastroenterology, Faculty of Medicine, Oita University, Yufu, Oita 879-5593, Japan
Author contributions: Mizukami K, Murakami K, and Fujioka T designed and performed research, analyzed data, and wrote the paper; Abe T, Inoue K, Uchida M, Okimoto T and Kodama M performed research and analyzed data.
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Correspondence to: Kazuhiro Mizukami, Dr., MD, Department of General Medicine and Gastroenterology, Faculty of Medicine, Oita University, 1-1, Idaigaoka, Hasama, Yufu, Oita 879-5593, Japan. mizkaz0809@oita-u.ac.jp
Telephone: +81-97-5866193 Fax: + 81-97-5866194
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Abstract

AIM: To evaluate the influence of taking low-dose aspirin for 4 wk on small intestinal complications and to examine the preventive effect of rebamipide.

METHODS: This study was conducted as a single-center, randomized, double-blind, cross-over, placebo-controlled study. Eleven healthy male subjects were enrolled. Each subject underwent video capsule endoscopy after 1 and 4 wk of taking aspirin and omeprazole, along with either rebamipide or placebo therapy. The primary endpoint was to evaluate small bowel damage in healthy subjects before and after taking low-dose aspirin for 4 wk.

RESULTS: The number of subjects with mucosal breaks (defined as multiple erosions and/or ulcers) were 1 at 1 wk and 1 at 4 wk on the jejunum, and 6 at 1 wk (P = 0.0061) and 7 at 4 wk on the ileum (P = 0.0019). Rebamipide significantly prevented mucosal breaks on the ileum compared with the placebo group (P = 0.0173 at 1 wk and P = 0.0266 at 4 wk).

CONCLUSION: Longer-term, low-dose aspirin administration induced damage in the small bowel. Rebamipide prevented this damage, and may be a candidate drug for treating aspirin-induced small bowel complications.

Key words: Healthy subjects; Low-dose aspirin; Small bowel injury; Capsule endoscopy; Rebamipide

Peer reviewer: Rami Eliakim, Professor, Department of Gastroenterology, Rambam Medical Center, Haifa 31096, Israel

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INTRODUCTION

Video capsule endoscopy (VCE)[1-2] is a practical technique that can be used to identify the causes and sites of obscure gastrointestinal bleeding. VCE allows for prospective investigation of small intestinal injuries, which frequently occur following the administration of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin. For example, Graham *et al*[3] used VCE and reported that small intestinal injury occurred in 71% of chronic NSAID users.

Low-dose aspirin is currently recommended for the secondary prevention of cardiovascular and cerebral diseases[4-6]. An observational registry reported that 70% to 80% of patients with a high risk of atherothrombosis

were receiving low-dose aspirin to prevent future vascular events[7]. Nonetheless, low-dose aspirin is not without risks. For instance, Lanas *et al*[8]reported that taking an anti-platelet agent induced lower, as well as upper, gastrointestinal (GI) events (16.9% and 15.5%, respectively). To date, there has been considerable interest in preventing upper gastrointestinal complications of NSAIDs; however, it has become clear that a strategy to prevent small bowel complications may also be needed[9].

It is not yet clear what duration of low-dose aspirin ingestion causes small bowel damage. Moreover, the frequency and severity of small bowel damage from taking low-dose aspirin is not yet known. Three recent reports investigating aspirin-induced small bowel damage were all short-term (1 to 2 wk) investigations[10-12]. These studies reported mild injuries (20% to 60%), such as erosion, in addition to more serious injuries (0 to 10%), such as ulcers[10-12]. The use of low-dose aspirin for cardiovascular prophylaxis is generally long-term, and it is clear that longer term observational studies are needed to examine damage to the small intestines.

The aims of this study were to investigate the frequency and type of small bowel damage associated with a 4 wk administration of low-dose aspirin in healthy subjects and to investigate the preventive effect of the cytoprotective agent, rebamipide, on aspirin-induced small bowel damage.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committees of Oita University, and written informed consent was obtained from all subjects. Eligible subjects were aged 20 to 65 years, who had taken no drugs during the one-month period prior to the start of this study and who had normal physical examinations. The exclusion criteria were as follows: (1) subjects who did not have a full length, small bowel VCE prior to the start of the study; (2) subjects with stenosis, tumors, ulcers, erosions, or bleeding in the small bowel; (3) subjects who had active GI disease or a history of ulcers, surgery, or bleeding; and (4) subjects who had used any medication, including NSAIDs or aspirin, within 4 wk of the start of the study.

Treatment protocol and post-treatment capsule endoscopy

This study was conducted using a cross-over design as shown in Figure 1. Medication groups A and B were defined as follows: group A: placebo plus aspirin (Bayer Pharmaceutical Co., Ltd., Tokyo, Japan) plus omeprazole (Sawai Pharmaceutical Co., Ltd., Osaka, Japan) for 4 wk; and group B: rebamipide (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) plus aspirin plus omeprazole for 4 wk (rebamipide 300 mg or placebo tid, aspirin 100 mg od, omeprazole 20 mg od). Omeprazole was used primarily for ethical reasons to avoid the effect of aspirin on the stomach.

Medications were administered for 4 wk. Then following a 4-wk washout period, the treatments were reversed for the two groups, and the medications were administered for a second 4-wk period. The washout period was designed according to a previous study by Niwa[13]. VCEs of the small bowel were performed five times: prior to the intervention, at 1 and 4 wk during the first period, and at 1 and 4 wk during the second treatment period.

Allocation and randomization were conducted by an independent pharmacologist of Yamanami Pharmacy who had no connection to our institution or the results of this study. The placebo was prepared by Yamanami Pharmacy.

Evaluation of small intestinal injuries

In this study, erosion and ulcer were defined according to Graham's report[3]. Red spots were defined as red areas without clear mucosal break. Erosions were defined as circumscribed areas of mucosal disruption denuded of villi with or without exudates or red color that involved a diameter equivalent to the valvulae conniventes. Ulcers were defined as erosions with a central area with exudates typically having a surrounding border of elevated mucosa, producing a target lesion or coral polyp appearance[3]. Typical cases of red spots and erosion are shown in Figures 2A and B, and an example of an ulcer is shown in Figure 2C.

Mucosal break was defined as two or more erosions and/or an ulcer. Red spots were scored, but were not considered as a significant injury, since they can be observed normally. The location of the injury was also scored in terms of the locations as proximal (jejunal) or distal (ileal) based on the transit time. The transit time from the pylorus to cecum was divided in half, and the first portion was arbitrarily defined as the jejunal section. Cases with erosion, multiple erosions, and ulcers were calculated. Multiple erosions were defined as more than 2 erosions in a subject. Subjects were only analyzed if the entire small bowel was observable by VCE.

Endpoints

The primary endpoint was to evaluate healthy subjects before and after 4 wk of low-dose aspirin-induced small bowel damage. The secondary endpoint was to evaluate the preventive effect of rebamipide.

Capsule endoscopy

We used the Olympus video capsule system (EndoCapsule, Olympus Ltd.; Tokyo, Japan) for this study. The capsule endoscopy procedure and the methodology for reviewing the images were conducted as previously described. All video images were analyzed twice by each skilled reviewer (KM, TA, and KI). These three investigators were instructed to mark any significant lesions under blinded conditions, and to evaluate lesions according to criteria for determination of the endpoints. If the results differed between the reviewers, then they consulted one

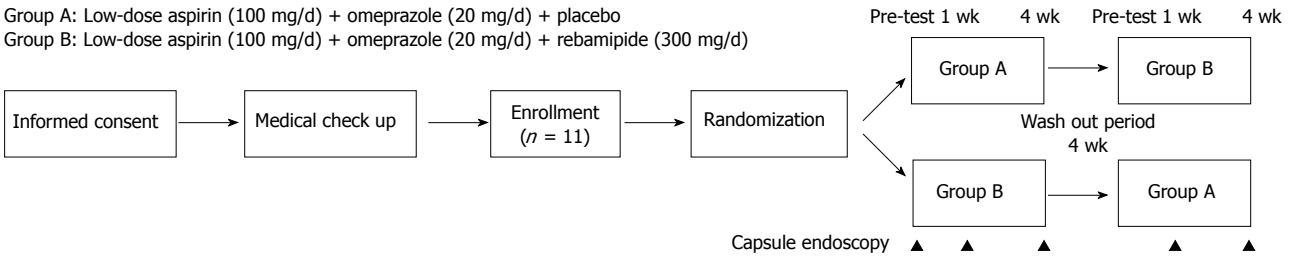

Figure 1 Study design.

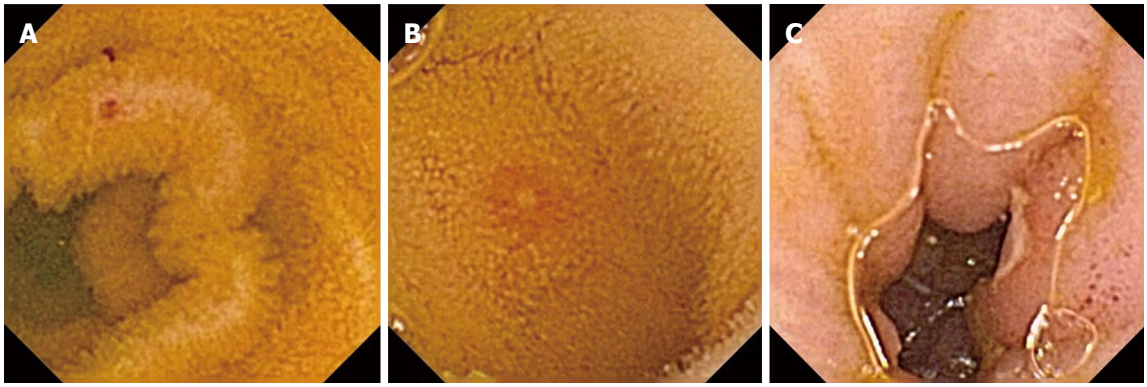

Figure 2 Typical small intestinal red spot, erosion and ulcer. A: Typical red spot; B: Typical erosion; C: Typical ulcer.

another to achieve a consensus. All images were saved for a final comprehensive analysis upon completion of all of the post-treatment capsule endoscopies.

Safety assessment

The subjects' symptoms were observed daily throughout the study periods, and the information was evaluated using a patient diary.

Statistical analysis

The primary endpoint was to evaluate the proportion of healthy subjects with small intestinal injury after 4 wk of low-dose aspirin therapy. The number of healthy subjects with small bowel mucosal breaks, ulcers, erosions, multiple erosions, and red spots were calculated and treated as parametric parameters. The small bowel area was divided into the jejunum and the ileum. The number of small bowel ulcers, erosions, and red spots were described as the mean ± SD. These values were analyzed at each measured point of the VCE in each group using Mann-Whitney's *U* test and compared with the evaluation prior to the aspirin administration.

The secondary endpoint was to compare the number of small intestinal injuries between the placebo and the rebamipide groups. The injuries were described as means ± SD and treated as nonparametric parameters. These injuries were analyzed at each measured point of the VCE in each group using Mann-Whitney's *U* test. In addition, the small intestinal injuries were compared between the placebo and rebamipide groups.

This study was a pilot study. Therefore, there were no reference data to calculate the sample size. A *P*-value < 0.05 was considered significant. All statistical analyses were performed using SAS version 8.2 (SAS Institute, Cary, NC, United States).

RESULTS

Eleven healthy subjects were enrolled in this study. The mean age of the subjects was 30 ± 6 years. The median age was 27 years, and the range was 24 to 43 years.

Influence of low-dose aspirin on small bowel mucosa in the placebo group

The number of subjects with multiple erosions, ulcers, and mucosal breaks of the small bowel are shown in Table 1. There were 8 subjects with red spots on the jejunum before the administration of aspirin, 10 subjects at 1 wk, and 10 at 4 wk. There were 8 subjects with red spots on the ileum before administration of aspirin, 11 subjects at 1 wk, and 11 at 4 wk. There were 2 subjects with small bowel erosion on the jejunum at 1 wk and 4 at 4 wk ($P = 0.0379$). There were 7 subjects with small bowel erosion on the ileum at 1 wk ($P = 0.0019$) and 9 at 4 wk ($P < 0.0001$).

The numbers of small bowel injuries before and after aspirin treatment are shown in Figure 3. There were 6.3 ± 6.9 small intestinal red spots at 1 wk and 9.6 ± 6.6 at 4 wk on the jejunum, and 68.0 ± 133.6 at 1 wk ($P = 0.0010$) and 48.4 ± 67.7 at 4 wk ($P = 0.0010$) on the ileum. There were 0.2 ± 0.6 small bowel erosions at 1 wk and 0.4 ± 0.6 at 4 wk on the jejunum, and 2.1 ± 2.6 at 1 wk ($P = 0.0156$)

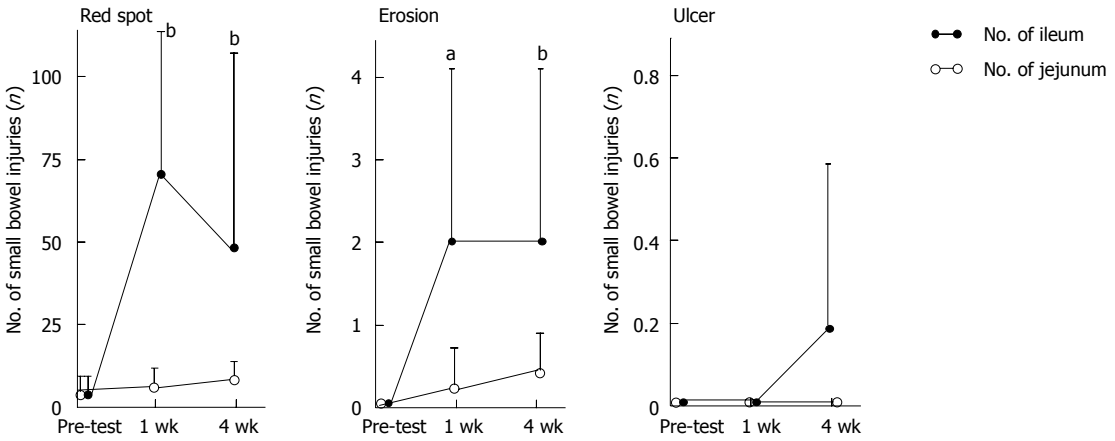

Figure 3 Low-dose aspirin-related small bowel injuries in the placebo group. The open circle indicates the number of low-dose aspirin-related small bowel injuries in the jejunum, and the closed circle indicates the number of injuries in the ileum at 4 wk. The data are represented as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.

Table 1 No. of healthy subjects presenting small bowel injuries following a 4 wk regimen of low dose aspirin in the placebo group and evaluation of preventive efficacy (n = 11)

	Jejunum			Ileum		
	Pre-test	1 wk	4 wk	Pre-test	1 wk	4 wk
Placebo group						
Multiple erosion (*n*)	0	1	1	0	6[b]	4[a]
Ulcer (n)	0	0	0	0	0	3
Mucosal break (*n*)	0	1	1	0	6[b]	7[b]
Rebamipide group						
Mucosal break (*n*)	0	0	1	0	1[c]	2[c]

Multiple erosion (*n*): No. of subjects with multiple erosions in jejunum or ileum; Ulcer (*n*): No. of subjects with ulcer in jejunum or ileum; Mucosal break (*n*): No. of subjects with multiple erosion and/or ulcer in jejunum or ileum. Statistical analysis was performed by Mann Whitney's *U* test, compared to before administration *vs* 1 wk and 4 wk. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.05$ compared with placebo and rebamipide group.

and 2.0 ± 2.5 at 4 wk ($P = 0.0039$) on the ileum. There were no small intestinal ulcers at 1 wk and at 4 wk on the jejunum, and there were no small intestinal ulcers at 1 wk and 0.2 ± 0.4 ulcers at 4 wk on the ileum.

Evaluation of the preventive effect of rebamipide

The preventive effect of rebamipide is shown in Table 1. There were no subjects with small bowel mucosal breaks on the jejunum at 1 wk and 1 subject at 4 wk, and there was 1 small bowel mucosal break on the ileum at 1 wk and 2 at 4 wk. Rebamipide significantly prevented small bowel mucosal breaks on the ileum compared with the placebo group ($P = 0.0173$ at 1 wk and $P = 0.0266$ at 4 wk). There were 15.7 ± 8.5 red spots on the ileum at 4 wk in the rebamipide group, which was significantly fewer than in the placebo group ($P = 0.0354$). There were 0.6 ± 1.2 erosions on the ileum at 4 wk, which was significantly fewer that in the placebo group ($P = 0.0362$).

Safety assessment

We examined symptoms daily for all subjects throughout the study period. Three subjects with ulcers were observed in the placebo group. Two of these subjects were symptomatic, but not anemic. The symptoms of these two subjects diminished 1 month after the study ended. One subject with an ulcer was observed in the rebamipide group, but the subject was neither symptomatic nor anemic.

DISCUSSION

In our study, we observed that the administration of low-dose aspirin induced small bowel red spots and erosions in healthy subjects in the early phase (1 wk). Red spots were observed in all subjects with a mean of 68.0 ± 133.6 at 1 wk and 48.4 ± 67.7 at 4 wk. There were 7 subjects with erosions on the ileum at 1 wk and 9 at 4 wk in the placebo group. These small bowel injuries were induced in the early phase and maintained while taking low-dose aspirin. On the other hand, small intestinal ulcers were observed in 3 subjects in the late phase (4 wk), although no ulcers were observed at 1 wk. These results indicate that serious small bowel injury, such as ulcers, may be induced by longer-term administration of low-dose aspirin. In addition, a very large ulcer was observed in one case at 4 wk (Figure 4), although no ulcer was observed in this subject at 1 wk. This may demonstrate a risk of longer-term low-dose aspirin ingestion. Patients taking low-dose aspirin should be given periodic management. In this study, small bowel bleeding was not observed; however, some patients may present small bowel bleeding due to the use of low-dose aspirin during treatment periods longer than 4 wk. The clinical implications of small bowel mucosal injuries are not yet clear. However, bleeding from mucosal injury can be fatal because aspirin is an anti-platelet agent. In this study, multiple erosions were also evaluated. They resulted in a more serious condition than a single erosion.

Our results indicate that most small bowel injuries occurred in the ileum rather than the jejunum area. There were 7 cases with a mucosal break in the ileum and 1 in the jejunum at 4 wk in the placebo group. Bjarnason *et al*[14] demonstrated that small bowel damage depended on various factors, such as microvascular aspects, neutrophil recruitment, mucosal prostaglandins,

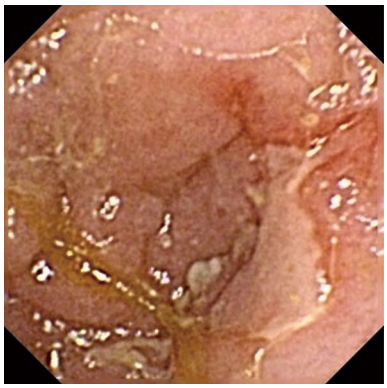

Figure 4 Case with a large ulcer of the small intestine induced by low-dose aspirin. A large ulcer was observed in the small intestine as a result of 4 wk of low-dose aspirin ingestion. The ulcer occupied one-third of the interior of the small intestine.

decreased blood flow, increased permeability, and bacteria. The number and variation of bacteria in the ileum is reported to be greater than in the jejunum[15]. The results of this study may be a reflection of the different bacterial environment in the ileum compared with the jejunum.

We conducted a comparative study with placebo and rebamipide to observe whether rebamipide could prevent aspirin-induced small bowel injuries. Niwa *et al*[13] reported that rebamipide had a preventive effect on diclofenac-induced small-bowel injury compared to the placebo. The preventive ratio of diclofenac-induced small bowel mucosal breaks was 60% in the placebo group and 20% in the rebamipide group, and this difference was statistically significant. Moreover, Fujimori *et al*[16] demonstrated that the prostaglandin analogue misoprostol prevented diclofenac-induced small-intestinal complications. These two reports indicate the importance of prostaglandins.

Rebamipide {2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinon-4-yl]-propionic acid} is a cytoprotective antiulcer drug that stimulates the production of endogenous prostaglandins[17]. Here, we observed that rebamipide significantly prevented small bowel mucosal breaks in the ileum area compared to the placebo group at both 1 wk and 4 wk. There were 3 subjects with ulcers in the ileum area at 4 wk in the placebo group, compared with 1 subject with an ulcer at 4 wk in the rebamipide group. The administration of rebamipide showed potential in preventing the incidence of ulcers; however, rebamipide does not have an anti-bacterial effect. The action of rebamipide may be explained by Bjarnason's hypothesis[14]. Previous reports have indicated that rebamipide induces the production of intracellular prostaglandins[14], improves blood flow[18], suppresses increases in permeability[19], scavenges free radicals[20], and has an anti-inflammatory action[21]. Presently, there is no drug to prevent aspirin-induced small bowel complications or treat patients with these complications, so it is essential to research drugs with these properties in the future.

A limitation of this study is that the study size was small. We did not use a large number of patients, since this was a preliminary study. In addition, since the small bowel is a long organ, it will be necessary to further investigate the appropriate dosage for this candidate drug.

In conclusion, the use of long-term low-dose aspirin induced small bowel damage. Rebamipide prevented this damage, and this candidate drug may be suitable for preventing aspirin-induced small bowel complications.

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COMMENTS

Background

Low-dose aspirin is currently recommended for the secondary prevention of cardiovascular and cerebral diseases. Recently, aspirin-induced small bowel complications have become the focus of investigations around the world.

Research frontiers

Few studies have observed healthy subjects with low-dose aspirin-induced, small bowel injuries using capsule endoscopy. These results demonstrated the prevalence of slight and low frequency small bowel injuries. However, these studies examined the effects of low-dose aspirin ingestion in the very short-term (1 or 2 wk). Here, we investigated the influence of small bowel damage following ingestion of low-dose aspirin for a longer period of 4 wk.

Innovations and breakthroughs

In this study, the ingestion of low-dose aspirin for 4 wk induced small bowel ulcers. In one case, a huge ulcer developed at 4 wk, although no ulcer was observed at 1 wk. Although it is common for patients to take low-dose aspirin for more than 4 wk, this duration of ingestion may be the limit in healthy subjects. Moreover, the results of this study demonstrate the differences in small bowel injuries after ingesting low-dose aspirin for 1 wk or 4 wk. Taking rebamipide was found to prevent aspirin-induced small bowel injuries.

Applications

The results of this study indicate that long-term use of low-dose aspirin can cause small intestinal injuries. Rebamipride could prevent this damage.

Terminology

Rebamipide is a cytoprotective antiulcer drug that stimulates the production of endogenous prostaglandins. Its actions include scavenging free radicals, elevating blood flow, and suppressing permeability. In this study, capsule endoscopy was performed using the Olympus video capsule system (EndoCapsule, Olympus Ltd.; Tokyo, Japan).

Peer review

This study focused on the influence of low-dose aspirin ingestion for 4 wk on small bowel damage in healthy subjects. The study was designed as a randomized, placebo-controlled, double-blind, cross-over study using video capsule endoscopy. The long-term (4 wk) use of low-dose aspirin induced small bowel damage. Rebamipide prevented this damage, and it may be a candidate drug for preventing aspirin-induced small bowel complications.

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BRIEF ARTICLE

Superiority of metastatic lymph node ratio to the 7th edition UICC N staging in gastric cancer

Long-Bin Xiao, Jian-Xing Yu, Wen-Hui Wu, Feng-Feng Xu, Shi-Bin Yang

Long-Bin Xiao, Jian-Xing Yu, Wen-Hui Wu, Feng-Feng Xu, Shi-Bin Yang, Department of Gastrointestinal-pancreatic Surgery, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China
Author contributions: Xiao LB and Yu JX contributed equally to this work; Yu JX and Wu WH performed the majority of data collection and wrote the manuscript; Xu FF and Yang SB performed the statistical analysis; Xiao LB designed the study and provided financial support for this work.
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Correspondence to: Long-Bin Xiao, MD, Department of Gastrointestinal-pancreatic Surgery, the First Affiliated Hospital, Sun Yat-Sen University, 58 Zhongshan Road Ⅱ, Guangzhou 510080, Guangdong Province, China. longbinxiao@yahoo.cn
Telephone: +86-20-82379585 Fax: +86-20-82379495
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Abstract

AIM: To compare and evaluate the appropriate prognostic indicators of lymph node basic staging in gastric cancer patients who underwent radical resection.

METHODS: A total of 1042 gastric cancer patients who underwent radical resection and D2 lymphadenectomy were staged using the 6th and 7th edition International Union Against Cancer (UICC) N staging methods and the metastatic lymph node ratio (MLNR) staging. Homogeneity, discriminatory ability, and gradient monotonicity of the various staging methods were compared using linear trend χ^2, likelihood ratio χ^2 statistics, and Akaike information criterion (AIC) calculations. The area under the curve (AUC) was calculated to compare the predictive ability of the aforementioned three staging methods.

RESULTS: Optimal cut-points of the MLNR were calculated as MLNR0 (0), MLNR1 (0.01-0.30), MLNR2 (0.31-0.50), and MLNR3 (0.51-1.00). In univariate, multivariate, and stratified analyses, MLNR staging was superior to the 6th and 7th edition UICC N staging methods. MLNR staging had a higher AUC, higher linear trend and likelihood ratio χ^2 scores and lower AIC values than the other two staging methods.

CONCLUSION: MLNR staging predicts survival after gastric cancer more precisely than the 6th and 7th edition UICC N classifications and should be considered as an alternative to current pathological N staging.

Key words: Gastric cancer; Metastatic lymph node ratio; Prognosis

Peer reviewer: Lygia Stewart, MD, Professor of Clinical Surgery, University of California San Francisco, 4150 Clement Street, San Francisco, CA 94121, United States

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INTRODUCTION

In recent years, more cases of gastric cancer have been diagnosed in China than in any other country[1]. Accurate prognosis prediction for gastric cancer patients enables doctors to determine the patients' expected clinical courses and to have more information when deciding whether to use adjuvant therapy and when comparing the therapeutic effects of different treatment modalities. A widely used classification proposed by the International

Union Against Cancer (UICC), the tumor-node-metastasis (TNM) system, combines the most powerful and reliable factors for analyzing tumor status[2,3]. Lymph node metastasis is one of the most important gastric cancer prognostic factors[4]. The identified number of involved lymph nodes depends on the number of lymph nodes removed and examined, which in turn depends on the surgical and pathologic procedures. Although TNM classification is a convenient and reproducible method for precise staging, it demands the examination of at least 15 lymph nodes. If the number of dissected and examined lymph nodes is small, downmigration of N stage may occur, and conversely, if the number is large, upmigration of N stage may occur, which is also referred to as stage migration in some references[5-10]. To improve prognosis prediction, the number of positive lymph nodes should be considered in the context of the number of nodes examined. The metastatic lymph node ratio (MLNR), defined as the number of positive lymph nodes divided by the number of lymph nodes retrieved, has been proposed as an alternative to classification systems that assess the absolute number of positive lymph nodes, such as the UICC (2002, 6th edition) or Japanese Gastric Cancer Association (JGCA) (1998, 2nd English edition) staging systems[11-16].

This year, gastric cancer lymph node metastasis staging was changed in both the UICC 7th edition and the JGCA 14th edition staging systems in that it now depends solely on the number of metastatic nodes found[3,17]. In the new UICC and JGCA systems, patients with one to two positive lymph nodes are classified as N1, patients with three to six positive lymph nodes are classified as N2, and patients with seven or more positive lymph nodes are classified as N3. Some authors have demonstrated that the 7th edition UICC staging system is superior to the 6th edition based on its homogeneity, discriminatory ability and prognostic value[18-20].

However, to date there has been no formal study that focused on comparing the prognostic significance of the MLNR with that of the 7th edition UICC N staging system. In the present article, we investigate whether patients with gastric cancer can be classified into meaningful risk categories based on MLNR by comparing this staging system with the 7th edition UICC N staging system.

MATERIALS AND METHODS

Patients

Between January 1996 and December 2007, 1042 patients with histologically diagnosed gastric cancer underwent surgery at the Department of Gastrointestinal-pancreatic Surgery, First Affiliated Hospital, Sun Yat-Sen University, China. The postoperative pathological results included tumor size, histological type, margin, adjacent tissues and neighboring organs, lymphatic/venous invasion, retrieved lymph nodes, metastatic lymph nodes, and pTNM staging. The inclusion criteria of the study were as follows: (1) gastric adenocarcinoma identified by histo-pathological examination; (2) histologically confirmed R0 resection, which was defined as no macroscopic or microscopic residual tumor; and (3) availability of complete follow-up data. Patients with distant metastases, a history of familial malignancy or other synchronous malignancy (such as gastrointestinal stromal tumor, esophageal cancer, colorectal cancer, *etc.*), or carcinoma of the gastric stump after gastric resection for benign disease or who died in the perioperative period were excluded from the study.

D2 lymphadenectomy was performed by experienced surgeons following the JGCA guidelines[21]. A total of 15 313 lymph nodes were retrieved, with a mean of 14.70 ± 10.25 lymph nodes per patient (25.14 ± 9.28 for patients with > 15 lymph nodes retrieved and 8.58 ± 3.87 for patients with ⩽ 15 lymph nodes retrieved) and a range from 3 to 66. The mean number of lymph nodes with evidence of metastasis was 6.40 ± 6.90 per patient (9.78 ± 9.42 for patients with > 15 lymph nodes retrieved and 4.15 ± 2.83 for patients with ⩽ 15 lymph nodes retrieved), with a range from 1 to 70. Lymph node involvement was classified according to the 7th edition UICC (2010) N staging system (N0: no metastasis; N1: 1-2 metastatic lymph nodes; N2: 3-6 metastatic lymph nodes; N3: ⩾ 7 metastatic lymph nodes) and 6th edition UICC (2002) N staging system (N0: no metastasis; N1: 1-6 metastatic lymph nodes; N2: 7-15 metastatic lymph nodes; N3: ⩾ 16 metastatic lymph nodes). All nodal material was separately dissected from the specimen by a surgeon at the end of the procedure. Our study does not include stage Ⅳ patients, graded according to the UICC 7th edition staging system, because all of the patients enrolled underwent radical resection and had no distant metastasis.

Follow-up

Postoperative follow-up at our outpatient department included clinical and laboratory examinations every 3 mo for the first 2 years, every 6 mo during the third to fifth years, and annually thereafter until at least 5 years after the operation or until the patient died, whichever came first. Overall patient survival, defined as the time from operation to death or final follow-up, was used as a measure of prognosis. The median follow-up for the entire cohort was 56 mo (range 3-178 mo).

Statistical analysis

To determine the appropriate MLNR cut-points in the entire cohort, our analysis for the best cut-points was conducted as follows: In the first step, we evaluated the prognostic value of the MLNR, adjusting for other clinicopathological covariates that are significantly associated with gastric cancer mortality. Second, patients having no involved lymph nodes (MLNR = 0) were assigned to one group because it has been well documented that their prognosis significantly differs from patients with metastatic lymph nodes[12,15,22,23]. After ascertaining that the MLNR was significantly associated with gastric cancer mortality, we determined two additional appropriate cut-points for categorizing the MLNR to make our cut-

points comparable with those for UICC N staging. For this, we recomputed the likelihood associated with all possible pairs of MLNR cutoffs ranging from 0.05 to 0.95 at intervals of 0.05. In our study, the two alternative cut-points for the MLNR were 0.30 and 0.50. Martingale residual analysis was also used to examine the function form of the MLNR, and our cut-points (0, 0.30 and 0.50) were found to be consistent. After extensive evaluations of our data, no other sets of cut-points performed better than those already described. Thus, four subgroups of the MLNR classification (MLNR0, 0%; MLNR1, 1%-30%; MLNR2, 31%-50%; MLNR3, 51%-100%) were used in our study.

To directly compare the 6th and 7th edition UICC N staging systems with the present MLNR staging system, we took advantage of two statistical methods. One method considers the homogeneity, discriminatory ability and monotonicity of the gradient test. Homogeneity was measured with the likelihood ratio χ^2 test related to the Cox regression model. The discriminatory ability and monotonicity of the gradient were measured with the linear trend χ^2 test. The likelihood ratio χ^2 test was used to assess homogeneity within each classification system and to estimate the gradient monotonicity. Additionally, the Akaike information criterion (AIC) value within a Cox proportional hazard regression model was used to measure the discriminatory ability of each system[24]. The AIC statistic was defined by AIC = -2 log maximum likelihood + 2 × the number of parameters in the model. A smaller AIC value indicates that the model is better at predicting outcome. The other method involves receiver operating characteristic (ROC) curves. ROC curves and the areas under the curves (AUC) were calculated for each of the aforementioned three N staging systems to assess the accuracy of their predictive ability. Differences between the AUC were tested for statistical significance based on the estimated areas and their standard errors[25].

The 5-year survival rate was calculated using the Kaplan-Meier method. The log-rank test was used to make statistical comparisons of different factors. Pearson correlations were examined with a two-tailed test. In multivariate analysis, forward stepwise regression analysis was performed with a Cox proportional hazards model. A P value of $\leqslant 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Of the 1042 patients, 708 were male (67.9%) and 334 were female (32.1%). The mean patient age was 57.4 ± 11.5 years (range 20-79 years). The overall 5-year survival rate for all patients was 47.5%, and 474 patients were alive when our follow-up was complete.

Univariate and multivariate survival analysis

After univariate analysis of the 1042 patients who underwent radical resection, ten factors were found to have statistically significant associations with overall survival (OS). They were: age, tumor location, tumor size, histological grade, lymphatic/venous invasion, pT, 6th edition UICC pN, 7th edition UICC pN, MLNR, and the number of retrieved lymph nodes (Table 1). We summarize the postoperative survival results as follows: (1) patients who were older had significantly shorter OS than those who were younger [hazard ratio (HR) = 1.019, $P < 0.001$]; (2) patients whose primary tumor was located in the distal third of the stomach had significantly longer OS than those whose primary tumor was located elsewhere in the stomach (HR = 0.735, $P < 0.001$); (3) patients with a larger primary tumor had significantly shorter OS than those with a smaller primary tumor (HR = 1.147, $P < 0.001$); (4) patients with poorly differentiated or undifferentiated adenocarcinoma had significantly shorter OS than those with well or moderately differentiated adenocarcinoma (HR = 1.254, $P < 0.001$); (5) patients with tumor lymphatic/venous invasion had significantly shorter OS than those without lymphatic/venous invasion (HR = 2.685, $P < 0.001$); (6) the deeper the primary tumor invasion, the shorter the OS of the gastric cancer patients (HR = 1.852, $P < 0.001$); (7) the higher the metastatic lymph node counts of the 6th edition UICC N stage, the shorter the OS of the gastric cancer patients (HR = 1.571, $P < 0.001$); (8) the higher the metastatic lymph node counts of the 7th edition UICC N stage, the shorter the OS of the gastric cancer patients (HR = 1.604, $P < 0.001$); (9) the higher the MLNR stage, the shorter the OS of the gastric cancer patients (HR = 1.776, $P < 0.001$); and (10) patients who had more than 15 lymph nodes retrieved had significantly longer OS than those who had $\leqslant$ 15 lymph nodes retrieved (HR = 0.616, $P < 0.001$). All of the aforementioned 10 variables were included in a multivariate Cox proportional hazards model (forward stepwise procedure) to adjust for the effects of covariates (Table 2). In that model, we demonstrated that age, tumor location, tumor size, histological grade, lymphatic/venous invasion, pT, the 7th edition UICC N staging, MLNR, and the number of retrieved lymph nodes were independent prognostic factors, while the 6th edition UICC N staging was excluded.

The survival curves developed according to the 6th and 7th edition UICC N staging systems and the MLNR staging system are shown in Figure 1. For all three staging systems, the Kaplan-Meier plot had good discriminatory ability in each group except in N2 and N3 of the 6th edition UICC N staging (Figure 1). The 5-year survival rates of N0, N1, N2, and N3 patients in the 6th edition UICC N staging were 71.1%, 43.3%, 21.4%, and 25.1%, respectively ($P < 0.001$, $P < 0.001$ and $P = 0.143$, respectively). The 5-year survival rates of N0, N1, N2, and N3 patients in the 7th edition UICC N staging were 71.1%, 50.7%, 37.5%, and 22.2%, respectively ($P < 0.001$, $P = 0.003$ and $P = 0.001$, respectively). The 5-year survival rates of MLNR0, MLNR1, MLNR2, and MLNR3 patients were 71.1%, 59.0%, 32.7% and 16.0%, respectively ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively).

Table 1 Univariate analysis of various clinicopathologic factors in 1042 cases of gastric cancer

Variable	*n* (%)	5-yr survival rate (%)	Log rank χ^2 value	Hazard ratio	*P* value
Gender			0.433	1.060	0.511
Male	708 (67.9)	48.6			
Female	334 (32.1)	45.1			
Age (continuous)	1042 (100)	47.5	124.704	1.019	< 0.001
Tumor location			78.529	0.735	< 0.001
Proximal	579 (55.6)	39.0			
Distal	418 (40.1)	62.0			
Two-thirds or more	45 (4.3)	17.5			
Tumor size (continuous)		47.5	124.704	1.147	< 0.001
Histological grade			18.407	1.254	< 0.001
Well/moderately differentiated adenocarcinoma	388 (37.2)	54.8			
Poorly differentiated adenocarcinoma	448 (43.0)	44.7			
Undifferentiated adenocarcinoma/signet-ring cell carcinoma/mucinous adenocarcinoma	206 (19.8)	39.8			
Lymphatic/venous invasion			65.905	2.685	< 0.001
No	954 (91.6)	50.0			
Yes	88 (8.4)	20.4			
Depth of invasion (7th edition)			172.456	1.852	< 0.001
T1	81 (7.8)	90.7			
T2	120 (11.5)	74.3			
T3	195 (18.7)	57.5			
T4a	538 (51.6)	35.9			
T4b	108 (10.4)	22.1			
The 7th edition UICC N			168.281	1.604	< 0.001
N0	332 (31.9)	71.1			
N1	211 (20.2)	50.7			
N2	268 (25.7)	37.5			
N3	231 (22.2)	22.2			
The 6th edition UICC N			160.982	1.571	< 0.001
N0	332 (31.9)	71.1			
N1	479 (46.0)	43.3			
N2	172 (16.5)	21.4			
N3	59 (5.7)	25.1			
Metastatic lymph node ratio			281.341	1.776	< 0.001
MLNR0	332 (31.9)	71.1			
MLNR1	277 (26.6)	59.0			
MLNR2	154 (14.8)	32.7			
MLNR3	279 (26.8)	16.0			
Retrieved lymph nodes			67.098	0.616	< 0.001
≤ 15	657 (63.1)	58.0			
> 15	385 (36.9)	68.4			

Table 2 Multivariate survival analysis results

Variables	Wald	*P* value	HR	95% CI
Age (continuous)	23.741	< 0.001	1.020	1.012-1.028
Tumor location	8.825	0.003	0.794	0.682-0.925
Tumor size (continuous)	29.678	< 0.001	1.085	1.054-1.118
Histological grade	11.542	0.001	1.222	1.089-1.372
Lymphatic/venous invasion	30.629	< 0.001	2.063	1.596-2.666
UICC 7th T	43.652	< 0.001	1.434	1.289-1.596
UICC 7th N	5.806	0.016	1.218	1.037-1.430
MLNR	14.693	< 0.001	1.330	1.149-1.538
Retrieved lymph nodes	29.666	< 0.001	0.548	0.441-0.680

CI: Confidence interval; UICC: International Union Against Cancer; HR: Hazard ratio; T: Tumor; N: Node; MLNR: Metastatic lymph node ratio.

We also investigated the impact of the number of lymph nodes retrieved on OS rates according to different N staging systems. In the 6th edition UICC N staging, the 5-year survival rate was significantly higher for patients with N0 compared with N1 and N2 in the ≤ 15 lymph nodes retrieved group, in which no patient was classified as N3 ($P < 0.001$ and $P < 0.001$, respectively). The Kaplan-Meier plot discriminated well between each N staging in the > 15 lymph nodes retrieved group ($P = 0.008$ and $P < 0.001$, respectively), except for N2 *vs* N3 ($P = 0.720$). As for the 7th edition UICC N staging, the Kaplan-Meier plot discriminated well between each N staging in both the ≤ 15 and > 15 lymph nodes retrieved groups, except for N0 *vs* N1 and N1 *vs* N2 ($P = 0.070$ and $P = 0.433$, respectively) in the > 15 lymph nodes retrieved group. When we investigated the MLNR in the ≤ 15 and > 15 lymph nodes retrieved groups, the Kaplan-Meier plot showed that the 5-year survival rate was significantly different for each MLNR stage. In the ≤ 15 lymph nodes retrieved group, the 5-year OS was 66.2%, 49.9%, 29.9%, and 11.2% for MLNR 0, 1, 2, and 3 ($P = 0.001$, $P < 0.001$ and $P < 0.001$, respectively). In the > 15

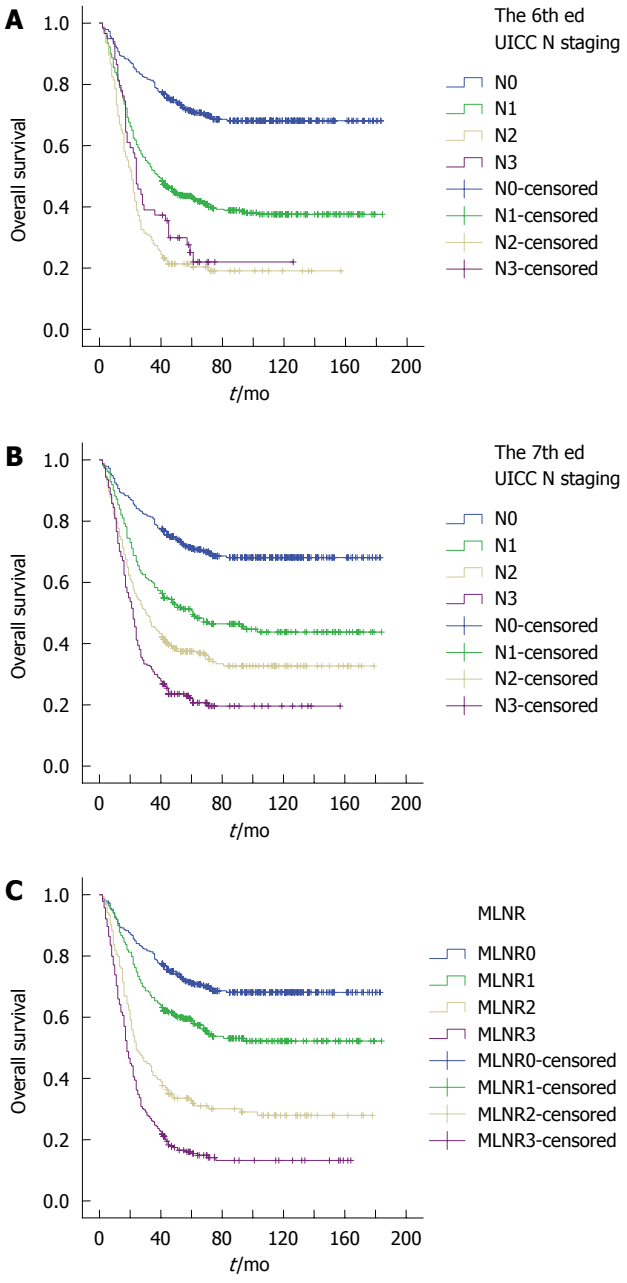

Figure 1 Impact of 6th and 7th International Union Against Cancer N staging systems (A and B) and metastatic lymph node ratio staging (C) on overall survival of gastric cancer patients who underwent radical resection. A: The 6th International Union Against Cancer (UICC) N staging: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P < 0.001$; N2 *vs* N3, $P = 0.143$; B: The 7th UICC N staging: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P = 0.003$; N2 *vs* N3, $P = 0.001$; C: Metastatic lymph node ratio (MLNR) staging: MLNR0 *vs* MLNR1, $P < 0.001$; MLNR1 *vs* MLNR2, $P < 0.001$; MLNR2 *vs* MLNR3, $P < 0.001$.

lymph nodes retrieved group, the 5-year OS was 82.5%, 68.3%, 38.7%, and 25.8% for MLNR 0, 1, 2, and 3 ($P = 0.001$, $P < 0.001$ and $P = 0.038$, respectively) (Figure 2).

The performance of the 6th and 7th edition UICC N staging systems and the MLNR staging, as assessed by the linear trend χ^2, likelihood ratio χ^2, and the AIC test, is described in Table 3. Compared with the 6th and 7th edition UICC N staging systems, the MLNR staging had better homogeneity (higher likelihood ratio χ^2 score), discriminatory ability, and monotonicity of gradients (higher linear trend χ^2 score). Furthermore, the MLNR staging had a smaller AIC value, representing the optimum prognostic stratification.

Table 3 Comparison of the performance of the 6th and 7th edition International Union Against Cancer node staging systems and the metastatic lymph node ratio staging system

Classification	Subgroups	Linear trend χ^2	Likelihood ratio χ^2	AIC
6th ed UICC N staging	N 0, 1, 2, 3	117.751	141.517	7364.073
7th ed UICC N staging	N 0, 1, 2, 3	138.342	146.796	7325.731
MLNR staging	MLNR 0, 1, 2, 3	203.476	219.912	7240.017

UICC: International Union Against Cancer; N: Node; MLNR: Metastatic lymph node ratio; AIC: Akaike information criterion.

Finally, we used the ROC curves of the three aforementioned N staging systems to calculate the AUC and thus to assess the accuracy of each system's predictive ability for gastric cancer patients who underwent radical resection (Figure 3). The AUC was 0.692 for the 6th edition UICC N staging, 0.705 for the 7th edition UICC N staging, and 0.754 for the MLNR staging, indicating that the MLNR staging was superior to the 6th and 7th edition UICC N staging systems and could be used as a more precise prognostic staging tool for gastric cancer patients.

DISCUSSION

Because the 6th edition UICC TNM staging system is simple, reliable, and reproducible, it is currently used all over the world. For cases in which < 15 lymph nodes are examined, N stage may be incorrect because of stage migration. A method for bypassing this problem is to consider the ratio between metastatic and examined lymph nodes. Studies have demonstrated that staging by the MLNR is superior to staging by the absolute number of metastatic lymph nodes (such as in the 6th edition UICC N staging) for predicting prognosis of gastric cancer patients[14,26,27]. Furthermore, some reports have demonstrated that the 7th edition UICC N staging is more suitable for prognosis than the 6th edition system[18,28]. For example, although all the patients in our study underwent D2 gastrectomy with R0 resection, the number of lymph nodes recovered in the majority of patients (63.1%) was no more than 15, and therefore, in these patients, the N stage cannot be classified as N3 according to the 6th edition UICC staging system. On the other hand, in the 7th edition system, patients may be classified as N3 as long as the number of retrieved lymph nodes is more than 7, and thus, this revised edition system may reduce stage migration. Whether the 7th edition UICC N staging is optimal is still unknown. To our knowledge, although a few documents claim that the 7th edition UICC N staging is superior to the 6th edition system, there are no formal studies

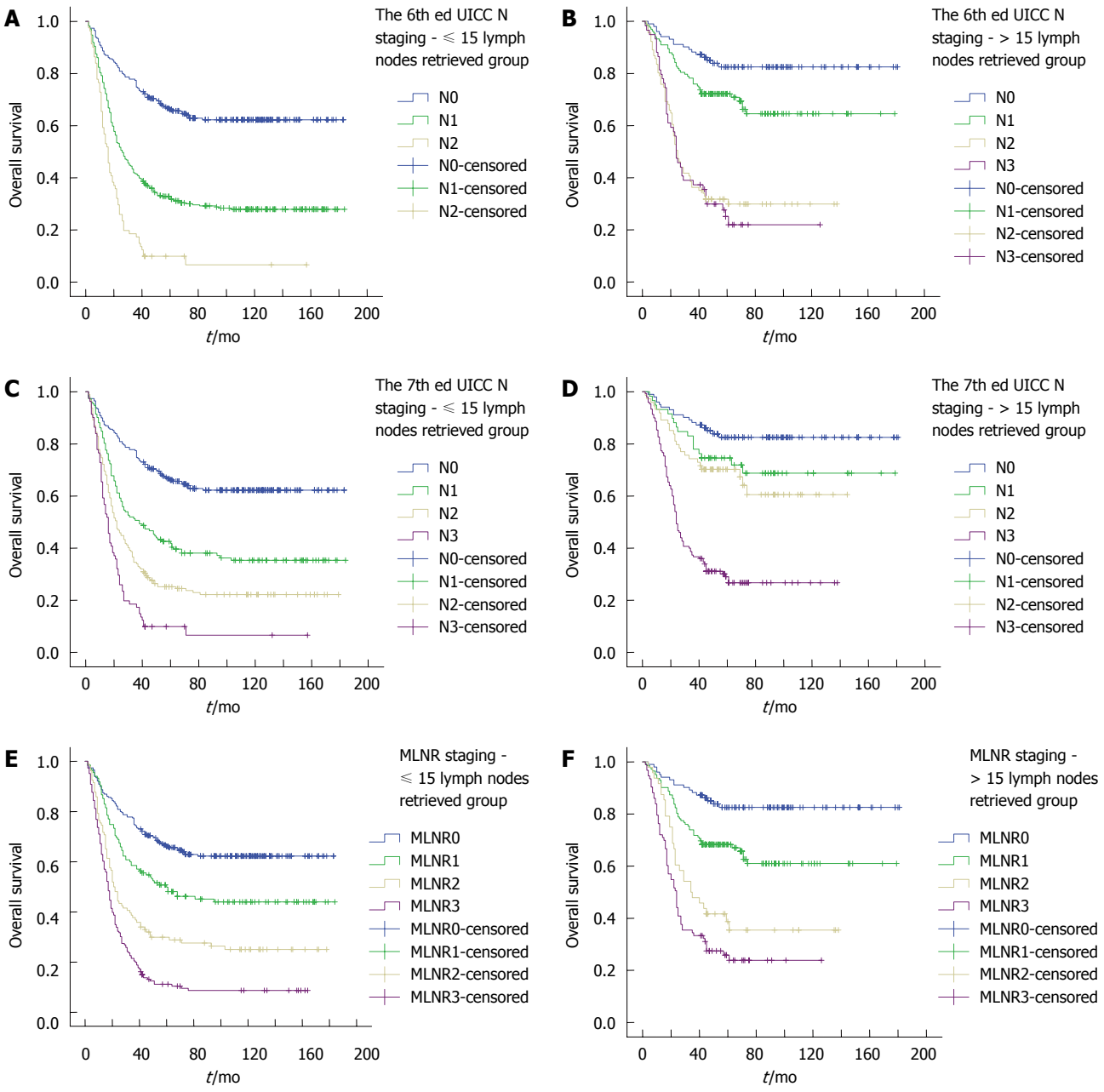

Figure 2 Analysis of the 6th (A and B) and 7th (C and D) International Union Against Cancer N staging systems and metastatic lymph node ratio staging (E and F) stratified according to the number of lymph nodes retrieved. A: The 6th International Union Against Cancer (UICC) N staging in the ≤ 15 lymph nodes retrieved group: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P < 0.001$; B: The 6th UICC N staging in the > 15 lymph nodes retrieved group: N0 *vs* N1, $P = 0.008$; N1 *vs* N2, $P < 0.001$; N2 *vs* N3, $P = 0.720$; C: The 7th UICC N staging in the ≤ 15 lymph nodes retrieved group: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P = 0.001$; N2 *vs* N3, $P < 0.001$; D: The 7th UICC N staging in the > 15 lymph nodes retrieved group: N0 *vs* N1, $P = 0.070$; N1 *vs* N2, $P = 0.433$; N2 *vs* N3, $P < 0.001$; E: Metastatic lymph node ratio (MLNR) stage in the ≤ 15 lymph nodes retrieved group: MLNR0 *vs* MLNR1, $P = 0.001$; MLNR1 *vs* MLNR2, $P < 0.001$; MLNR2 *vs* MLNR3, $P < 0.001$; F: MLNR stage in the > 15 lymph nodes retrieved group: MLNR0 *vs* MLNR1, $P = 0.001$; MLNR1 *vs* MLNR2, $P < 0.001$; MLNR2 *vs* MLNR3, $P = 0.038$.

that have examined the superiority of the MLNR to the 7th edition UICC N staging to date.

In this study, the MLNR was one of the most important prognostic factors of gastric cancer mortality. The MLNR provided a better classification of patient prognostic risk profiles than the 6th and 7th edition UICC N classification systems, particularly in the analysis stratified by the number of lymph nodes retrieved. The MLNR shows a clear advantage over the 6th and 7th edition UICC N staging systems for the following reasons: first, in univariate analysis, the log-rank χ^2 associated with the MLNR ($\chi^2 = 281.341$) was larger than that of the 6th and 7th edition UICC N staging systems ($\chi^2 = 160.982$ and $\chi^2 = 168.281$, respectively), indicating a higher statistical significance (Table 1); second, in multivariate analysis, the HR was higher in the MLNR (HR = 1.330, 95% CI: 1.149-1.538) staging than in the 7th edition UICC N staging (HR = 1.218, 95% CI: 1.037-1.430) (Table 2); third, although the 6th and 7th edition UICC N classifications discriminated well between each group,

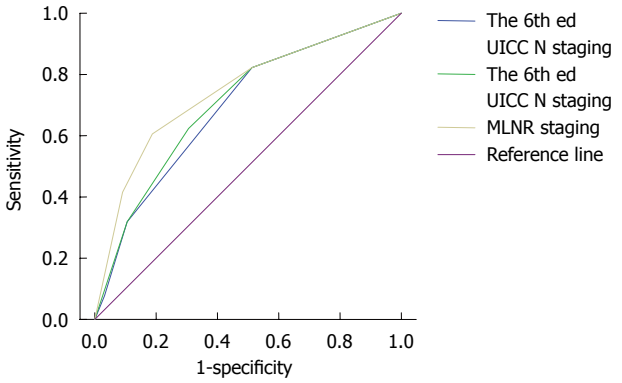

Figure 3 Receiver operating characteristics curve of the 6th and 7th edition International Union Against Cancer N staging systems and metastatic lymph node ratio staging for predicting survival of gastric cancer patients with radical resection. Area under the curve (AUC) of 1.0 represents a "perfect" diagnostic test that lacks false negative and false positive results. The AUC for the 6th and 7th edition International Union Against Cancer (UICC) N staging systems and metastatic lymph node ratio (MLNR) staging was 0.692, 0.705 and 0.754, respectively.

the MLNR provided a better classification of patient prognostic risk profiles than the pN stage, particularly in the analysis stratified by the number of lymph nodes retrieved (Figure 2); fourth, the MLNR staging had better homogeneity (higher likelihood ratio χ^2 score, 219.912 *vs* 146.796 *vs* 141.517), discriminatory ability, and monotonicity of the gradients (higher linear trend χ^2 score, 203.476 *vs* 138.342 *vs* 117.751) and a smaller AIC value (7240.017 *vs* 7325.731 *vs* 7364.073) (Table 3); and finally, the AUC under the ROC curve was larger in the MLNR staging (0.754) than in the 6th and 7th edition UICC N staging systems (0.692 and 0.705, respectively), indicating that MLNR staging was superior to the 6th and 7th edition UICC N staging methods and could be used as a more precise prognostic staging tool for gastric cancer patients.

According to Ueno *et al*[29], the performance of a staging system can be evaluated as follows: homogeneity within subgroups (small differences in survival among patients with the same stage), discriminatory ability between different groups (large differences in survival among patients in different stages), and monotonicity of the gradients shown in the correlation between stages and survival rates (within the same system, patients in earlier stages have longer survival than those in later stages). In our study, the MLNR staging had better homogeneity (higher likelihood ratio χ^2 score), discriminatory ability, and monotonicity of the gradients (higher linear trend χ^2 score) than did the 6th and 7th edition UICC N staging systems. Furthermore, in our study, the MLNR staging had a smaller AIC value, indicating that the MLNR staging has the optimum prognostic stratification and smallest loss of information for predicting outcome[30,31]. Additionally, the AUC under the ROC curve was larger in the MLNR staging than the aforementioned two N staging systems. These results demonstrate that the MLNR staging has better prognostic stratification and more precise prediction than do the 6th and 7th edition TNM staging systems.

Although the body of literature regarding the MLNR is growing, many studies have been performed using diverse patient groups and different surgical techniques. The cut-points for the MLNR have not necessarily been discussed adequately or validated in alternative data sets. We believe that systematic MLNR analyses of multi-institutional, randomized patient data with validation in similar independent data sets are required to clearly demonstrate the importance of the MLNR. Although the current UICC TNM staging system is the most basic and prevalent for predicting the survival of gastric cancer patients with radical resection, we believe that it will be essential to consider a staging system that includes accurate prognostic variables such as the MLNR in the near future. For all these reasons, the potential advantages of incorporating the MLNR in staging systems should be investigated in large, prospective data sets.

In conclusion, our study compared three lymph node based N staging systems for gastric cancer patients who underwent radical resection and D2 lymphadenectomy and then demonstrated that the MLNR categories could define gastric cancer prognosis more adequately and precisely than the 6th and 7th edition UICC N categories. We propose that nodal ratios should be considered as an alternative to the current UICC N staging.

COMMENTS

Background

In recent years, more new cases of gastric cancer are diagnosed in China each year than in any other country. The most powerful and reliable factors which have been widely used are the tumor-node-metastasis classification proposed by the International Union Against Cancer (UICC).

Research frontiers

Although UICC N staging is a convenient and reproducible method for precise staging, it demands the examination of at least 15 lymph nodes. If the number of dissected and examined lymph nodes is small or large, downmigration or upmigration of N stage may occur. The metastatic lymph node ratio (MLNR), defined as the number of positive lymph nodes divided by the number of lymph nodes retrieved, has been considered as an alternative to the absolute number of positive lymph nodes.

Innovations and breakthroughs

In the year 2010, staging of lymph node metastasis in gastric cancer has changed in both the UICC 7th edition staging system and in the Japanese Gastric Cancer Association 14th edition system to depend solely on the number of metastatic nodes found. Some authors have demonstrated that the 7th edition UICC staging system was superior to the 6th edition in aspects of homogeneity and discriminatory ability with prognostic value. However, there has been no formal proposal to date focused on comparing the prognostic significance between the MLNR and the 7th edition UICC N staging systems. In the present article, we investigated whether patients with gastric cancer can be classified into meaningful risk categories based on LNR, by comparing this staging with the 7th edition UICC N staging.

Applications

This study compared three lymph node based N staging systems for gastric cancer patients with radical resection and D2 lymphadenectomy, and then demonstrated that the MLNR categories could define gastric cancer prognosis more adequately and precisely than the 6th and 7th edition UICC N categories. The authors suggest that nodal ratios should be considered as a countermeasure to the current UICC N staging.

Peer review

This is a large study of 1042 gastric cancer patients undergoing radical resection plus D2 lymphadenectomy, with a mean follow-up of 56 mo. The authors have analyzed patient outcomes in considerable depth, their data is well characterized. They provide an in depth analysis of factors contributing to survival and have utilized multivariate analysis in doing this. The information in the manuscript is highly relevant and useful.

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Portal vein cannulation: An uncommon complication of endoscopic retrograde cholangiopancreatography

Evangelos Kalaitzakis, Nicholas Stern, Richard Sturgess

Evangelos Kalaitzakis, Nicholas Stern, Richard Sturgess, Digestive Disease Unit, Department of Gastroenterology, Aintree University Hospitals, Liverpool, L9 7AL, United Kingdom
Author contributions: Kalaitzakis E drafted the paper; all authors contributed to conception and analysis of clinical data, critical revision of the draft, and approval of the final version of the paper; and Sturgess R contributed to supervision.
Correspondence to: Evangelos Kalaitzakis, MD, PhD, Digestive Disease Unit, Department of Gastroenterology, Aintree University Hospitals, Liverpool, L9 7AL, United Kingdom. evangelos.kalaitzakis@medicine.gu.se
Telephone: +44-151-5298157 Fax: +44-151-5292490
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Abstract

Portal vein cannulation is a rare complication of endoscopic retrograde cholangiopancreatography (ERCP). It has been reported that it usually occurs after endoscopic sphincterotomy, whereas in cases without prior sphincterotomy, the presence of portobiliary fistulas has been shown. Here, we present a case in which cannulation of the portal vein occurred despite careful wire-guided cannulation and the absence of sphincterotomy. Although fatal cases of cerebral and pulmonary air and/or bile embolism have been reported in patients with combined portal and hepatic vein trauma after ERCP and sphincterotomy, isolated portal vein cannulation, as in the current case, does not usually result in mortality or serious morbidity. However, awareness of this rare complication is important so that no further intervention is performed.

Key words: Endoscopic retrograde cholangiopancreatography; Complications; Portal vein cannulation

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

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TO THE EDITOR

A 68-year-old man presented acutely with a 2-wk history of right upper quadrant (RUQ) pain, fever, and dark urine. Physical examination revealed mild RUQ tenderness. Investigations demonstrated deranged liver function tests with raised alkaline phosphatase (481 U/L, reference < 130 U/L), alanine aminotransferase (371 U/L, reference < 55 U/L) and bilirubin (55 μg/L, reference < 22 μg/L). C-reactive protein was elevated (195 mg/L, reference < 10 mg/L) but full blood count and renal profile were normal. Transabdominal ultrasound scan showed dilated intrahepatic bile ducts and multiple calculi in the gallbladder. Endoscopic retrograde cholangiopancreatography (ERCP) was undertaken. The papilla was edematous (prior to instrumentation), which made cannulation difficult. When wire-guided cannulation was achieved, injection of 10 mL contrast failed to opacify the biliary tree (Figure 1). The procedure was aborted and abdominal computed tomography showed air in the portal venous system (Figure 2). The patient remained asymptomatic and was hemodynamically stable. He was observed as an inpatient, and 4 d later had a repeat ERCP. The common bile duct was cannulated and a cholangiogram showed multiple filling defects consistent with stones, but no opacification of the portal venous system occurred. All stones were removed after endoscopic sphincterotomy and the patient was referred for elective laparoscopic cholecystectomy.

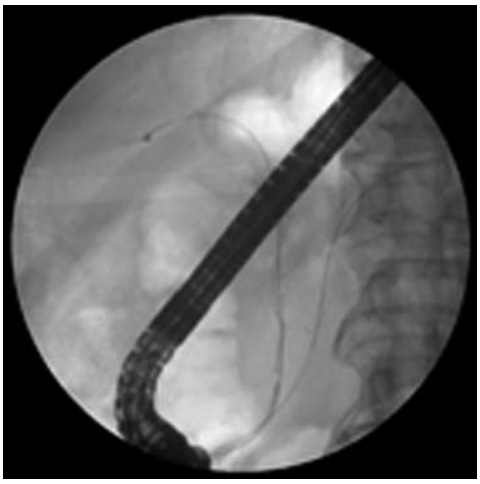

Figure 1 Sphincterotome in the portal vein after guide-wire cannulation. A two-wire technique was used as, initially, the pancreatic duct was cannulated.

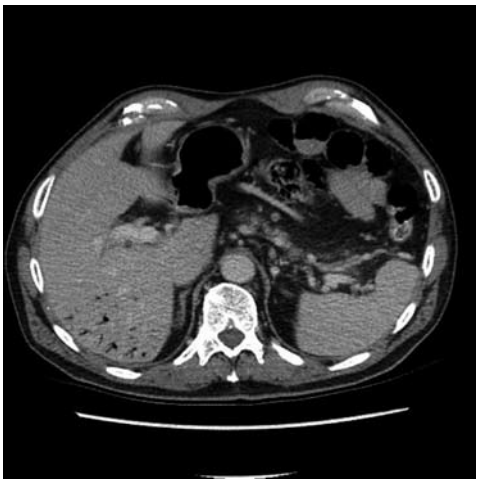

Figure 2 Abdominal computed tomography scan obtained following index endoscopic retrograde cholangiopancreatography showing air in the portal venous system.

Opacification or deep cannulation of the portal vein is a rare complication of ERCP, which occurs in 1 of 6000-8000 procedures[1]. It has been reported to occur mainly, but not solely, in patients with pancreatic cancer[1-5]; usually after pre-cut and/or conventional sphincterotomy[1,3]. Portal vein cannulation may occur as a result of direct trauma to the papilla or mucosal and vascular laceration[1]. In cases in which sphincterotomy was not performed, investigators have noted the presence of portobiliary fistulas due to presumed tumor infiltration[2] or erosion by abscesses[4]. In the current case, cannulation of the portal vein occurred despite careful wire-guided cannulation and absence of sphincterotomy. Although fatal cases of cerebral and pulmonary air and/or bile embolism have been reported in patients with combined portal and hepatic vein trauma after ERCP and sphincterotomy[6], isolated portal vein cannulation, as in the current case, has not been reported to result in mortality or serious morbidity[5]. However, in the event of large defects in the portal vein with serious bleeding, balloon tamponade or stenting with a fully covered stent can be performed, prior to considering surgical repair. Finally, awareness of this rare complication is important, so that it is readily recognized by endoscopists, and no further intervention (e.g., sphincterotomy or stent insertion into the portal vein) is performed.

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Raquel Almeida, Dr., MS, Institute of Molecular Pathology and Immunology, University of Porto, Rua Dr Roberto Frias s/n, Porto 4200, Portugal

Akira Andoh, MD, Department of Internal Medicine, Shiga University of Medical Science, Seta Tukinowa, Otsu 520-2192, Japan

Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Rosemary Joyce Burnett, MPH, Department of Epidemiology National School of Public Health, University of Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204, South Africa

Clifford S Cho, MD, Assistant Professor, Department of Surgery, Section of Surgical Oncology, University of Wisconsin School of Medicine and Public Health, H4/724 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792-7375, United States

Hugh J Freeman, Professor, Department of Medicine, University of British Columbia, UBC Hospital2211 Wesbrook Mall, Vancouver, BC V6T 1W5, Canada

Morten Frisch, Dr., MD, PhD, DSc, Department of Epidemiology Research, Statens Serum Institut, Building 206, Room 317, 5 Artillerivej, DK-2300 Copenhagen S, Denmark

Diego Garcia-Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

Evangelos Kalaitzakis, MD, PhD, Associate professor, Institute of Internal Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg 41345, Sweden

Leonidas G Koniaris, Professor, Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

Ezio Laconi, MD, PhD, Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4-IV Piano, 09125 Cagliari, Italy

Emanuel K Manesis, MD, Professor of Medicine, Athens University School of Medicine, Liver Unit, Euroclinic, 19 Mavromateon Street, Athens 1034, Greece

Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

Mark S Pearce, Dr., Paediatric and Lifecourse Epidemiology Research Group School of Clinical Medical Sciences, University of Newcastle, Sir James Spence Institute, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, United Kingdom

Guida Portela-Gomes, MD, PhD, Professor, Faculty of Medicine, University of Lisbon, Rua Domingos Sequeira-128, Estoril 2765-525, Portugal

Lima Ribeiro Marcelo, PhD, Clinical Pharmacology and Gastroenterolgy Unit, Laboratory of Microbiology and Molecular Biology, Sao Francisco University Medical School, Av Sao Francisco de Assis, 218, Braganca Paulista-SP 12916-900, Brazil

Casadesus Rodriguez Damian, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City 999075, Cuba

Ekihiro Seki, MD, PhD, Department of Medicine, University of California San Diego, Leichag Biomedical Research Building Rm 349H, 9500 Gilman Drive MC No. 0702, La Jolla, CA 92093-0702, United States

Arne Søreide Jon, Professor, MD, PhD, FACS, Department of Surgery, Stavanger University Hospital, N-4068 Stavanger, Norway

Thomas Wild, Dr., MD, Department of Surgery, Paracelsus Medical University, Feldgasse 88, Kapellerfeld 2201, Austria

Mitsunori Yamakawa, Professor, Department of Pathological Diagnostics, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

Yuichi Yoshida, MD, PhD, Assistant Professor, Department of Gastroenterology and Hepatology, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Katsutoshi Yoshizato, PhD, Academic Advisor, Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima 739-0046, Japan

Run Yu, MD, PhD, Division of Endocrinology, Diabetes and Metabolism, Cedars-Sinai Medical Center, 8700 Beverly Blvd, B-131, Los Angeles, CA 90048, United States

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January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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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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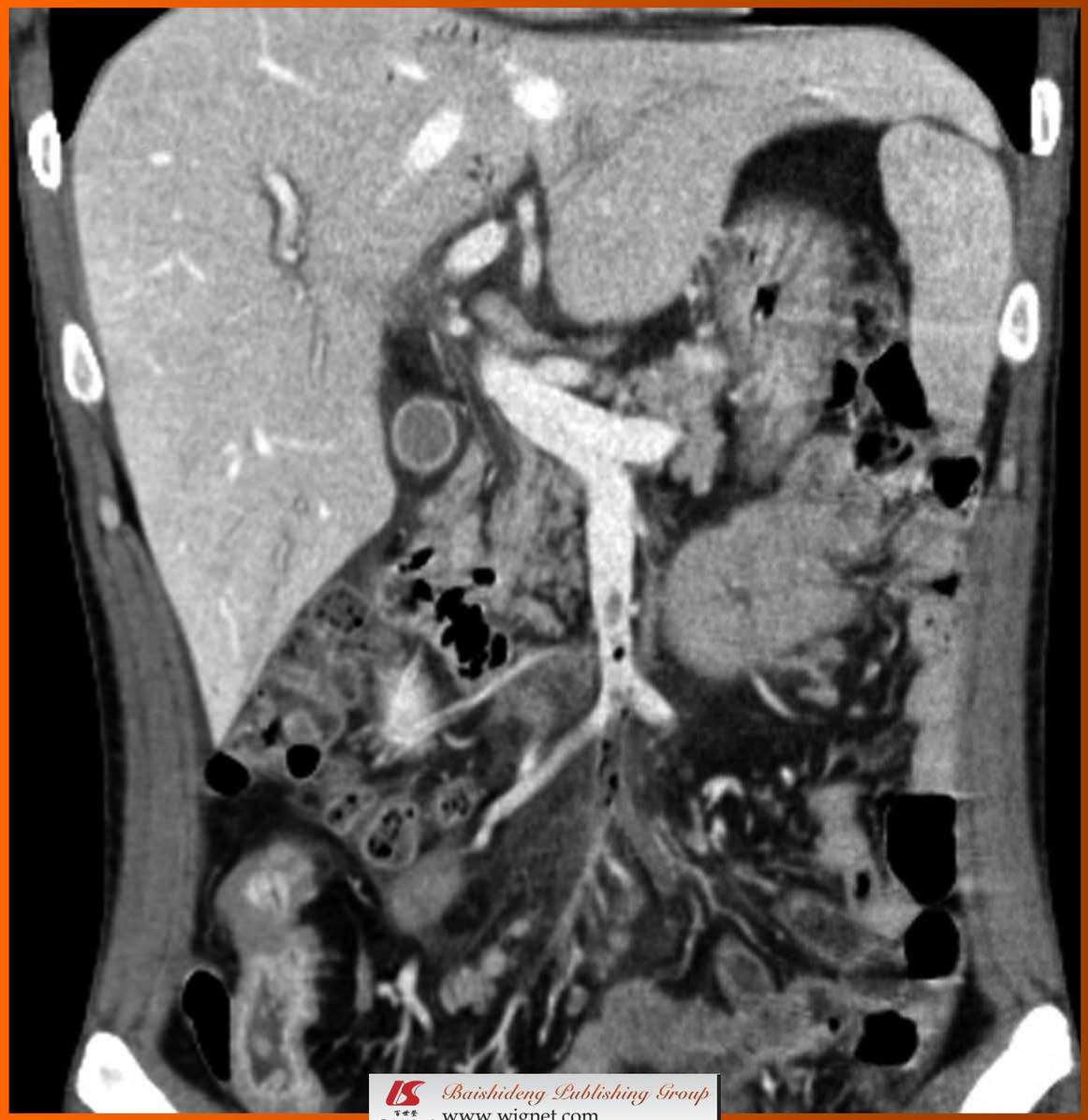

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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Baishideng Publishing Group Co., Limited
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No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
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Beijing Baishideng BioMed Scientific Co., Ltd.
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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EDITORIAL

In vivo magnetic resonance spectroscopy of liver tumors and metastases

EGW ter Voert, L Heijmen, HWM van Laarhoven, A Heerschap

EGW ter Voert, A Heerschap, Department of Radiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
L Heijmen, HWM van Laarhoven, Department of Medical Oncology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
Author contributions: ter Voert EGW wrote the manuscript; Heijmen L discussed and commented on the manuscript and the included articles; van Laarhoven HWM and Heerschap A supervised the project, discussed and commented on the manuscript and the included articles.
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Correspondence to: EGW ter Voert, MSc, Department of Radiology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen,
The Netherlands. e.tervoert@rad.umcn.nl
Telephone: +31-24-3668392 Fax: +31-24-3540866
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Abstract

Primary liver cancer is the fifth most common malignancy in men and the eighth in women worldwide. The liver is also the second most common site for metastatic spread of cancer. To assist in the diagnosis of these liver lesions non-invasive advanced imaging techniques are desirable. Magnetic resonance (MR) is commonly used to identify anatomical lesions, but it is a very versatile technique and also can provide specific information on tumor pathophysiology and metabolism, in particular with the application of MR spectroscopy (MRS). This may include data on the type, grade and stage of tumors, and thus assist in further management of the disease. The purpose of this review is to summarize and discuss the available literature on proton, phosphorus and carbon-13-MRS as performed on primary liver tumors and metastases, with human applications as the main perspective. Upcoming MRS approaches with potential applications to liver tumors are also included. Since knowledge of some technical background is indispensable to understand the results, a basic introduction of MRS and some technical issues of MRS as applied to tumors and metastases in the liver are described as well. *In vivo* MR spectroscopy of tumors in a metabolically active organ such as the liver has been demonstrated to provide important information on tumor metabolism, but it also is challenging as compared to applications on some other tissues, in particular in humans, mostly because of its abdominal location where movement may be a disturbing factor.

Key words: Metastasis; Metabolism; Proton magnetic resonance spectroscopy; Phosphorus magnetic resonance spectroscopy; Carbon-13 magnetic resonance spectroscopy; Magnetic resonance spectroscopy; Magnetic resonance

Peer reviewer: Xiao-Peng Zhang, Professor, Department of Radiology, Peking University School of Oncology, Beijing Cancer Hospital and Institute, No.52 Haidian District, Beijing 100142, China

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INTRODUCTION

Primary liver cancer is the fifth most common malignancy in men and the eighth in women worldwide. In 2000, it was estimated that there were about 564 000 new cases of liver cancer worldwide, and a similar number of patients died as a result of this disease[1]. Also, the liver is

the second most common site for metastatic spread of cancer[2]. In fact, liver lesions are more likely to represent a metastatic tumor than a primary liver tumor[3,4]. For further clinical management a proper diagnosis is crucial. Currently tissue sample analysis by histopathology is the golden standard for the diagnosis of suspected cancer in the liver. However, taking biopsies for histopathological analysis has some disadvantages. Besides patient discomfort, there is a change that the needle misses the cancer foci. Also, the needle might loosen cancerous cells which could result in tumor dissemination outside the liver along the needle track[5].

Thus, non-invasive advanced imaging techniques are desirable to assist in the diagnosis of liver lesions. A major modality to obtain anatomical information is magnetic resonance (MR). The MR technique is very versatile and it also offers many possibilities to acquire more functional information. Among these, MR spectroscopy (MRS) is particular interesting as it can provide specific information on tumor pathophysiology and metabolism. This may include data on the type, grade and stage of the tumor, which thus can assist in further management of the disease.

In this review, we will summarize and discuss the available literature on MRS of primary liver tumors and metastases. We will focus on the main nuclei employed in MRS, proton (^{1}H), phosphorus-31 (^{31}P) and carbon-13 (^{13}C). Since knowledge of some technical background is indispensable to appreciate the impact of the results described in this paper we first give an introduction into the basics of MRS and some technical issues of MRS as applied to tumors and metastases in the liver.

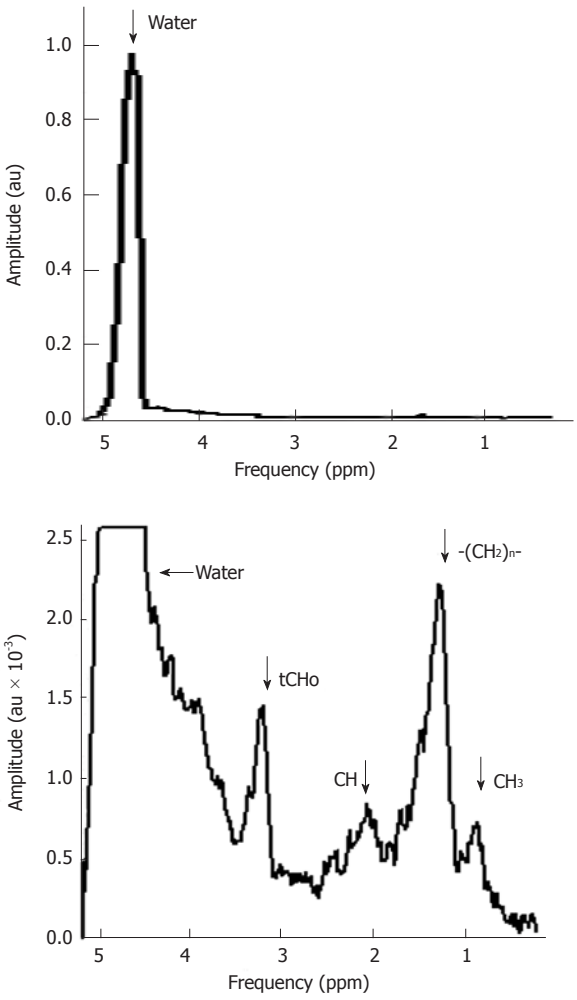

Figure 1 *In vivo* ^{1}H magnetic resonance spectra of human liver tissue obtained from a healthy volunteer on a 3.0T magnetic resonance system. Above: Spectrum with unsuppressed water signal; Below: Spectrum with partial suppressed water signal, showing the overlapping resonances for N-(CH$_3$)$_3$ protons at about 3.2 ppm occurring in choline compounds (tCho) and resonances for specific protons in lipids.

BASIC CONCEPTS OF MAGNETIC RESONANCE SPECTROSCOPY

In vivo MRS allows for the noninvasive measurement of the levels of some compounds in body tissues. It exploits the magnetic properties of certain atomic nuclei that are present in these molecules. The nuclei that are best accessible for *in vivo* MRS experiments are those of proton (^{1}H), phosphorus (^{31}P) and carbon-13 (^{13}C) atoms.

The following sections present a short introduction of MR spectroscopy to provide the reader with sufficient background to understand the biological and clinical applications of MR spectroscopy. For more in-depth information the reader is referred to other publications, see for example[6].

MR spectra

The key feature of MR spectroscopy is that certain biochemical compounds, mostly metabolites, can be identified in an MR spectrum by their specific spectral pattern, which is composed of one or more distinct signals. The intensity of the signal is proportional to the tissue amount of a certain nuclei and thus reflects the tissue levels of the compound in which it is present. An example of a typical *in vivo* ^{1}H MR spectrum of a healthy liver is shown in Figure 1. The horizontal axis of a spectrum represents the resonance frequency or chemical shift (both terms are explained below), the vertical axis represents the signal intensity. This spectrum is dominated by three main signals and in addition there are some smaller peaks, and broad underlying resonances. In the next few sections we explain in more detail how this spectrum is obtained and what particular information can be extracted from it.

Larmor frequency

Atomic nuclei with unpaired neutrons and/or protons, are detectable by nuclear magnetic resonance (NMR). As the nucleus is spinning around its axes and bears an electric charge it is associated with a magnetic dipole that can be seen as a tiny bar magnet (Figure 2). Outside a magnetic field these nuclear spins or tiny magnets have a random orientation; however, when placed in a strong constant external magnetic field B_0 they will become

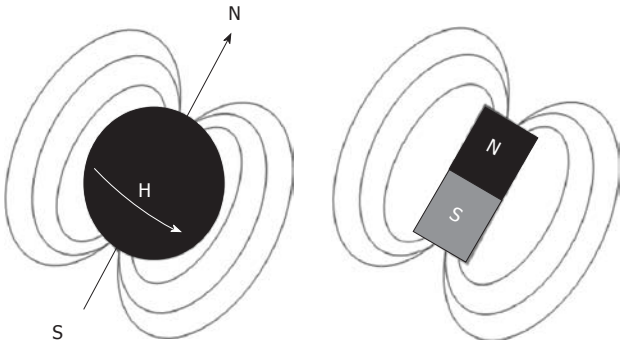

Figure 2 A ^{1}H nucleus spinning around its axes (left) can be regarded as a tiny bar magnet (right).

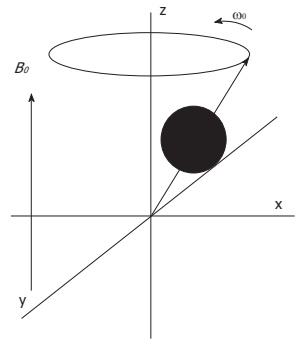

Figure 4 A spin precessing with the Larmor frequency around the axis of B_0 at a small angle with respect to this axis.

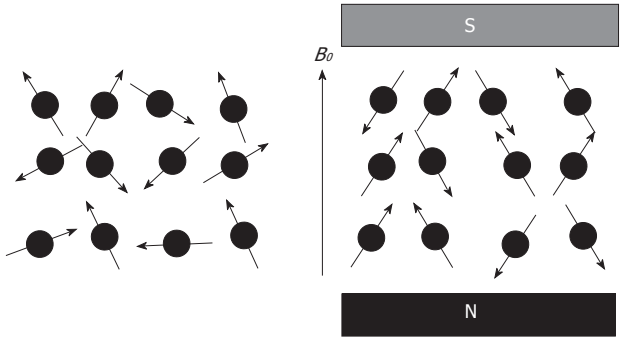

Figure 3 Outside a magnetic field spins have a random orientation (left) but spins inside a strong constant magnetic field B_0 will become aligned (right).

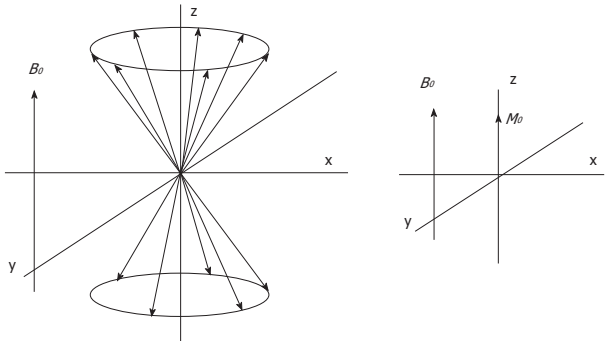

Figure 5 The direction of the main magnetic field B_0, is commonly placed along the z-axis and the magnetization along this axis is M_z, which at equilibrium, equals M_0. Left: Multiple individual spins precessing with the Larmor frequency around the axis of B_0. The spin vectors have a random, incoherent phase with respect to each other. Right: Magnetization vector M_z, which at equilibrium, equals M_0 (right).

aligned (Figure 3). The nuclear spins of the atoms ^{1}H, ^{31}P and ^{13}C can be oriented parallel or anti-parallel to B_0. However, the spins do not exactly align but are at an angle to B_0. This causes them to precess around the axis of B_0 with the so-called Larmor frequency $\nu_0 = \omega_0/2\pi = \gamma B_0/2\pi$ where the gyromagnetic ratio γ has a specific value for each nucleus (Figure 4). This implies that every type of nucleus has a different precession frequency, proportional to the B_0 field strength. At a field strength of 3T, which is commonly used for human applications, these frequencies for ^{1}H, ^{31}P and ^{13}C are: 127.7 MHz, 51.8 MHz and 32.1 MHz respectively, which is in the radiofrequency range.

Energy levels

The parallel and anti-parallel orientations are associated with a low and a high energy state respectively. The energy difference between the two spin states equals

$$E = h\,\gamma\,B_0 \qquad \text{[Equation 1]},$$

where h is Planck's constant ($h = 6.626 \times 10^{-34}$ J/s). At room temperature, there are slightly more spins in the lower energy level, N^{α}, than in the upper level, N^{β}. The distribution over these energy levels is given by Boltzmann statistics

$$N^{\beta}/N^{\alpha} = e^{-E/kT} \qquad \text{[Equation 2]},$$

where k is Boltzmann's constant (1.3805×10^{-23} J/K) and T is the temperature in Kelvin. The population difference results in a net macroscopic magnetization M_0. This so-called longitudinal magnetization is aligned parallel to B_0 (Figure 5). Only the net magnetization is detectable and its extent determines the achievable signal-to-noise ratio (SNR). At 1.5T and 37 ℃ (310 K), the population difference represents only a small fraction (about 10^{-6}) of the total spin population, which explains why MR is a relatively insensitive technique. It follows from Equations 1 and 2 that sensitivity can be improved with a higher magnetic field B_0 or a lower temperature.

Flip angle, free-induction decay, T_1 and T_2

The direction of the main magnetic field B_0, is commonly placed along the z-axis and the magnetization along this axis is M_z, which at equilibrium, equals M_0 (Figure 5). The longitudinal magnetization however is not detectable as it is "overruled" by the main magnetic field B_0. To detect the net macroscopic magnetization M_0, an radio frequency (RF) pulse with a magnetic field perpendicular to the main magnetic field B_0 and with a frequency equal to the precession frequency is sent with an RF transmitter coil. As a consequence of the applied RF pulse M_0 magnetization will rotate away from the z-axis toward the transverse plane (x-y plane). The angle to which the net magnetization is rotated relative to the main magnetic field direction is called the flip angle. A

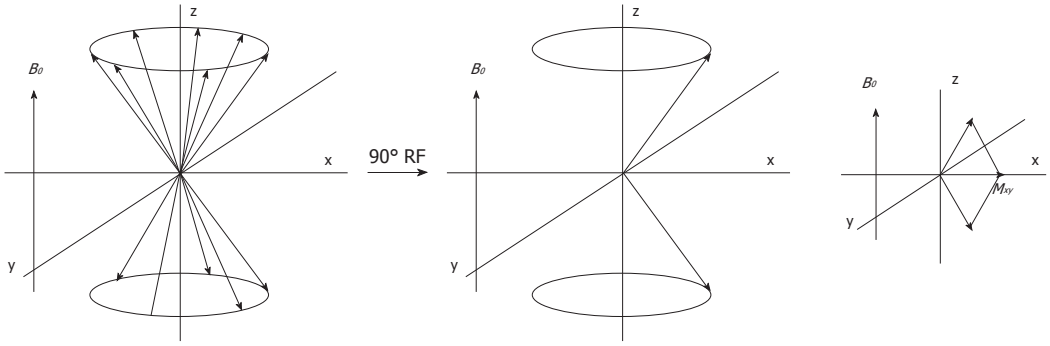

Figure 6 Multiple spins precessing with the Larmor frequency around the direction of B_0 (left). After a 90° radio frequency (RF) pulse, the spin population of the energy levels become equal and the spins precess coherently (middle). The magnetization vector M_0, rotates into the transverse (x-y) plane (right).

so called 90° RF excitation pulse will therefore rotate M_0 into the transverse plane. The spin population of the energy levels becomes equal and spins precess coherently (Figure 6). However, after the RF excitation pulse the spins start to return towards the original energy level distribution and towards incoherent precession (dephasing) and after a while the system will be in equilibrium again. The time constant which describes how the magnetization returns to the original longitudinal alignment is called the spin lattice relaxation time T_1: $M_z = M_0 (1\text{-}e^{-t/T1})$. The time constant which describes the return to incoherent precession is called the spin-spin relaxation time T_2: $M_{xy} = M_{xy0}\, e^{-t/T2}$. By definition T_1 is longer than T_2.

After the RF pulse, the transverse component of the M_0 magnetization precesses with the Larmor frequency at resonance and will induce a current in the RF coil which is now switched to receive mode. The decaying signal of this component is called the free-induction decay (FID) response signal. This digitally recorded FID signal is mathematically converted by a Fourier transform from the time to the frequency domain, which results in a so called MR spectrum, that may contain one or more resonances, signals or peaks at particular frequencies.

Shielding and chemical shift

An MR spectrum of the liver would not be very interesting if all nuclei of a certain type resonate at the same frequency. Fortunately, each nucleus in a given molecule is shielded from the main field by a weak opposing field from the surrounding electrons, induced by and also proportional to B_0. The amount of shielding by these electrons highly depends on the chemical environment of the nucleus. This shielding, which is generally unique for each chemically distinguishable site in a molecule, is expressed as the shielding constant σ, and the total effective field experienced by a given nucleus is: $B_{eff} = B_0 (1\text{-}\sigma)$. The resulting change in resonance frequency $\nu_{eff} = \omega_{eff}/2\pi = \gamma B_0 (1\text{-}\sigma)/2\pi$ relative to that of a chosen reference compound, ν_{ref}, is generally referred to as the chemical shift $\sigma = (\nu_{eff} - \nu_{ref})/\nu_{ref}$ expressed in units of ppm (1 ppm = 100 Hz at ν_0 = 100 MHz). Thus the chemical shift is the key property of MR spectroscopy which enables detection of a wide variety of chemical groups and metabolites containing these groups. Special RF pulses are used to excite a band of frequencies covering the chemical shift range of a particular nucleus in a biological sample.

^{1}H MR SPECTRUM OF THE LIVER EXPLAINED

As shown in Figure 1 the ^{1}H spectrum of the liver is dominated by 3 peaks. The peak on the right originates from the ^{1}H nuclei in methylene groups (-CH_2-) in lipids, the peak in the middle originates from the ^{1}H nuclei in the three methyl groups (CH_3) of choline containing compounds [$(CH_3)_3N^+CH_2CH_2O$-], and the peak on the left originates from the ^{1}H nuclei in water (H_2O). The electronegative oxygen atom in the water molecule shifts the electron density away from the ^{1}H nuclei, leading to a reduced shielding and thus to a higher resonance frequency compared to ^{1}H nuclei in the methylene groups of lipids. Thus ^{1}H nuclei in water have a higher chemical shift value then the ^{1}H nuclei in the methylene groups from lipids (compared to ^{1}H nuclei in a reference compound at 0 ppm) and appear on the left side. Note that the chemical shift scale on the horizontal axis increases from right to left.

The peak area rather than its amplitude is proportional to the amount of ^{1}H nuclei in the same chemical environment and thus the tissue content of that chemical group or metabolite. Quantification of metabolite concentrations is performed by comparing peak areas with those of known substances and concentrations.

The content of water in the liver is much higher than that of metabolites. Hence, the proton signals of the latter have a much lower SNR (Figure 1). For this reason signal averaging is usually required and it is also needed to measure larger voxels than commonly done with MR imaging of water.

WATER SIGNAL SUPPRESSION

As the water resonance in ^{1}H MR spectra is at least 200 times more intense than the resonances of hydrogen

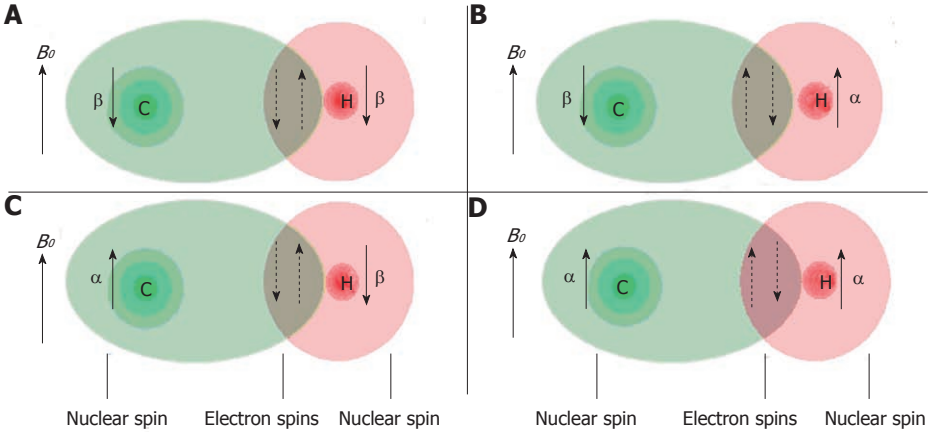

Figure 7 Two bonded nuclei, carbon (C) and hydrogen (H) in decreasing energy levels associated with spin states: ββ (A), βα (B), αβ (C) and αα (D). The electron clouds around each nucleus are indicated in light green and red. Arrows indicate individual spin states.

containing metabolites (Figure 1) it may hamper the proper detection of metabolite signals, e.g., by artifacts such as side bands of this huge signal[7], the suppression of the water signal is commonly performed. There are many ways to do this: a well-known sequence is chemical shift selective water suppression[8]. A frequency selective RF pulse excites the water spin magnetization into the transverse plane after which all coherences are dephased by magnetic field gradients. The spectrum shown in Figure 1 was obtained with only partial water signal suppression.

MAGNETIC FIELD HOMOGENEITY

As the purpose of MR spectroscopy is to separate signals with different chemical shift it is crucial for its proper application that good magnetic field homogeneity is obtained over the object of interest. However, due to the many different tissue types and air containing compartments, the magnetic field in the human body is usually not very homogeneous, leading to broadened spectral lines, which may overlap. Therefore optimizing field homogeneity (a process called shimming) is usually a required step in an MR spectroscopy experiment. Good homogeneity is most important in ^{1}H MRS as it has a relatively small chemical shift range and needs a well separated and defined water signal for proper suppression and to avoid artifacts. By selecting small volumes of interest, a limited amount of different tissues are included which will result in a more homogeneous magnetic field. The requirements for homogeneity are much less strict for ^{31}P and ^{13}C MRS because resonances in their spectra are more separated (larger spectral dispersion).

SPIN-SPIN COUPLING AND DECOUPLING

Nuclei which are close to one another exert an influence on each other's effective magnetic field through electrons in chemical bonds (Figure 7). If the distance between non-equivalent nuclei is less than or equal to three bond lengths, this effect may be observable in the *in vivo* MR spectrum. Instead of one peak for a certain nucleus several smaller peaks are observed in the spectrum. This interaction of nuclei is often referred to as scalar coupling, J coupling or spin-spin coupling. Because of this splitting the signal to noise decreases and spectral interpretation may become more complicated. However, the specific pattern may also be helpful to identify specific molecular groups and with special pulse sequences the phenomenon of spin-spin coupling may be used to identify the resonances of these groups in the presence of other overlapping resonances (so-called editing).

Spin-spin couplings are expressed in Hz. A typical value for a three bond proton-proton coupling in metabolites is 7 Hz. For example this can be used to identify the methyl doublet resonance of lactate in ^{1}H MR spectra. At magnetic fields above about 2T, line width broadening will obscure the direct visualization of this coupling. Two bond ^{31}P - ^{31}P couplings occur at about 17 Hz in adenosine triphosphate (ATP). Heteronuclear couplings commonly dealt with in *in vivo* MRS are between protons and ^{31}P or ^{13}C. To improve signal to noise and spectral resolution the spin-spin splitting can be removed by a technique called decoupling. Special RF pulses are used to irradiate selected resonance(s) of nuclear spins such that their field directionality is averaged out. As a result nearby spins in a molecule experience no field of these irradiated spins anymore and the resonance splitting disappears.

Decoupling is important in MRS of ^{31}P and ^{13}C as these atoms often show split resonances due to nearby hydrogen atoms. Irradiation of these protons for decoupling increases the signal to noise ratio and improves spectral resolution. Irradiation also induces a through space effect called nuclear overhauser enhancement (NOE), which can increase signal intensity even more[9-11]. Typical enhancement values reached *in vivo* by NOE are 1.3-2.9 and 1.4-1.8 for ^{13}C-^{1}H and ^{31}P-^{1}H interactions respectively[6].

DYNAMIC NUCLEAR POLARIZATION

^{13}C labeled (enriched) substrates combined with ^{13}C MRS have traditionally been used to monitor metabolic conversions during steady-state conditions and even has been used to assess active metabolism in human tumors[12-14]. The relative low sensitivity of ^{13}C MRS prevented imaging of these processes. However, with polarization transfer techniques sensitivity can be enhanced[14-16].

Recently a new method has been introduced in biomedical MR, dynamic nuclear polarization (DNP), that mostly makes use of ^{13}C MRS, to image metabolic conversions at reduced acquisition times (in an order of minutes or less). Inspection of Equation 2 (above) reveals that decreasing the temperature also will increase the population differences between energy levels. DNP is a hyperpolarization technique that increases the spin polarization obtained at room or body temperature in traditional MR, by orders of magnitude by cooling the sample to very low temperatures and using selective microwave irradiation for efficient transfer of spin polarization from electron spin to nuclear spin[6,17,18]. The substrate is then rapidly transferred to body temperature for administration. The major disadvantage of DNP is that the polarization decay is determined by the spin-lattice relaxation time T_1 of the nucleus (20-40 s for a ^{13}C nucleus in a carboxyl group). The consequence is that only conversions in which substrates are rapidly taken up by tissues and metabolized within minutes can be imaged successful. With efficient uptake usually one or two metabolic steps can be imaged, before the signal has decayed away.

LOCALISATION

To analyze different regions of interest in the liver with healthy tissue or tumor lesions by *in vivo* MRS it is required that only signals that originate from these locations appear in the spectra. This can be achieved in different ways. The most rudimentary one is to use only a surface coil on the body adjacent to the liver with a simple pulse sequence globally selecting the area next to the coil. However, in most cases a better localization is desired. More advanced spatial localization is possible with single voxel or multi-voxel methods.

Single voxel localization

The most common single voxel localization techniques are Image Selected *In Vivo* Spectroscopy (ISIS)[19], Stimulated Echo Acquisition Mode (STEAM)[20] and Point Resolved Spectroscopy (PRESS)[21].

ISIS uses three frequency-selective inversion pulses, in the presence of three orthogonal magnetic field gradients. By turning on and off the inversion pulses, according to an encoding scheme, eight different scans are recorded. Adding and subtracting the different scans will add signal from the desired location while canceling signal from other locations. A disadvantage of ISIS is its sensitivity to motion as eight scans need to be obtained for a full 3D localization. ISIS is rarely used for ^{1}H MRS, because of potential artifacts such as those arising from incomplete water signal suppression. However, it is the favored method in ^{31}P MRS localization as effects due to relatively rapid T_2 decay and to J-coupling are avoided.

For ^{1}H MRS the most common single voxel localization techniques are STEAM and PRESS, which both use three spatially slice selective RF pulses to produce an echo signal from a well-defined region. STEAM uses 90°-90°-90° pulses and PRESS 90°-180°-180° pulses to define three orthogonal slices. Only signal from the volume of interest remains in the final echo. In STEAM 50% of the original signal is lost as the second 90° pulse only rotates half of the transverse magnetization to the longitudinal axis, while the other half is dephased by crushers. The PRESS technique retains full signal intensity, but the minimal possible TE is commonly larger than for STEAM.

Multi voxel localization

Multi voxel localization allows the detection of localized spectra from a multidimensional array of locations. Disadvantages compared to single voxel localization concerns some more magnetic field inhomogeneities due to the many different tissue types in the field of view, intervoxel contamination, and the minimally required number of scans that may end up in long acquisition times. Spectroscopic imaging techniques acquire the signal from multiple voxels by using phase encoding gradients[22], analogous to the phase encoding technique used in MR imaging. The nominal voxel size is the field of view divided by the number of phase encoding gradient steps. The actual voxel size can deviate substantially from the nominal value as the signal is sampled only over a finite time. This introduces intervoxel contamination due to the characteristics of the Fourier transform. This voxel bleeding can be decreased by apodization functions like those with Gaussian or Hamming shapes, however at the expense of decreased spatial resolution.

Conventional encoding of $N_1 \times N_2 \times N_3$ volume elements (voxels) requires $N_1 \times N_2 \times N_3$ acquisitions. A typical $16 \times 16 \times 16$ dataset obtained with a repetition time of 2000 ms and 4 averages would require a measuring time of $(16 \times 16 \times 16 \times 2000 \times 4/3600 = 9$ h). Therefore techniques are developed that increase the temporal resolution, e.g., by special k-space trajectories. For example "circular 2D or spherical 3D k-space sampling with k-space apodization during acquisition". This also reduces the total acquisition time by spending less time acquiring the high k-space coordinates and more time acquiring the low k-space coordinates. Other methods are based on fast magnetic resonance imaging (MRI) sequences, e.g., EPI, RARE, spiral and steady-state sequences[6].

QUANTIFICATION

Although the tissue level of metabolites is proportional

to the area under its signal curve in the spectrum, it commonly requires some corrections and calibration with a signal of known concentration to obtain an absolute number (e.g., in mmol/L).

Such a reference signal maybe that of water or of another metabolite in the liver assuming a stable and known value for its tissue level. In the liver, the unsuppressed water signal is often used as an internal reference after correction for T_1 and T_2 relaxation. However, dietary regimes and liver pathologies may affect the amount of water. Li *et al*[23] reported a 1.8 fold difference in five normal liver studies between the largest and smallest water signal intensity obtained from localized liver tissues. In four hepatocellular carcinoma studies, they observed a 3.2-fold difference between the largest and smallest water signal intensities obtained from the localized liver tumors. Lipid peaks exhibited even larger variations than did the water peaks.

External phantoms with known concentrations sometimes are also used for calibration purposes, but this may be not so practical in a clinical environment. In addition differences in coil loading have to be taken into account in this approach. To avoid correction and calibration issues spectral quantities are sometimes also assessed as ratio's between integrals of signals of different compounds.

MOTION AND OTHER ARTEFACTS

Motion can lead to voxel misregistration and outervoxel contamination. Motion of tissue through inhomogeneous fields (e.g., air in the lungs) results in broadening of the spectral resonances. Broadening of the resonances increases the risk of signal overlap and also lowers the signal to noise ratio. Compared to other organs like brain and skeletal muscle, MR spectroscopy of the liver is challenging as there are potential field inhomogeneities and artifacts caused by respiratory movement, cardiac and aortic pulsations[24-26]. Although often applied, breath-hold acquisitions may be problematic. Long acquisition times are needed to increase the SNR and, since a breath-hold period can only last for about 15 s in patients, the acquisition will require multiple breath-hold periods. Even when the acquisition is performed at end-expiration, there is no guarantee that the tissue is at exactly the same position, leading to outervoxel contamination. Respiratory and cardiac gating can be applied to reduce motion artifacts at the cost of an increased scan time. Another option is to align individual spectra and/or exclude bad spectra before averaging during post processing. Some liver pathologies, e.g., due to long-term total parenteral nutrition may induce iron accumulation in the liver, which will result in field inhomogeneities and broadening of the spectral resonances.

REPRODUCIBILITY

In order to predict treatment outcome or monitor therapy, differences in *in vivo* MR spectroscopy outcome parameters should reflect true differences in tumor biology and not differences induced by variations in the MRS protocol or interfering body physiology. This issue is relevant, since the time of MR scanning during the day (e.g., before or after a meal) or differences in eating patterns might already influence the metabolic activity and thus the concentrations of metabolites in the liver. Large inter- and intrapatient variability of MRS outcome parameters have been described[23].

^{1}H MR SPECTROSCOPY

In MR, hydrogen (proton) is the most commonly studied nucleus. Compared to other MR sensitive nuclei it has the highest sensitivity and occurs at 100% isotopic abundance. Almost all metabolites in the human body contain protons. Therefore, in principle a large amount of metabolites can be investigated. However, in practice, sensitivity restrictions set the *in vivo* detection limit of metabolites to a minimum tissue concentration of about 0.1 mmol/L. An advantage of ^{1}H MRS is that it uses the same nucleus as MRI techniques. Therefore, it can be performed with the same hardware and there is no need for special equipment. The major drawback of ^{1}H MRS is the relatively small chemical shift range (about 10 ppm) for the many resonances of *in vivo* detectable compounds, resulting in limited spectral resolution. Moreover, these have to be resolved from a dominating water peak, in certain cases a large lipid peak, and at short echo times a high baseline due to macro-molecules.

Metabolites visible in ^{1}H MR spectra of liver tumors

Lipids: Outside the brain ^{1}H MR spectra of tissues usually show large signals of mobile lipids, mostly triglycerides: in particular a methylene peak at 1.2 ppm with smaller methylene peaks between 2.1 and 2.4 ppm and a peak for methyl protons at 0.9 ppm (Figure 1). Triglycerides occur in fatty liver, but also may be a marker of membrane breakdown and can be seen in tumors, abscesses and other pathological processes[27].

Lactate: Due to the Warburg effect tumor cells obtain relative less energy than normal cells from oxidative phosphorylation and have a more glycolytic character[28,29]. Pyruvate, the end product of glycolysis, is converted into lactate, which is further promoted by hypoxic conditions. Therefore MR spectra of tumor tissue often show signals for lactate. The three equivalent methyl protons of lactate give rise to a resonance at 1.31 ppm, which is a doublet due to coupling with the methylene proton, while the single methylene proton resonates as a quartet at 4.10 ppm due to coupling with the methyl protons. In liver and tumor tissue, the lactate signal at 1.3 ppm will overlap with large lipid resonances. However, with so-called spectral editing techniques it is possible to separate the lactate signal from the lipid signals.

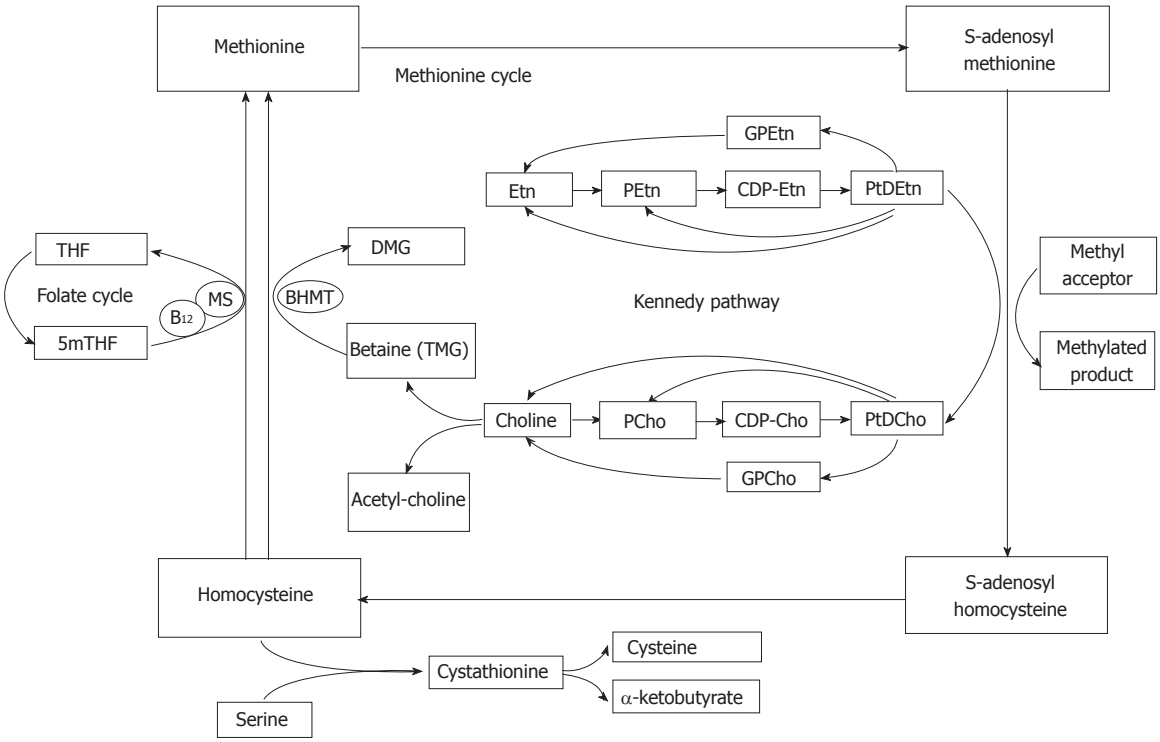

Figure 8 Simplified schematic overview of choline and ethanolamine metabolism including parts of the Kennedy pathway, the methionine and folate cycle. B12: Vitamin B12; BHMT: Betaine-homocysteine methyltransferase; CDP-Cho: Cytidine diphosphate-choline; CDP-Etn: Cytidine diphosphate-ethanolamine; DMG: Dimethylglycine; Etn: Ethanolamine; GPCho: Glycerol-3-phosphorylcholine; GPEtn: Glycerol-3-phosphorylethanolamine; MS: Methionine synthase; PCho: Phosphorylcholine; PEtn: Phosphorylethanolamine; PtdCho: Phosphatidylcholine; PtdEtn: Phosphatidylethanolamine; THF: Tetrahydrofolate; 5mTHF: 5-methyltetrahydrofolate; TMG: Trimethylglycine (betaine).

Creatine: Creatine (Cr) and phosphorylated creatine (PCr) play an important role in energy metabolism of many tissues. PCr serves as a spatio-temporal energy buffer, maintaining a constant level of ATP, facilitated by the creatine kinase reaction. The methyl protons of Cr and PCr resonate at about 3.03 ppm and the methylene protons resonate at approximately 3.9 ppm. Under normal conditions, the concentration of total creatine is relatively constant in muscle and brain and therefore often used as an internal reference. However, decreased Cr levels have been observed in tumors and other pathologies. Furthermore, hepatocytes do not express creatine kinase under normal circumstances[30], and therefore no creatine peak will be visible in the spectrum of healthy liver tissue. *In vitro* experiments at 9.4T have shown 5-10 times increased levels of Cr in liver metastasis compared to normal liver tissue[31].

Choline and ethanolamine containing compounds

The signals of choline containing compounds in MR spectra have been used as key biomarkers to identify malignant tumors[32-34]. *In vivo* ^{1}H MR spectra of the liver show the N-trimethyl [$^{+}N(CH_3)_3$] resonances of choline compounds at about 3.2 ppm. This resonance is also known as the total choline (tCho) peak as it may originate from several different choline compounds. The relative increase in the tCho signal seen in human tumors is due to an abnormal choline uptake and/or metabolism related to cell membrane turnover. However, metabolism of choline containing compounds in tissue cells is complex. Although far less prominent in ^{1}H MR spectra than choline, ethanolamine signals may also contribute to a characteristic spectral profile of tumor tissue. Therefore some important biochemical pathways involving choline metabolism and the closely related metabolism of ethanolamine in the liver are briefly described (Figure 8).

Choline and ethanolamine metabolism

Choline is a key precursor molecule in several metabolic pathways. It can be acetylated, oxidized, phosphorylated or hydrolyzed. Choline oxidation plays a major role in the provision of methyl groups *via* its metabolite, trimethylglycine (betaine) that participates in the synthesis of S-adenosylmethionine (SAM). Methylation reactions are involved in the biosynthesis of lipids, the regulation of several metabolic pathways, and detoxification in the body. Choline phosphorylation results in compounds such as phosphatidylcholine (PtdCho), lysophosphatidylcholine, choline plasmalogen, and sphingomyelin which are essential for structural integrity and signaling in cell membranes[35-38].

PtdCho, the major phospholipid component of cells is derived from the Kennedy pathway[39], which has two branches, one *via* cytidine 5'diphospho (CDP)-choline and the other *via* CDP-ethanolamine (Figure 8). In the CDP-choline branch choline is initially converted

to phosphorylcholine (PCho) and after some steps to PtdCho which can be converted into choline or into PCho again[36]. Alternatively, phosphatidylethanolamine (PtdEtn) is generated *via* the CDP-ethanolamine branch, employing similar biochemical reaction steps. The resulting PtdEtn can be methylated, using SAM as the methyl donor, to PtdCho[36-38,40]. The methylation pathway is, however, only relevant in liver. In rat hepatocytes it accounts for 20%-40% of PtdCho synthesis[41]. Besides entering the CDP-choline branch of the Kennedy pathway, choline can also enter another major pathway in the liver in which it is oxidized into betaine[42-44].

Contributions to the tCho peak

The contribution of the nine methyl protons of free choline, which resonate at 3.19 ppm, to the tCho peak is limited as the concentration of free choline is usually low. Another potential contribution to the tCho peak may come from PtdCho, which makes up a very high proportion of the cell plasma membrane. However, it is a large molecule with a relatively short T_2 relaxation time that becomes even shorter by being incorporated into a membrane. Therefore, it is almost invisible in the *in vivo* ^{1}H MR spectrum. Nevertheless, some evidence suggests that PtdCho may contribute to the tCho signal[45]. Precursors of PtdCho such as PCho and phosphorylethanolamine (PEtn) are more likely to contribute to ^{1}H MR spectra as these are small molecules with long T_2 relaxation times. Experimental evidence suggests that the tissue levels of PCho and PEtn increase during cell proliferation and tCho levels also have been correlated with tumor aggressiveness[46]. In addition to PCho and PEtn [together called phosphomonoesters (PME)] their glycerol derivatives glycerol-3-phosphorylcholine (GPCho) and glycerol-3-phosphorylethanolamine (GPEtn) [together called phosphodiesters (PDE)] also contribute to the tCho signal. Besides protons of choline and ethanolamine containing compounds, protons from other metabolites might also resonate around 3.2 ppm, e.g., glucose at 3.23 ppm, myo-inositol at 3.27 ppm, and taurine at 3.25 ppm. In liver and kidney the resonance at about 3.26 ppm is almost entirely composed of proton signals of betaine (trimethylglycine)[6].

Liver tumors and metastases

***In vitro* high field ^{1}H MR spectra of the liver:** Soper *et al*[47] performed a diagnostic correlation between MR spectra and histopathology. They analyzed liver tissue specimens from 54 patients undergoing partial or total hepatectomy. The samples included 31 normal, 59 cirrhotic and 32 hepatocellular carcinoma (HCC) histologically confirmed tissues and were analyzed by ^{1}H MRS at 8.5 Tesla. They found reduced amounts of lipids and carbohydrate residues and increased tCho in HCC compared to all normal and all cirrhotic liver tissue. Cirrhotic liver tissue and HCC were distinguished with a sensitivity and specificity of 95.8% and 88.9%, respectively. Lactate signals of variable intensity were found at 1.3 ppm, probably resulting from anaerobic metabolism after excision.

***In vivo* liver ^{1}H MR spectrum:** *In vivo* ^{1}H MRS is characterized by a much poorer spectral resolution and SNR than *in vitro* ^{1}H MRS (see above, technical issues). Kuo *et al*[48] investigated the value of *in vivo* ^{1}H MRS in the assessment of large focal hepatic lesions. They included 43 consecutive patients and 8 normal volunteers in a prospective MRS study. MRS was performed at 3.0T with shallow and regular breathing. Single voxel PRESS with TE = 30 ms, TR = 1500 ms, 256 averages, was used to select a volume of 2 cm × 2 cm × 3 cm. The voxel of interest was located in the largest solid portion of hepatic tumors in patients. Healthy liver data was collected from an area at the centre of the right hepatic lobe for normal volunteers, or in an uninvolved area of the right hepatic lobe in patients. Patients with diffuse-type HCC, with focal nodular hyperplasia and obvious fatty infiltration, and histological unconfirmed lesions were excluded. Thirty-three lesions (21 HCC, 2 angiosarcomas, 1 lymphoma and 9 hemangiomas) were included. They found that malignant tumors had elevated tCho resonances compared to uninvolved liver or benign tumors, but the difference in mean tCho/lipid ratio between malignant tumors or uninvolved liver did not reach statistical significance. Several factors may have contributed to these results. First of all, the tumors in this study may have contained significant necrotic areas with less viable cells. This may have diluted more prominent changes observed in areas of rapid cell turnover, within viable tumor tissue and may have caused low signal to noise for metabolite signals leading to larger errors. Physiological motion due to breathing and cardiac movement will have contributed, especially if the tumor was located in the left lobe or at the extreme end of the right hepatic lobe. Finally, three different tumor types were included in the malignant group, which may have resulted in a variation of different metabolites in phospholipid metabolism, and thus in a more variable tCho resonance. Thus, *in vivo* ^{1}H MRS is technically feasible at 3.0T for the evaluation of focal hepatic lesions, but the clinical application of the measurement protocol used by Kuo *et al*[48] is limited as normal liver, benign and malignant tumors cannot be clearly differentiated.

In the second part of their study Kuo *et al*[48] attempted to measure metabolic changes in HCC after transcatheter arterial chemoembolization (TACE). Eight HCC were evaluated before and two to five days after TACE. The tCho peak at 3.2 ppm was significantly decreased while the lipid and water signals at 1.3 and 4.7 ppm respectively, were increased. The mean tCho/lipid ratio significantly decreased from 0.23 ± 0.11 before to 0.01 ± 0.00 after TACE treatment. One of the post-TACE lesions showed recurrence three months later and MRS also revealed an elevated tCho/lipid ratio at that stage. Therefore, ^{1}H MRS at 3.0T may be used for treatment monitoring.

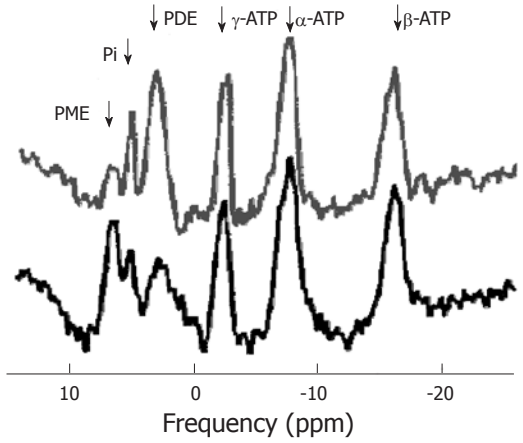

Figure 9 ***In vivo*** **^{31}P magnetic resonance spectra of human liver tissue obtained from a healthy volunteer (top) and from a patient with hepatocellular carcinoma (bottom).** PME: Phosphomonoesters; PDE: Phosphodiesters; Pi: Inorganic phosphate; ATP: Adenosine triphosphate. (Reproduced with permission of John Wiley and Sons, www.interscience.wiley.com, from[64].)

Fishbach *et al*[49] improved the MRS acquisition and processing protocol compared to previous studies by introducing a control of respiratory motion using breath-hold acquisitions and an abdominal compression belt. They also applied dedicated pre- and post-processing including automatic phase and frequency correction based on the residual and the unsuppressed water signal in order to remove potential distortions mainly introduced by motion. Apart from 39 volunteers, they included 55 patients with advanced cancer with lesions of more than 3 cm in diameter in their study (22 metastases of colorectal cancer, 11 hepatocellular carcinomas, 9 metastases of breast cancer, 3 metastases of pancreatic cancer and 1 metastasis of prostate cancer). Liver spectra were acquired at 3.0T using a body transmit/receive coil. Breath-hold at end-expiration spectra were acquired with the single voxel (2 cm × 2 cm × 2 cm) PRESS technique with TE=35 ms, TR=2000 ms, 128 averages and 16 additional unsuppressed water reference lines. The intra-individual reproducibility of this ^{1}H MRS acquisition in the liver was tested in 25 patients and volunteers and judged to be satisfactory. In total 186 spectra were acquired and 27 spectra had to be discarded because they did not meet the predefined quality specifications. The remaining 113 spectra were measured in normal-appearing parenchyma of 37 patients and 39 volunteers.

Although, remarkably, tCho signals relative to those of water seemed to be lower in metastatic lesions compared to normal liver tissue, no significant differences were observed between malignant liver tumors and normal liver parenchyma for any of the parameters analyzed, in particular tCho signals relative to water and lipid signals. This was attributed to the large variability of normal values.

The divergent results observed in the above mentioned studies might also be due to the multiple contributions from the unresolved tCho signal. In normal liver tissue low concentrations of PMEs and high concentrations of PDEs have been shown while in tumor tissue elevated levels of PMEs and decreased levels of PDEs have been observed[50]. This implies that in tumor tissue increased levels of PMEs may be canceled out by decreased PDEs, resulting in an unchanged overall tCho level. Different tumor types might also have divergent contributions to the unresolved tCho signal. In addition, alterations of the metabolite concentrations might not be due to malignancy, but due to proliferating healthy tissue such as a regenerating liver, benign tumors, and even some degenerative pathologies. With ^{31}P MRS PME and PDE signals can be studied separately as discussed below.

^{31}P MR SPECTROSCOPY

After ^{1}H MRS, phosphorus-31 MR spectroscopy is the most commonly used MRS technique to study tumors *in vivo*. Phosphorus has an MR sensitivity of 6.6% compared to proton. However, the chemical shift dispersion of its signals observed *in vivo* is larger (about 30 ppm *vs* about 10 ppm), resulting in a better spectral resolution. Also, ^{31}P MRS is capable of detecting some key metabolites in tissue energy metabolism such as ATP, PCr, and inorganic phosphate (Pi). In addition some important metabolites involved in membrane metabolism such as PCho and PEtn (together called PME) and their glycerol derivatives GPCho and GPE (together called PDE) may be resolved. In this respect ^{31}P spectra (Figure 9) are more informative than ^{1}H MR spectra in which signals of all choline compounds (tCho) usually are observed unresolved at about 3.2 ppm. Furthermore, from ^{31}P MR spectra physiological parameters like intracellular pH can be deduced from the chemical shift of the Pi resonance. The phosphoryl resonances from large and membrane bound compounds may only show up in a phosphorous-31 MR spectrum as broad underlying baseline signals due to their very short T_2 values[51].

Unfortunately, due to a lower sensitivity and less favorable spin relaxation the spatial resolution of ^{31}P MR spectra is an order of magnitude less than that of ^{1}H MRS. Higher magnetic fields provide improved experimental conditions for ^{31}P MRS.

Metabolites visible in ^{31}P MR spectra of liver tumors

Phosphocreatine: The largest peak in ^{31}P MR spectra of muscle, brain and other tissues originates from PCr. Its spectral position is used as an internal chemical shift reference and commonly has been assigned a chemical shift of 0.00 ppm. PCr is, however, not detectable in spectra of healthy liver since hepatocytes do not express creatine kinase under normal circumstances[30]. Tumors, however, might express creatine kinase and show some PCr.

Adenosine triphosphate: ATP is the main direct energy supply within cells. ATP consists of adenosine and three phosphate groups. The phosphoryl groups, starting with those closest to the adenosine moiety, are referred to as α, β,

and γ phosphates. ATP is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Multiple processes in the cell can split ATP into ADP or AMP and inorganic phosphate, and use the energy that is released. At a pH of 7.2, with full magnesium complexation, the resonances of ATP appear at -7.52 ppm (α), -16.26 ppm (β), and -2.48 ppm (γ). The ATP resonances may overlap with the signals of other nucleotides: uridine triphosphate (UTP), guanosine triphosphate (GTP) and cytidine triphosphate (CTP). Therefore these resonances are sometimes referred to as nucleoside triphosphate (NTP), although the others usually occur at much lower concentrations.

Inorganic phosphate: Like the level of ATP that of inorganic phosphate (Pi) reflects the cellular phosphorylation potential. The chemical shift of Pi and some other phosphorus containing compounds is dependent on the intracellular pH (pHi) and magnesium concentrations[52]. The protonation or complexation with magnesium of phosphate affects the chemical environment of the ^{31}P nucleus and hence its chemical shift. As proton exchange is fast on the NMR timescale, the resonance frequency is indicative of the relative amount of protonated and unprotonated molecules, and hence the pH can be deduced. The shift in resonance of Pi relative to PCr is most commonly used as it has a large dependence in the physiological pH range whereas the chemical shift of PCr is constant in this range. At a pH of 7.2 and normal magnesium level it occurs at 5.02 ppm. The accuracy of pH determination from the Pi-PCr shift is typically 0.05 pH units[6]. However, as PCr is not detectable in the normal liver the α-ATP resonance is used instead as a reference.

Tumor pH: Due to the Warburg effect and/or hypoxic conditions tumor cells preferentially convert glucose to lactic acid. Lactic acid is largely dissociated *in vivo* to H^+ and lactate$^-$. Normal, as well as tumor cells, have multiple systems to continuously export H^+ ions to maintain a constant pHi, as well as a system for exporting lactic acid (but not lactate). This results in a neutral, or a slightly alkaline pHi of intact (tumor) cells. However, since tumors may be poorly vascularized the extracellular tumor pH (pHe) of tumors is more commonly acidic[53-59].

Phosphodiesters: Signals of PDE occur around 3 ppm in ^{31}P MR spectra. Tumor tissue sometimes contains significantly lower concentrations of PDE than healthy liver tissue. Some *in vitro* studies show that the PDE levels increase with decreasing growth fraction of the tumor. This suggest that PDE signals may be dominated by breakdown products of phospholipids[60], and the concentration may be an indicator of the necrotic fraction in tumors associated with phospholipid catabolism[61]. The phospholipid derivatives, particularly GPCho and GPEtn, were found to contribute to the PDE resonance[61]. Their phosphor spins resonate at 2.76 ppm and 3.20 ppm respectively.

Phosphomonoesters: Resonances of PME occur at about 6 ppm in ^{31}P MR spectra. Increased levels of PME have been hypothesized to be associated with intensified cell membrane synthesis, cellular growth, cell nutritional state and rate of cell replication. Several studies identified increased PME signals as a possible diagnostic marker for tumors. PME/PDE ratios were suggested to represent altered relative rates of membrane synthesis, catabolism and metabolic turnover[36,60,61]. Phospholipid derivatives, particularly PCho and PEtn, contribute to the PME resonance[61]. PCho and PEtn resonate at 5.88 ppm and 6.78 ppm respectively.

Liver tumors and metastases

***In vitro* high field ^{31}P MR spectroscopy of liver:** Many *in vitro* ^{31}P MRS animal studies and several *in vitro* ^{31}P MRS studies on human hepatic tumor tissues have been performed. Obtaining a fully representative human hepatic tissue sample for ^{31}P MRS is difficult as the surgical removal and extraction usually results in a period of ischemia/hypoxia, which affects metabolic processes resulting in decreased ATP and increased Pi levels. Nevertheless, *in vitro* ^{31}P MRS may be used to study signals in the PME and PDE peaks that are still unresolved in *in vivo* ^{31}P MR spectra, as these are less affected by short periods of hypoxia.

Bell *et al*[50] investigated the metabolic changes arising in hepatic tumors and the possible systemic effects of these tumors on the liver as a whole. Ten biopsy specimens were obtained from hepatic tumors (one cystadenoma, four hepatocellular carcinomas, four metastatic colonic adenocarcinomas and one metastatic squamous cell carcinoma from the lungs). Five histologically proven normal tissue samples from the same tumor-bearing hepatic lobe were obtained immediately after the blood supply had been clamped and before partial hepatectomy. Six control samples were obtained from morphologically normal liver tissue from patients with histologically proven chronic pancreatitis and known to be free of any hepatic malignancy. Both ^{31}P spectra with proton decoupling and ^{1}H MR spectra with partially water suppression were acquired using high-resolution 11.7T systems. Betaine is the most prominent resonance in the *in vitro* rat liver ^{1}H spectrum but no resonance for betaine was observed in any of the human biopsy samples, suggesting that its presence is species related. The *in vitro* ^{31}P MRS spectrum showed that over 10 different compounds contributed to the PME resonance. The five principal resonances were: PCho, PEtn, glucose-6-phosphate, AMP, and glycerol-3-phosphate. The PDE region included at least 3 different compounds. The two main components were: GPCho and GPEtn. Compared to control tissue, tumor tissue showed significantly lower concentrations of GPCho (0.59 ± 0.15 *vs* 2.46 ± 0.37) and GPEtn (0.57 ± 0.17 *vs* 2.25 ± 0.46), and elevated levels of PCho (1.36 ± 0.50 *vs* 0.17 ± 0.11) and PEtn (2.47 ± 0.84 *vs* 0.16 ± 0.10). It was suggested that the increase in PME/NTP observed in *in vivo* spectra of HCC and

Table 1 Number of different cases

	High PME	High PDE	High Pi	Low PCr
HCC	91% (of 11 cases)	75% (of 4 cases)	0% (of 4 cases)	100% (of 11 cases)
Liver metastasis	100% (of 7 cases)	33% (of 6 cases)	17% (of 6 cases)	100% (of 4 cases)
Liver lymphoma	100% (of 6 cases)	17% (of 6 cases)	100% (of 6 cases)	100% (of 6 cases)

"High" and "low" levels are relative to the amount in normal or benign tissue (from Negendank[70]). PME: Phosphomonoesters; PDE: Phosphodiesters; Pi: Inorganic phosphate; PCr: Phosphorylated creatine; HCC: Hepatocellular carcinoma.

liver metastasis (see next section) is due to increased levels of PCho and PEtn. The decrease in concentration in GPC and GPE observed in this study might be responsible for the change in PDE/NTP seen in *in vivo* spectra. However, the underlying cause of these changes remains partially unknown and requires further study.

Bell *et al*[50] also observed that spectra from histologically normal tissue from the liver tumor-bearing hepatic lobe contained more PCho (0.32 ± 0.18 *vs* 0.17 ± 0.06) and PEtn (0.34 ± 0.12 *vs* 0.16 ± 0.07) than spectra obtained from control tissue. The levels of GPC and GPE showed no significant change.

Previously, in 1993 Dagnelie *et al*[62] studied liver metabolic changes in rats bearing subcutaneous Dunning prostate tumors by *in vivo* and *in vitro* ^{31}P MRS. Although absence of metastatic tumor cells in the liver of all tumor-bearing animals was confirmed by histological examination, hepatic phosphorylation status, phospholipid metabolism, and gluconeogenesis was significantly affected in the tumor-bearing animals. Dagnelie *et al*[63] also investigated liver metabolism in humans with metastatic cancer without evidence of liver metastases by ^{31}P *in vivo* MRS. They included 23 cancer patients and 12 healthy subjects and found markedly elevated PME and reduced PDE levels in the non-metastatic liver compared to controls.

Thus this may complicate the use of increased PME (PCho and PEtn) levels as a sole diagnostic biomarker to detect (metastatic) liver cancer. In addition, as no significant differences between HCC and liver metastasis were observed in biopsy samples by *in vitro* ^{31}P MRS[50,64], the use of *in vivo* ^{31}P MRS seems limited in this differentiation.

***In vivo* ^{31}P MRS of the liver:** In 1985 Maris *et al*[65] combined the results of *in vivo* and *in vitro* ^{31}P MR spectroscopy studies to compare the spectral characteristics of the liver of 2 children, one infant with neuroblastoma stage Ⅳ-S and the other with neuroblastoma stage Ⅳ disease. The ^{31}P MR spectra from the primary tumor in the latter infant, and the spectra from the infiltrated liver regions in both the infants showed substantially elevated PME/β-NTP ratios compared with a spectrum from a normal control. The ratio increased during periods of rapid progression and persisted until treatment became effective. The PME/β-NTP ratio decreased to normal values, during either spontaneous or therapy-induced regression of the disease[66]. It was suggested that the increased PME (corresponding to PEtn and PCho) was due to the need for increased phospholipid synthesis in these tissues. This study demonstrated that ^{31}P MRS could be used to detect tumors and to monitor their response to treatment.

Dixon *et al*[67,68] studied whether hepatic involvement in lymphoma produced biochemical changes that could be detected by *in vivo* ^{31}P MRS of the liver. Twenty-two patients were included. Lymph node biopsies showed that eight patients had Hodgkin's disease and 14 non-Hodgkin's lymphoma. Eleven patients of these 14 had high grade lymphoma and three low grade disease. Six patients, diagnosed with lymphomatous infiltration of the liver on the basis of liver function tests and either ultrasound or CT imaging, had a significantly higher PME/Pi ratio (1.43 ± 0.37) or PME/ATP ratio (0.94 ± 0.27) compared to 25 controls (ages 20-50 years) (0.58 ± 0.11 and 0.37 ± 0.10, respectively) with normal livers. The PME/Pi ratio decreased following chemotherapy to an average of 66% of the initial value (range 40%-82%). In two patients the ratio fell to the normal range and these patients showed clinical remission. Four patients whose liver spectra showed persistently high ratios after therapy died subsequently of progressive disease. *In vitro* ^{31}P MRS studies of extracts of lymphomatous lymph nodes suggested that PEtn was largely responsible for the increased PME signal. In this study, however, no difference was observed between Hodgkin's and non-Hodgkin's lymphoma and between histological grades.

Meyerhoff *et al*[69] used ^{31}P MRS to assess the metabolic state of hepatic cancers and their metabolic response to chemoembolization. Their preliminary report described studies on five patients (two with colon cancer metastasis, two with HCC, and one with adenocarcinoma of unknown primary) and thirteen healthy volunteers. Untreated hepatic tumors showed elevated PME/ATP ratios, reduced ATP and Pi content, and normal PDE levels compared to normal controls. ATP, PME, and/or PDE levels diminished as an acute response to chemoembolization, whereas Pi content increased or stayed relatively constant. This could point to tumor regression and/or necrosis. Long-term follow-up after treatment showed decreased PME/ATP and increased ATP levels, even in the absence of changes on standard imaging. This could be the result of returning normal liver tissue and thus recovery.

Negendank[70] reviewed hundreds of cancer cases in ^{31}P and/or ^{1}H MRS studies that were published up to early 1992. In general he found that human cancers, other than brain, of different types in different locations had similar metabolic characteristics: 173 of 194 cases had high PME and levels, 121 of 166 cases had high PDE levels, 59 of 125 cases had high Pi levels and 96 of 117 cases had low PCr. Table 1 lists the cases with HCC, metastatic liver cancer and lymphoma in the

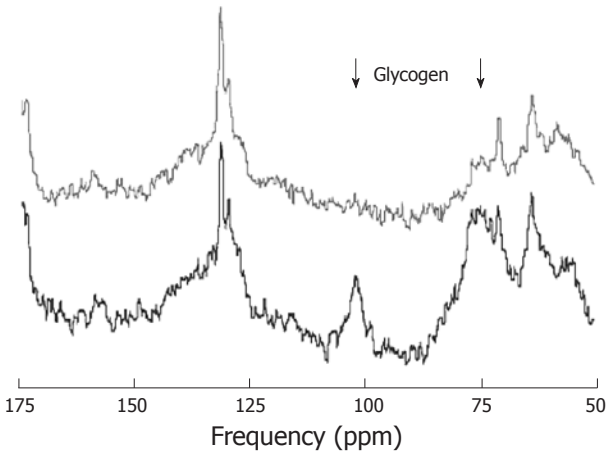

Figure 10 *In vivo* ^{13}C magnetic resonance spectra (1.5T) of human liver tissue obtained from a healthy volunteer before (bottom) and after exercise (top). Resonances of glycogen (101 ppm and around 75 ppm) are reduced after exercise. Other resonances are mainly lipids.

liver. The most frequently used characteristic that differentiated healthy liver tissue from liver lymphoma was an increased PME level, and an increased PME/Pi and increased PME/Pi for HCC, and increased PME/NTP and increased PDE/NTP ratios for metastasis in the liver. Also, an early decrease in PME (or in the PME/PDE ratio) was a good predictor of response to whatever treatment.

Concluding remarks

From these studies it appears that the increase of PME levels is associated with tumor progression and that successful treatment is associated with its decrease. Therefore ^{31}P MRS seems very suitable for treatment response monitoring. Since 2003, however, only a limited number of *in vivo* ^{31}P MRS studies on liver tumors and metastasis have been reported. The reason for this could be the low spatial and time resolution of *in vivo* ^{31}P MRS on 1.5T MR systems. Currently, 3T MR systems have become widely available, dual tuned multi-channel ^{31}P/^{1}H coils have been developed and several techniques, e.g., ^{1}H decoupling, nuclear overhauser enhancement, polarization transfer, have been demonstrated[16] to improve ^{31}P MRS sensitivity and spectral resolution. Finally, high field *in vitro* ^{31}P MRS of cell cultures might establish new markers to distinguish different tumor types and to separate benign from malignant tumors.

^{13}C MR SPECTROSCOPY

As carbon-12 has no net nuclear spin it cannot be detected by MR spectroscopy. In contrast, ^{13}C can be detected by MRS but it has a natural abundance of only 1.11%. Therefore ^{13}C has a relatively low MR sensitivity. In addition, the signal to noise ratio may be negatively affected by ^{1}H coupling. To obtain spectra with acceptable signal to noise it is needed to apply averaging, polarization transfer, and ^{1}H decoupling. The chemical shift dispersion of ^{13}C MRS *in vivo* is large (about 200 ppm) and is also characterized by narrow line widths, resulting in a very good spectral resolution (Figure 10). Although ^{13}C MR spectroscopy is primarily known for MRS of ^{13}C-labeled substrates (e.g., glucose), natural abundance ^{13}C MRS can also be applied.

Metabolites visible in natural abundance ^{13}C MR spectra

Almost all metabolites in the human body contain carbon and therefore in principle a large amount of metabolites can be investigated with ^{13}C MRS, but because of the 1.11% natural abundance this is restricted to only a few highly concentrated compounds such as lipids. Chemical shifts above 150 ppm are indicative of carbonyl groups; carbons adjacent to hydroxyl groups typically resonate in the 60-100 ppm range; CH, CH_2, CH_3 groups resonate in the 45-60 ppm, 25-45 ppm and < 25 ppm ranges, respectively. The resonances of -CH = CH- groups are located around 125 ppm. Dominant lipid resonances are usually found in non-brain tissue. Two distinct resonances at 63 and 73 ppm originate from the glycerol backbone[6].

^{13}C labled metabolites visible in ^{13}C MR spectra

^{13}C MRS with labeled substrates is the only way to study metabolic conversions in the living intact body. Substrates enriched with ^{13}C are usually administrated intravenously to reach a high and stable level in the blood. One of the most commonly used enriched substrate in humans is [1-^{13}C]glucose. Other known substrates are [1,2-$^{13}C_2$]-choline, [1,2-$^{13}C_2$]-ethanolamine, [3-^{13}C]pyruvate and lactate, [2-^{13}C]acetate, [2-^{13}C]glucose and [1,6-$^{13}C_2$]glucose. The latter, often used in studies involving rats and mice, results in two labeled [3-^{13}C]-pyruvate molecules, and thus more signal. Pyruvate is an intermediate common to three major metabolic and catabolic pathways. After i.v. injection, pyruvate is rapidly distributed in the body and taken up by most cells, and then converted into alanine, lactate, or carbon dioxide, depending on the intracellular energy status.

Liver tumors and metastases

Natural abundance ^{13}C MR liver spectrum: In 1983 Bottomley *et al*[71] demonstrated the feasibility of natural abundance ^{13}C MRS at 1.5T. The low sensitivity and the fact that most information could also be obtained from ^{1}H and ^{31}P MRS spectra has prevented widespread application of natural abundance ^{13}C MRS. However, there are areas where it has some advantages such as in the detection of glycogen. Carbohydrate reserves are mainly stored as glycogen in animals and humans, in particular in muscle and liver. Natural abundance ^{13}C MRS detection of glycogen is typically performed *via* the glycogen-C_1 resonance at 100.5 ppm[72]. Some studies on rats have indicated that glycogen levels in hepatic tumors were markedly less than those observed in livers of control animals[73,74].

^{13}C labled ^{13}C MR liver spectrum

^{13}C-labeled glucose: Infusion of enriched ^{13}C-labeled glucose in combination with ^{13}C MRS can provide highly specific information on metabolites and metabolic rates involved in energy metabolism. The ^{13}C-labeled glucose is transported into the cell in the same way as ^{18}FDG used in PET[75]. Where the derivative of ^{18}FDG is trapped inside the cell, indicating areas of high glucose transport and thus indirectly indicating glycolytic activity, the ^{13}C-labeled glucose will enter metabolic pathways like glycolysis and the TCA cycle. This allows the direct study of glucose uptake, the flux through labeled metabolites, relative contributions of glycolytic pathways and oxidative phosphorylation, as well as oxygen consumption[12,76].

^{13}C labeled glucose combined with ^{13}C MRS in human liver tissue is, however, not without difficulties and the number of studies with this technique is still very limited. In 2008 Tomiyasu *et al*[77] monitored liver glycogen synthesis in diabetic patients using ^{13}C MRS on a 3.0T system. The MR signals of liver [1-^{13}C]-glucose and glycogen were assessed and a correlation between the quantity of liver glycogen and the fasting plasma glucose levels was found. To investigate glucose metabolism of liver tumors would be of great interest, but such studies are still lacking.

^{13}C-labeled ethanolamine and choline in experimental tumors: Dixon *et al*[67,78] administrated [^{13}C$_2$]-ethanolamine to mice with lymphomatous liver to study the kinetics of PtdEtn synthesis[79]. The newly synthesized PEtn and PtdEtn could be distinguished from naturally-abundant compounds by their ^{13}C label. The results showed that PtdEtn synthesis in the normal liver largely follows the Kennedy pathway. The data extracted from the ^{13}C MR spectra were fitted to a kinetic model representing this pathway, which allowed them to determine the approximate rates of the various enzymes in the synthetic pathway *in vivo*. They also showed that the overall rate of PtdEtn synthesis from Etn was not increased in lymphomatous liver.

Katz-Brull *et al*[35] investigated the distribution of metabolites following infusion of [1,2-^{13}C]-choline by ^{13}C MRS in mice. In their study MCF7 human breast cancer cells were inoculated s.c. in the right flank of CD-1 female athymic mice. In the tumors significantly more PCho (labeled and unlabeled) was observed than in normal liver and kidney tissue. Therefore, ^{13}C MRS combined with modeling can be used to study choline and ethanolamine metabolism and enzymes rates in mice.

Hyperpolarization and tumor metabolism: Gallagher *et al*[80] performed a ^{13}C MRS *in vitro* study of glutaminase activity in human hepatocellular carcinoma cells using DNP hyperpolarized ^{13}C-labeled glutamine. They showed that the conversion of hyperpolarized [5-^{13}C]glutamine to glutamate senses intramitochondrial glutaminase activity in hepatoma cells. These results represented the first step in the development of an imaging technique for the detection of glutamine metabolism *in vivo*. The rate of glutamine uptake and metabolism to glutamate in HCC cells was shown to be up to 30-fold higher than in normal hepatocytes. And thus this approach has clinical potential such as in the detection of small HCC in the presence of a cirrhotic liver. This study also suggests a new technique to detect changes in tumor cell proliferation in response to cytotoxic treatment since glutamine utilization has been correlated with cell proliferation.

Golman *et al*[75] conducted a metabolic imaging study in P22 tumors implanted on the back of rats. The high signal, due to DNP hyperpolarization, allowed mapping of pyruvate, lactate and alanine in a 5 mm × 5 mm × 10 mm imaging voxel using a 1.5T scanner. Tumor tissue showed a significantly higher lactate content than normal tissue, possibly explained by the Warburg effect. The results indicate that fast noninvasive quantification may be possible. To show that the DNP technique can be used both in small and larger animals, Golman *et al*[81] also conducted nearly similar real-time metabolic imaging studies in rats and pigs. The pig study would provide important information as to whether [1-^{13}C] enriched hyperpolarized dynamics also could be visualized in a more clinically relevant setting. They showed that in both species, where pharmacokinetic parameters widely differ, it is possible to map pyruvate and some of its metabolites in resting skeletal muscle within a clinically useful time frame of about 10 seconds. This indicates the technique may work in humans as well.

Concluding remarks

Hyperpolarization is a promising technique in MR cancer research. Similar images as those in ^{18}FDG-PET can be obtained, but with the further advantages that, without radiation, real (glucose) metabolism is observed. Although this new MR method is far more sensitive than conventional ^{13}C MRS it only allows measurement of single step metabolic conversions associated with rapid cellular uptake of the administered substrate. Therefore, conventional ^{13}C MRS studies will remain valuable to understand and complement results from hyperpolarized ^{13}C MR imaging.

CONCLUSION

Hydrogen is the most commonly studied nucleus, as it has the highest sensitivity compared to ^{31}P and ^{13}C, and essentially can be performed with the same hardware as for standard MR imaging. In liver tumor studies the lactate resonance is related to energy metabolism (Warburg effect) of the tumor. Proton resonances of mobile lipids and the peak of total choline (tCho) have been explored as biomarkers to identify malignant tumors. However, the tCho peak is composed of several unresolved resonances of different choline containing compounds, which makes changes in this signal difficult to interpret.

With ^{31}P MRS phosphorylated choline and ethanolamine containing compounds can be resolved. From

several studies it is known that the increase of PME is associated with tumor progression and that successful treatment is associated with a decrease of PME levels. Therefore ^{31}P MRS could very well be used for treatment response monitoring. Besides the signals from phospholipid metabolism, ^{31}P MR spectra of liver also show signals of ATP and Pi which can be used to investigate tumor energy metabolism.

MRS with ^{13}C as label is a unique method to measure the dynamics of metabolic conversions *in vivo*, but it has hardly been used to examine human liver metabolism due to its technical complexity and relatively low sensitivity. However, developments such as hyperpolarization may open new ways of liver assessment and imaging.

In vivo MR spectroscopy provides a number of adequate research tools to study metabolism in liver tumors and metastasis. However, they are not yet applied often in a clinical setting for diagnosis and treatment monitoring. This may be due to technical challenges associated with the body location of the liver, relatively long scan times needed for a good signal to noise ratio, the need for additional hardware (except ^{1}H MRS) and the need for expertise in spectral interpretation. With higher magnet fields becoming available, new multi-element detection probes, new acquisition techniques for improved spatial and time resolution, better postprocessing, and with new biomarkers it is expected that the research and clinical usefulness of MRS of liver and tumors therein will increase.

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EDITORIAL

Utility of co-transplanting mesenchymal stem cells in islet transplantation

Naoaki Sakata, Masafumi Goto, Gumpei Yoshimatsu, Shinichi Egawa, Michiaki Unno

Naoaki Sakata, Gumpei Yoshimatsu, Shinichi Egawa, Michiaki Unno, Division of Hepato-Biliary-Pancreatic Surgery, Department of Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan
Masafumi Goto, Division of Advanced Surgical Science and Technology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan
Masafumi Goto, New Industry Creation Hatchery Center, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan
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Correspondence to: Naoaki Sakata, MD, PhD, Assistant Professor of Division of Hepato-Biliary Pancreatic Surgery, Department of Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan. n-sakata@surg1.med.tohoku.ac.jp
Telephone: +81-22-7177205 Fax: +81-22-7177209
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Abstract

Islet transplantation is characterized by the transplantation of isolated islets from donor pancreata into a diabetic recipient. Although it is a viable choice in the treatment of insulin dependent diabetes mellitus, most patients (approximately 90%) require insulin five years after transplantation. Recently, the co-transplantation of mesenchymal stem cells (MSCs) and islets in animal studies has revealed the effectiveness of MSCs co-transplantation for improving islet function. The mechanisms underlying the beneficial impact of MSCs include immunomodulation and the promotion of angiogenesis. In this review, we discuss MSCs and how they support improved graft survival and function.

Key words: Mesenchymal stem cell; Islet transplantation; Bone marrow; Immunomodulatory; Regulatory T cell; Angiogenesis; Vascular endothelial growth factor; Diabetes mellitus

Peer reviewer: Ming Li, Associate Professor, Tulane University Health Sciences Center, 1430 Tulane Ave Sl-83, New Orleans, LA 70112, United States

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INTRODUCTION

According to the International Diabetes Federation (IDF) database, the number of patients with diabetes mellitus (DM) worldwide is 285 million, indicating that 6.4% of the global population have DM. Furthermore, the IDF predict that the number will increase to 438 million by 2030. DM is a serious disease; approximately 4 million people die each year from DM. In addition, DM is a major cause of serious complications such as blindness, renal failure, and ischemic heart disease. Type 1 diabetes is characterized by the irreversible autoimmune destruction of pancreatic β cells and is usually diagnosed in children and young adults[1]. Islet transplantation consists of the transplantation of pancreatic islets that have been isolated from a donor pancreas[2]. The therapeutic effect was regarded as insufficient for a long time; however,

since the use of the "Edmonton Protocol"[2], a markedly improved islet transplant protocol developed at Alberta University, islet transplantation has been performed widely for 10 years. However, according to a recent report, although approximately 70% of patients did not need daily insulin one year after transplantation, approximately 90% of patients required insulin after five years[3]. Therefore, studies aimed at improving the outcome of islet transplantation are still required.

One of the reasons for failure of insulin independence is islet graft loss due to a variety of causes including instant blood-mediated inflammatory reaction[4], acute rejection[5], islet toxicity by immunosuppressive agents[6], and ischemia caused by poor vascularity at transplantation[7] and the embolization effect of the islets[8]. Moreover, islet transplantation also faces the problem of a limited supply of suitable donor human pancreata[9]. Thus, to promote islet transplantation in the future, there is a need to establish a novel donor source and develop more effective treatments to prolong the function of transplanted islets.

WHAT ARE MESENCHYMAL STEM CELLS?

Mesenchymal stem cells (MSCs) are multipotent cells capable of self-renewal and differentiation into a various cell lineages. They are derived from many organs such as bone marrow, adipose tissue, skin, fetal liver, and umbilical cord blood[10,11]. Although the number of MSCs in the bone marrow is very small compared with other component cells (only 0.01%-0.001%)[12], MSCs regulate the maintenance and proliferation of hematopoietic stem cells (HSCs) in the bone marrow[13]. MSCs are able to differentiate into various cells derived from ectoderm (epithelial cells and neurons), mesoderm (connective stroma, cartilage, fat and bone cells), and endoderm (muscle cells, gut epithelial cells, and lung cells)[13]. A number of groups have demonstrated that insulin-producing cells could also be differentiated from MSCs[14-18]. The regeneration of insulin-producing cells is an important theme in research aiming to improve the outcome of cell replacement therapy and has been the focus of some groups. However, in an alternative approach, many studies have examined the effect of transplanting islets with MSCs and have demonstrated improved islet function when co-transplanted with MSCs[7,19-22]. The beneficial effects of MSCs in the context of islet transplantation could be attributed to immunomodulation and angiogenesis.

IMMUNOMODULATORY EFFECT OF MSCS

The pancreata used for clinical islet transplantation are allogeneic and recipients therefore require immunosuppressive drugs to prevent rejection. Recently, Solari *et al*[23] demonstrated that MSCs could promote the prolonged survival of allograft islets in a rat model with limited treatment with immunosuppressive agents. Allogeneic islets transplanted to diabetic rats with syngeneic MSCs survived for over one month whilst islets transplanted alone survived for only seven days. They also demonstrated prolonged graft survival of allogeneic islets transplanted with allogeneic MSCs compared to allogeneic islets transplanted alone. T cell production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α was decreased in allogeneic islets transplanted with syngeneic MSCs. Furthermore, *in vitro* studies of cocultured MSCs and islets generated interleukin (IL)-10 which inhibited $CD4^+$ T cells. Melzi *et al*[20] performed allogeneic islet and allogeneic neural stem cell (NSC) transplantation in a murine model and showed significantly longer graft survival in allogeneic islet/NSC transplanted mice in the absence of immunosuppression, compared to mice transplanted with islets alone (> 100 d survival). Intriguingly, they detected expansion of regulatory T cells in the spleen of co-transplanted mice. These results indicate that MSCs exert an immunomodulatory role and can actively limit the rejection of co-transplanted islets.

The mechanism underlying the immunomodulatory effect of MSCs is likely to be multifactorial and result from the communication between various immune cells and cytokine generation (Figure 1). For example, MSCs can inhibit the proliferation and cytotoxicity of resting natural killer (NK) cells, which are key effector cells of the innate immune system and play an important role in antiviral and anti-tumor immune responses[24]. Spaggiari *et al*[25] demonstrated that the cytokine-induced proliferation of freshly isolated NK cells was inhibited by the presence of MSCs. MSCs also inhibited NK cell activation, cytotoxic activity, and IFN-γ production[26]. These effects are mediated by prostaglandin E_2 (PGE_2) and indoleamine 2,3-dioxygenase (IDO)[13,26].

Another important effect of MSCs is to inhibit the differentiation of monocytes to dendritic cells (DCs) that, following DC maturation, present antigens to naïve T cells[27,28]. MSCs also inhibit TNF-α production by DCs and upregulate IL-10 production by plasmacytoid DCs (pDCs)[29] - effects modulated by PGE_2. These effects of MSCs upon DC function undoubtedly contribute to their anti-inflammatory and immunoregulatory effects.

MSCs may also directly inhibit $CD4^+$ T cells, $CD8^+$ T cells, and B cells, immune cells involved in rejection of allogeneic cells, by releasing soluble mediators, including PGE_2, IDO, or soluble human leukocyte antigen (sHLA)-G5. Inhibition of $CD4^+$ T cells impairs B cell proliferation and antibody production[13]. $CD8^+$ cytotoxic T cells are involved in killing virus-infected or allogeneic cells, and MSCs are capable of inhibiting the induction of $CD8^+$ T cell responses and preventing cytotoxicity[30]. MSCs inhibit B cell proliferation and antibody secretion, as well as their differentiation to plasma cells[31]. On the other hand, MSCs may induce the generation of regulatory T cells, which suppress immune cell activation, and help to maintain homeostasis and promote self tolerance by inducing production of IL-10 from pDCs and by releasing HLA-G5[29,32]. In

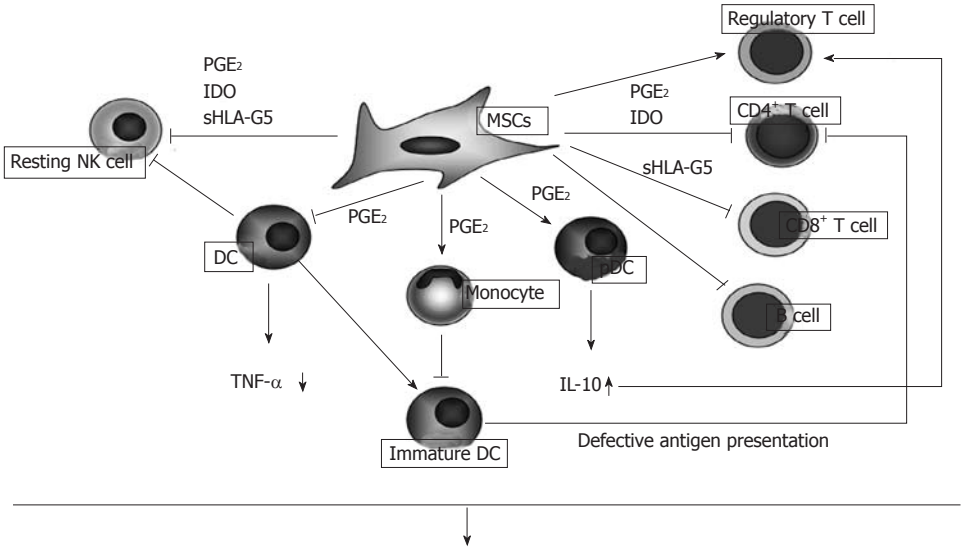

Figure 1 Immunomodulatory effect of mesenchymal stem cells (modified and quoted from Uccelli *et al*[13]). Mesenchymal stem cells (MSCs) can inhibit the proliferation and cytotoxicity of resting natural killer (NK) cells *via* the generation of mediators, including prostaglandin E_2 (PGE_2), indoleamine 2,3-dioxygenase (IDO) and soluble human leukocyte antigen (sHLA)-G5; MSCs inhibit the differentiation of monocyte to antigen presenting dendritic cells (DCs). MSCs also inhibit TNF-α production by DCs and upregulate IL-10 production by plasmacytoid DCs (pDCs): effects modulated by PGE_2; MSCs directly inhibit $CD4^+$ T cell, $CD8^+$ T cell, and B cells that are involved in allogeneic cell rejection by releasing PGE_2, IDO, or sHLA-G5. $CD4^+$ T cell inhibition limits B cell proliferation and antibody production whilst $CD8^+$ T cell inhibition prevents cytotoxicity. MSCs induce generation of immunomodulatory regulatory T cells that suppress immune activation, help to maintain homeostasis, and promote self tolerance by production of IL-10 from pDCs and by releasing HLA-G5. Thus, MSCs can promote immunotolerance and facilitate the engraftment of allogeneic islets.

summary, MSCs can promote immunological tolerance and facilitate the survival and function of allogeneic islets. It is likely, however, that the immunomodulatory roles of MSCs have not been fully clarified.

ANGIOGENIC EFFECT OF MSCS

Pancreatic islets have a rich vascular supply in the pancreas, with some reports indicating that islets receive 5%-10% of pancreatic blood flow, despite the islet mass only comprising 1%-2% of the total pancreas[33,34]. However, isolated islets are avascular, as the process of islet isolation destroys the vascular network between the islet and surrounding tissue[35]. As a result, islets undergo prolonged ischemia during the reconstruction of the vascular network, which may take approximately 14 d[36] and many islets become damaged. It is thus apparent that strategies to limit islet ischemia are necessary to improve the outcome of islet transplantation.

Some studies suggest that angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1, are required to generate a vascular network around transplanted islets[37,38]. Recently, the pro-angiogenic effects of MSCs have been examined (Figure 2). The process of revascularization consists of proteolytic digestion of the vascular wall and subsequent migration, proliferation, and differentiation of endothelial cells (ECs)[39]. MSCs express platelet-derived growth factor (PDGF) receptors and respond to PDGF production by ECs during revascularization[40]. MSCs promote EC migration by producing proteases that facilitate immature EC sprouting[41] and upregulating the expression of angiopoietin and VEGF in ECs, as these factors promote angiogenesis and stability of the developing vasculature[42]. The roles of MSCs in angiogenesis have been explored in experimental models of ischemia. Martens *et al*[43] demonstrated that MSCs produced VEGF and induced neovascularization in the ischemic myocardium. Jiang *et al*[44] showed that transplantation of MSCs into ischemic limbs promoted angiogenesis. Johansson *et al*[45] explored the enhancement of angiogenesis by MSCs in the context of islets. They first examined cultures of human islets and ECs in the presence or absence of MSCs. The study indicated that the inclusion of MSCs promoted EC proliferation and migration of ECs to the surface of islets to form a "coat"[45]. Islets with this surrounding "coat" of endothelial cells survived for a long time in culture and exhibited improved insulin release[45]. The coated islets had many sprouts and were connected to other endogenous islets by vessel-like structures[45]. These findings indicated that MSCs act to promote EC proliferation in both donor and recipient sides, together with sprout formation and growth of ECs into the islet. Thus, MSCs may exert a potent angiogenic function and contribute to islet engraftment by promoting islet vascularization.

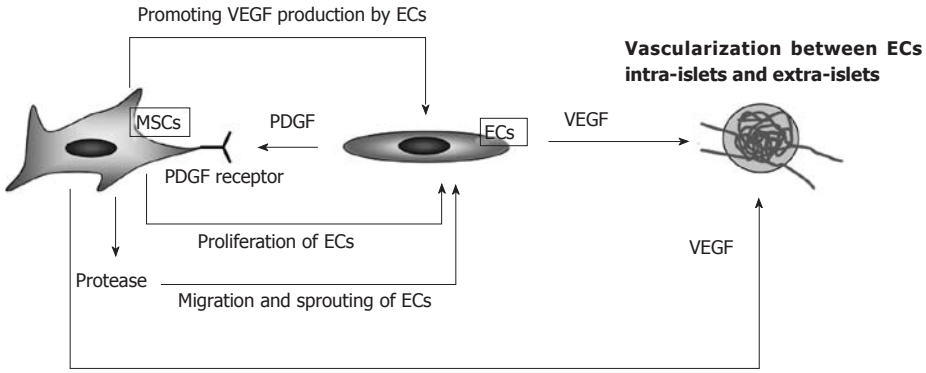

Figure 2 Pro-angiogenic effect of mesenchymal stem cells. The process of revascularization requires proteolytic digestion of the vascular wall and subsequent migration, proliferation, and differentiation of endothelial cells (ECs). Mesenchymal stem cells (MSCs) express platelet-derived growth factor (PDGF) receptors and respond to PDGF production by ECs during revascularization. MSC-derived proteases promote EC migration and immature EC sprouts and upregulate the expression of angiopoietin and vascular endothelial growth factor (VEGF) in ECs, thereby promoting both angiogenesis and vascular stability. MSCs also produce VEGF and induce neovascularization. MSCs promote both donor and recipient EC proliferation, EC sprout formation, the ingrowth of ECs into islets and the formation of a vascular network between intra- and extra-islet vessels.

Recently, Ito *et al*[21] co-transplanted rat islets and MSCs into diabetic severe combined immunodeficiency mice and evaluated the rate of normoglycemia and extent of islet vascularization. All the diabetic mice that received 500 islets with 10^7 MSCs exhibited normoglycemia, compared to 30% of mice transplanted with islets alone. Neovascular density was also increased in the islet/MSCs co-transplanted group and was associated with strong expression of VEGF and endothelial von Willebrand factor. Sakata *et al*[7] and Figliuzzi *et al*[22] also evaluated the impact of MSCs upon transplanted islets, and found a similar improvement of islet function and vascularization. The beneficial impact of vascularization is in accord with our previous work indicating that hyperbaric oxygen therapy prevented cellular apoptosis of islets[46]. These data indicate that promoting islet vascularization by co-transplanting MSCs acts to limit the duration and severity of islet ischemia, thereby limiting islet cell apoptosis and promoting islet integrity and function.

CONCLUSION

It is likely that MSCs may exert beneficial effects in addition to those previously outlined. For example, Olerud *et al*[19] demonstrated that neural crest stem cells, a kind of MSCs, augment islet cell proliferation and improve islet function. In addition, Melzi *et al*[20] showed that transplanted bone marrow cells stimulated pancreatic β-cell proliferation after streptozotocin-induced pancreatic injury.

In conclusion, MSCs may exert beneficial immunomodulatory and pro-angiogenic effects when co-transplanted with islets. Immunomodulatory effects include the functional inhibition of immunocompetent cells, such as NK cells, DCs, cytotoxic T cells, and B cells. MSCs may also induce the generation of regulatory T cells that promote immunological tolerance. The pro-angiogenic effects of MSCs result from the release of angiogenic factors and promotion of the vascular network linking islets to the surrounding tissue. This pro-angiogenic role limits the duration and severity of islet ischemia and improves islet function. We therefore believe that the co-transplantation of MSCs represents a viable method for improving islet transplantation. Recently, a clinical trial of combined islet and hematopoietic stem cell allotransplantation was performed[47]. The data did not support the effectiveness of HSCs co-transplantation in prevention of graft rejection and in avoiding side effects of immunosuppression. Moreover, Melzi *et al*[20] reported the risk of inducing cancer because of NSC co-transplantation, as well as proving the effectiveness of this strategy to prevent islet graft rejection. Thus, further studies examining the effect of MSCs in a clinical setting should be undertaken.

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ORIGINAL ARTICLE

Helicobacter pylori's virulence and infection persistence define pre-eclampsia complicated by fetal growth retardation

Simona Cardaropoli, Alessandro Rolfo, Annalisa Piazzese, Antonio Ponzetto, Tullia Todros

Simona Cardaropoli, Alessandro Rolfo, Annalisa Piazzese, Tullia Todros, Department of Obstetric and Gynecology, University of Turin, Turin 10126, Italy
Antonio Ponzetto, Department of Internal Medicine, University of Turin, Turin 10126, Italy
Author contributions: Ponzetto A and Todros T designed the research, supervised, edited and proof read the manuscript; Cardaropoli S analyzed the data; Piazzese A recruited patients and collected samples; and Cardaropoli S and Rolfo A performed the research and wrote the paper.
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Correspondence to: Simona Cardaropoli, PhD, Department of Obstetric and Gynecology, University of Turin, Turin 10126, Italy. simona.cardaropoli@unito.it
Telephone: +39-011-3134433 Fax: +39-011-3134450
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Abstract

AIM: To better understand the pathogenic role of *Helicobacter pylori* (*H. pylori*) in pre-eclampsia (PE), and whether it is associated or not with fetal growth retardation (FGR).

METHODS: Maternal blood samples were collected from 62 consecutive pregnant women with a diagnosis of PE and/or FGR, and from 49 women with uneventful pregnancies (controls). Serum samples were evaluated by immunoblot assay for presence of specific antibodies against *H. pylori* antigens [virulence: cytotoxin-associated antigen A (CagA); ureases; heat shock protein B; flagellin A; persistence: vacuolating cytotoxin A (VacA)]. Maternal complete blood count and liver enzymes levels were assessed at delivery by an automated analyzer.

RESULTS: A significantly higher percentage of *H. pylori* seropositive women were found among PE cases (85.7%) compared to controls (42.9%, $P < 0.001$). There were no differences between pregnancies complicated by FGR without maternal hypertension (46.2%) and controls. Importantly, persistent and virulent infections (VacA/CagA seropositive patients, intermediate leukocyte blood count and aspartate aminotransferase levels) were exclusively associated with pre-eclampsia complicated by FGR, while virulent but acute infections (CagA positive/VacA negative patients, highest leukocyte blood count and aspartate aminotransferase levels) specifically correlated with PE without FGR.

CONCLUSION: Our data strongly indicate that persistent and virulent *H. pylori* infections cause or contribute to PE complicated by FGR, but not to PE without feto-placental compromise.

Key words: *Helicobacter pylori*; Virulence factors; Pre-eclampsia; Fetal growth retardation; Cytotoxin-associated antigen A; Vacuolating cytotoxin A

Peer reviewer: Zeinab Nabil Ahmed, Professor of Microbiology, Microbiology and Immunology Department, Faculty of Medicine, Al-Azhar University, Nasr City, 1047 Cairo, Egypt

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INTRODUCTION

Pre-eclampsia (PE) is a severe hypertensive pregnancy-re-

lated disorder that affects 5%-8% of women worldwide, thus representing the main cause of feto-maternal mortality and morbidity[1,2]. PE is often associated with fetal growth retardation (FGR), defined as failure of the fetus to achieve its genetically determined growth potential[3,4]. FGR is commonly considered a severe complication of PE, but whether or not PE and FGR are manifestations of the same disorder, or two distinct pathologies, still remains unclear.

PE is characterized by excessive maternal inflammatory response, with high circulating levels of pro-inflammatory cytokines and endothelial injury[1,2]. Despite being an object of intense investigation, the etiopathogenetic mechanisms of PE are still poorly understood. Several lines of evidence suggest that subclinical infections could play a role in the onset of PE[5,6].

We previously reported a strong association between *Helicobacter pylori* (*H. pylori*) infection and PE[7]. *H. pylori* is a Gram-negative bacterium responsible for the large majority of peptic ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma[8]. It has been demonstrated that this pathogen enhances platelets activation and thrombus formation[9,10], thus inducing endothelial inflammation and injury. Therefore, *H. pylori* could directly cause or intensify the generalized inflammation and endothelial dysfunction typical of PE[7]. Furthermore, it was recently observed that *H. pylori* seropositive PE subjects are characterized by a more severe inflammatory status[11] and lipid peroxidation[12].

The role of cytotoxin-associated antigen A (CagA) in inducing a severe immunogenic response in patients infected by *H. pylori* is now well established[13]. Nevertheless, other virulence factors could be involved in the severe inflammatory response mediated by this bacterium. The vacuolating cytotoxin A (VacA) is a protein produced by *H. pylori* with several effects on vulnerable cells, such as vacuolation with alteration of the endo-lysosomal function and mitochondrial damage accompanied by cytochrome C release and apoptosis[14].

Ureases allow colonization of the gastric mucosa by catalyzing the hydrolysis of urea and help to recruit neutrophils and monocytes in the mucosa, thus inducing pro-inflammatory cytokines production[15].

Heat shock protein B (HspB) has been shown to increase the risk of gastric carcinoma, by directly inducing hyper-proliferation of gastric cells[16]. Moreover, it strongly activates the immune system and stimulates a massive immune response in patients with gastritis and gastric cancer[17-19].

To better understand the pathogenic role of *H. pylori* in pre-eclampsia, we investigated maternal serum positivity for antibodies against CagA, VacA, HspB, ureases A, C, E and H (UreA, UreC, UreE, UreH), and for flagellin A (FlagA). FlagA is the major *H. pylori* flagellin isoform, mainly expressed during late exponential growth phase and represents a good *H. pylori* virulence index[20].

To correlate *H. pylori* virulence with PE severity, and to detect differences in *H. pylori* profiles between PE and FGR pregnancies, we determined seropositivity for the above mentioned antigens in three populations: PE without FGR, PE complicated by FGR, and FGR without PE.

Finally, we verified the reported association between *H. pylori* infection and elevated leukocyte blood count and serum amino-transferases levels[21].

MATERIALS AND METHODS

Population and samples

The study was approved by our Hospital Ethics Committee "Comitato Etico Interaziendale AA.OO O.I.R.M./S.Anna di Torino and Ordine Mauriziano di Torino" and written informed consent was obtained from each participating woman.

Maternal blood samples (5 mL) were collected before delivery from 62 consecutive pregnant women with diagnosis of PE and/or FGR, and from 49 women with normotensive pregnancies with normal fetal growth and normal uterine and umbilical Doppler flow velocimetry (FVW).

PE was diagnosed when hypertension (systolic blood pressure $\geqslant$ 140 mmHg or diastolic blood pressure $\geqslant$ 90 mmHg) and proteinuria ($\geqslant$ 300 mg/24 h) appeared after 20 wk of gestational age in previously normotensive women, according to the American College of Obstetricians and Gynecologists criteria[22]. PE was considered severe when one or more of the following criteria were present: systolic pressure $\geqslant$ 160 mmHg or diastolic pressure $\geqslant$ 110 mmHg on two occasions at least 6 h apart, or significant proteinuria ($\geqslant$ 3 + on urine dipstick or > 5 g in a 24-h urine)[22]. Patients with PE were further classified as either having early-onset ($\geqslant$ 34 wk), or late-onset (> 34 wk) disease according to the gestational age of PE diagnosis.

The hemolysis-elevated liver enzymes-low platelets (HELLP) syndrome was defined by the following criteria: hemolysis (characteristic peripheral blood smear and serum lactate dehydrogenase $\geqslant$ 600 U/L), elevated liver enzymes (serum aspartate aminotransferase $\geqslant$ 70 U/L), and low platelet count (< 100 000/μL)[23].

The diagnosis of FGR was made according to the following criteria: ultrasound measurement of fetal abdominal circumference below the 10th centile[24] or growth velocity below the 10th percentile[25] and/or birth weight below the 10th centile, according to Italian reference values[26] with abnormal umbilical arteries Doppler FVWs[27] and/or abnormal uterine artery Doppler FVWs (resistance index of > 0.58)[28]. Exclusion criteria were: multiple pregnancies, congenital malformations, and prenatal or postnatal diagnosis of chromosomal anomalies in number and/or structure.

For all cases and controls, the following data were collected: maternal age at delivery, gestational age at birth, week of PE onset, mode of delivery, neonatal sex, birth weight, placental weight, parity, blood pressure, urinary protein, complete blood count and differentials (count and percentage of neutrophils, lymphocytes, monocytes, eosinophils, and basophils), liver enzymes levels, risk factors for PE (previous pregnancy with PE, autoim-

Table 1 Clinical characteristics of study populations (continuous variables)

Variable	Controls (n = 49) Median (25th-75th)	PE-only (n = 17) Median (25th-75th)	PE FGR (n = 32) Median (25th-75th)	FGR-only (n = 13) Median (25th-75th)	P value[1]
Maternal age at delivery (yr)	30 (28-33)[4]	29 (24-32)	30 (26-34)[7]	25 (24-26)[4,7]	[4]< 0.001; [7]0.002
Gestational age at delivery (wk)	40 (39-41)[2,3,4]	30 (28-31)[2,5,6]	32 (31-34)[3,5]	34 (32-39)[4,6]	[2,3,4]< 0.001; [5]0.045; [6]0.009
Neonatal weight (g)	3380 (3170-3700)[2,3,4]	1140 (1045-1570)[2]	1278 (920-1668)[3]	1600 (1060-2730)[4]	[2,3,4]< 0.001
Placental weight (g)	600 (500-650)[2,3,4]	300 (240-410)[2]	280 (215-360)[3]	345 (300-470)[4]	[2,3,4]< 0.001
Systolic blood pressure (mmHg)	120 (110-120)[2,3]	160 (150-160)[2,6]	150 (148-160)[3,7]	120 (120-125)[6,7]	[2,3,6,7]< 0.001
Diastolic blood pressure (mmHg)	75 (70-80)[2,3]	100 (100-100)[2,6]	100 (95-105)[3,7]	77 (75-80) [6,7]	[2,3,6,7]< 0.001
Proteinuria (g/24 h)	0 (0-0)[2,3]	2.21 (1.52-3)[2,6]	1.34 (0.79-2.38)[3,7]	0 (0-0)[6,7]	[2,3,6,7]< 0.001

[1]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. [2]Comparison between controls and PE-only group; [3]Comparison between Controls and PE FGR group; [4]Comparison between controls and FGR-only group; [5]Comparison between PE-only and PE FGR groups; [6]Comparison between PE-only and FGR-only groups; [7]Comparison between PE-only and FGR-only groups. PE: Pre-eclampsia; FGR: Fetal growth retardation.

mune diseases, diabetes, cardiovascular diseases, or other common risk factors for PE), and family history of pre-eclampsia and/or cardiovascular diseases.

Venous blood samples were collected into Vacutainer tubes (Becton Dickinson, Plymouth, United Kingdom) without anticoagulant. Serum was separated by centrifugation immediately after clotting and stored at -30 ℃ until assayed.

Serology

Serum samples were evaluated for specific antibodies against *H. pylori* antigens by commercially available Heli-Blot assay (Nurex; Sassari, Italy). *H. pylori* seropositivity was determined according to manufacturer instructions. Briefly, diluted serum samples (1:100) were incubated with Heli-Blot strips for 30 min. The strips were then incubated consecutively with anti-IgG for 30 min, with substrate for range of minutes, and then dried. Results were read according to the standard control protein bands provided. The standard *H. pylori* antigens available in the strip included: 120 kDa (CagA), 89 kDa (VacA), 60 kDa (Urease C), 54 kDa (HSP), 35 kDa (Flagellin), 30 kDa (Urease H), 26 kDa (Urease A), and 19 kDa (Urease E). The presence of one of the three most specific antigens (CagA, VacA, or flagellin) or the presence of two of the three smallest antigens was considered a positive test for the diagnosis of *H. pylori* infection.

Statistical analysis

Data analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, Illinois, United States). Continuous variables were reported as medians and interquartile ranges (25th-75th percentiles). Medians among groups were analyzed by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. Categorical variables are presented as frequencies (percentages) and the comparison between different groups was done with a χ^2 test by means of a 2 × 2 contingency table; Fisher's exact test was used for small sample sizes. All tests were 2-tailed and results were considered significant for a P value less than 0.05. The odds ratios (OR) and 95% confidence intervals (CI), adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, presence of maternal and family risk factors, were calculated using logistic regression analysis to assess the risk of PE and/or FGR associated with *H. pylori* infection.

RESULTS

Population

A total of 111 serum samples from pregnant women were examined: 49 uneventful pregnancies (Ctrl) and 62 pathological pregnancies complicated by fetal growth retardation (FGR-only, n = 13), pre-eclampsia (PE-only, n = 17), or both (PE-FGR, n = 32). Characteristics of the study population are summarized in Tables 1 and 2.

We found that normotensive women with pregnancy complicated by FGR were significantly younger (median of 25 years with an interquartile range of 24-26 years) compared to controls and PE women (both with a median age of 30 years). As expected, pregnancies complicated by PE and/or FGR were delivered more often by caesarean section. Moreover, pathological cases led to lower neonatal and placental weight compared to controls, due to lower gestational age at delivery and reduced fetal growth.

Pre-eclamptic mothers presented higher blood pressure values and urine protein concentrations. The presence of family risk factors was increased in PE cases without FGR (Table 2), while maternal risk factors for PE did not differ among groups (Table 2). The percentage of nulliparous women was significantly higher in the PE group than in controls. In 45 PE mothers, hypertension and proteinuria appeared early (before 34 wk) and in 32 of them these symptoms were severe; moreover five PE pregnancies were complicated by HELLP syndrome.

Leukocyte blood count, platelet count, and serum amino-transferases values in normal and pathologic pregnancies

Pre-eclamptic pregnancies were characterized by significantly higher values of total leukocyte count (P = 0.004) and serum amino-transferases [alanine aminotransferase

Table 2 Clinical characteristics of study populations (categorical variables) *n* (%)

Variable		Controls (*n* = 49)	PE-only (*n* = 17)	PE FGR (*n* = 32)	FGR-only (*n* = 13)	*P* value[5]
Cesarean section delivery		15 (30.6)[6,7,8]	16 (94.1)[6]	29 (90.6)[7]	9 (69.2)[8]	[6,7]< 0.001; [8]0.022
Neonatal sex	Male	19 (38.8)[7]	7 (41.2)	20 (62.5)[7]	6 (46.2)	[7]0.043
	Female	30 (61.2)	10 (58.8)	12 (37.5)	7 (53.8)	NS
Nulliparae		31 (63.3)[6,7]	16 (94.1)[6]	27 (84.4)[7]	10 (76.9)	[6]0.015; [7]0.047
Maternal risk factors		4 (8.2)	2 (11.8)[1]	8 (25.0)	1 (7.7)	NS
	Autoimmune diseases	1 (2.0)	2 (11.8)	4 (12.5)	0 (0)	NS
	Cardiovascular diseases	3 (6.1)	1 (5.9)	4 (12.5)	1 (7.7)	NS
Family risk factors		20[2] (40.8)[6]	12[3] (70.6)[6,10]	13[4] (40.6)	2 (15.4)[10]	[6]0.049; [10]0.004
	Hypertension	9 (18.4)	8 (47.1)	10 (31.3)	2 (15.4)	NS
	Diabetes	10 (20.4)	3 (17.6)	5 (15.6)	0 (0)	NS
	Cardiovascular diseases	5 (10.2)	2 (11.8)	3 (9.4)	0 (0)	NS
Other complications:	FGR	0 (0.0)[7,8]	0 (0.0)[9,10]	32 (100)[7,9]	13 (100)[8,10]	[7,8,9,10]< 0.001
	Early onset PE	-	16 (94.1)	29 (90.6)	-	NS
	Severe PE	-	13 (76.5)	19 (59.4)	-	NS
	HELLP syndrome	-	3 (17.6)	2 (6.3)	-	NS

[1]One patient presented both maternal risk factors (autoimmune and cardiovascular diseases); [2]Four patients presented two family risk factors (3 hypertension and diabetes; 1 diabetes and cardiovascular disease); [3]One patient presented two family risk factors (hypertension and diabetes); [4]Five patients presented two family risk factors (4 hypertension and diabetes; 1 hypertension and cardiovascular disease). [5]*P* values were calculated by chi-square test (χ^2). [6]Comparison between controls and PE-only group; [7]Comparison between controls and PE FGR group; [8]Comparison between controls and FGR-only group; [9]Comparison between PE-only and PE FGR groups; [10]Comparison between PE-only and FGR-only groups. NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation; HELLP: Hemolysis-elevated liver enzymes-low platelets.

(ALT), aspartate aminotransferase (AST) $P = 0.006$ and $P = 0.029$, respectively], while eosinophil count and percentage were significantly lower ($P = 0.028$ and $P = 0.02$, respectively) compared to controls. However, if we exclude pathological cases complicated by HELLP syndrome, only ALT levels remained significantly higher in PE (Table 3). Normotensive pregnancies complicated by FGR showed significantly higher leukocyte levels compared to controls ($P = 0.045$, Table 3). Moreover, the FGR-only lymphocyte percentage was significantly higher relative to PE-only ($P = 0.047$, Table 3).

H. pylori seropositivity was increased in PE-FGR but not in FGR-only pregnancies

H. pylori seropositivity was significantly more frequent in PE women with or without FGR (85.7%) ($P < 0.001$; OR 9.22, 95% CI: 2.83-30.04), while it did not differ between FGR-only (46.2%) and controls (42.9%) (Table 4, Figure 1A). Further subdivision of PE group showed a higher prevalence of seropositive subjects among PE-FGR cases (93.8%) ($P < 0.001$; OR 35.56, 95% CI: 5.22-242.43) compared to controls; while in the PE-only group, the percentage of *H. pylori* seropositive women was higher, but not statistically significant (70.6%), relative to controls (Table 4, Figure 1A).

CagA and VacA seropositivity was increased in pre-eclamptic but not in FGR-only pregnancies

Similar to *H. pylori* seropositivity, the presence of antibodies against CagA antigen was prevalent only in PE pregnant women (81.6%) relative to controls (22.4%) ($P < 0.001$; OR 17.66, 95% CI: 5.25-59.49), while there were no differences between FGR-only cases (38.5%) and controls (Table 4, Figure 1B). CagA seropositivity was significantly more frequent in both PE-FGR (90.6%) ($P < 0.001$; OR 54.97, 95% CI: 9.24-326.88) and PE-only groups (64.7%) ($P = 0.038$; OR 5.20, 95% CI: 1.09-24.69), relative to controls. VacA seropositivity was significantly higher in PE-FGR cases (87.5%) ($P < 0.001$; OR 19.64, 95% CI: 3.75-102.98), while there were no differences between PE-only (55.6%) and FGR-only cases (53.8%), relative to controls (40%) (Table 4, Figure 1C).

Seropositivity for both CagA and VacA antibodies was associated with higher risk of PE-FGR (OR 45.44; 95% CI: 7.79-265.18). In fact, 87.5% of PE-FGR pregnancies were CagA and VacA seropositive, compared to 22.4% in Ctrl group (Table 5, Figure 1D). Patients seropositive for VacA, but not for CagA, were nine controls (18.4%), one FGR (7.7%), and no PE women, while seropositivity for CagA only was a specific feature of the PE-only group (Table 5). Seronegative women for both anti-CagA and VacA antibodies were only 9.4% in the PE-FGR group, while they were 59.2%, 35.3% and 53.8% in the Ctrl, PE-only and FGR-only groups, respectively (Table 5, Figure 1D). Importantly, CagA and VacA seronegativity was associated with a lower risk of developing pre-eclampsia complicated by fetal growth retardation (OR 0.04; 95% CI: 0.01-0.22).

UreC and UreE seropositivity was higher in PE-FGR pregnancies

We found significantly higher UreC and UreE seropositivity in PE-FGR patients (46.9%; $P = 0.018$ and 56.3%; $P = 0.003$, respectively) relative to controls (26.5% and 24.5%, respectively) (Figure 2B and C), while there were no differences among groups for HspB, FlagA, UreA, and UreH (Table 4, Figure 2A and D-F). Odds ratios calculation showed higher risk of developing PE-FGR in

Table 3 Leukocytes, platelets and liver enzymes in normal and pathological pregnancies

Variable	Normal values in Italian female population range	Controls (n = 49) Median (25th-75th)	All PE (n = 49) Median (25th-75th)	PE-only (n = 17) Median (25th-75th)	PE FGR (n = 32) Median (25th-75th)	FGR-only (n = 13) Median (25th-75th)	P value[2]
Total leukocyte count ($1 \times 10^3/\mu L$)	4.00-11.00	10.56 (9.21-11.65)[3,7,5,6]	12.03 (10.69-14.1)[3]	12.34 (10.71-13.83)[5]	11.83 (10.2-14.51)[6]	12.27 (11.21-13.47)[7]	[3]0.004; [7]0.045 [5]0.007; [6]0.024
Neutrophils ($1 \times 10^3/\mu L$)		8.27 (7.45-9.19)	10.09 (7.30-11.6)	10.15 (7.81-12.10)	9.92 (7.30-11.51)	9.34 (6.27-9.48)	NS
(%)	45.0-73.0	75.8 (68.1-78.8)	76.7 (68.37-87.1)	80 (72.5-90.1)	72.1 (68.2-83.6)	64.4 (57.3-77.3)	NS
Lymphocytes ($1 \times 10^3/\mu L$)		2.02 (1.68-2.26)	2.08 (1.3-3.06)	1.86 (1.22-2.34)	2.08 (1.31-3.07)	3.15 (2.13-3.79)	NS
(%)	19.0-47.0	18.7 (14-23.5)	17.4 (10.8-22.43)	14.55 (8.9-18.15)[8]	18.8 (11.1-23.2)	25.9 (17.4-34.3)[8]	[8]0.047
Monocytes ($1 \times 10^3/\mu L$)		0.54 (0.47-0.74)	0.59 (0.3-0.85)	0.61 (0.25-0.81)	0.58 (0.3-0.88)	0.89 (0.54-1.05)	NS
(%)	3.0-9.0	5.4 (4.5-6)	5.3 (3-7.3)	4.15 (2.15-7.75)	5.4 (3.5-7.3)	6.1 (4.9-7.9)	NS
Eosinophils ($1 \times 10^3/\mu L$)		0.15 (0.09-0.19)[3]	0.05 (0.02-0.1)[3]	0.04 (0.02-0.11)	0.05 (0.03-0.1)	0.17 (0.03-0.33)	[3]0.028
(%)	0.2-4.4	1.2 (0.9-1.8)[3]	0.5 (0.2-0.8)[3]	0.35 (0.1-0.8)	0.5 (0.2-0.8)	1.7 (0.2-2.4)	[3]0.020
Basophils ($1 \times 10^3/\mu L$)		0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.03 (0.01-0.03)	0.02 (0.01-0.03)	0.01 (0-0.04)	NS
(%)	0.1-1.3	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.15-0.25)	0.2 (0.1-0.3)	0.1 (0-0.3)	NS
Platelets ($1 \times 10^3/\mu L$)	150-400	219 (170-240)[5]	187 (126-228)	170 (111-214)[5,8]	191 (143-234)	235 (177-285)[8]	[5]0.024; [8]0.031
Platelets[1] ($1 \times 10^3/\mu L$)	150-400	219 (170-240)	191 (161-234)	180.5 (154-228)	191 (165-242)	235 (177-285)	NS
ALT (U/L)	< 34	15 (10-19)[3,5]	23 (14-46)[3]	26 (19-150)[5,8]	19.5 (13.5-35)	14 (10-21.5)[8]	[3]0.006; [5]0.002; [8]0.026
ALT[1] (U/L)	< 34	15 (10-19)[3]	20 (14-31)[3]	25 (15-46)	18 (13-27)	14 (10-21.5)	[3]0.023
AST (U/L)	< 31	17.5 (14-19)[3]	21 (16-39)[3,4]	25 (16-123)[8]	20 (16-35.5)[9]	14 (12-18)[4,8,9]	[3]0.029; [4]0.018; [8]0.031; [9]0.026
AST[1] (U/L)	< 31	17.5 (14-19)	19 (15.5-33)	19 (15-39)	18.5 (16-32)	14 (12-18)	NS

[1]Hemolysis-elevated liver enzymes-low platelets cases excluded; [2]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. [3]Comparison between controls and all PE group; [4]Comparison between all PE and FGR-only groups; [5]Comparison between controls and PE-only group; [6]Comparison between controls and PE FGR group; [7]Comparison between controls and FGR-only group; [8]Comparison between PE-only and FGR-only groups; [9]Comparison between PE-only and FGR-only groups. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation.

Table 4 Seropositivity against *Helicobacter pylori*, cytotoxin-associated antigen A, vacuolating cytotoxin A, ureases A, C, E and H, heat shock protein B and flagellin A n (%)

	Controls (n = 49)	All PE (n = 49)	PE-only (n = 17)	PE FGR (n = 32)	FGR-only (n = 13)	P value[1,2]	Odds ratio[1] (95% CI)
Helicobacter pylori	21 (42.9)[3,5]	42 (85.7)[3]	12 (70.6)	30 (93.8)[5]	6 (46.2)	[3,5]< 0.001	[3]9.22 (2.83-30.04) [5]35.56 (5.22-242.43)
CagA	11 (22.4)[3,4,5]	40 (81.6)[3]	11 (64.7)[4]	29 (90.6)[5]	5 (38.5)	[3,5]< 0.001 [4]0.038	[3]17.66 (5.25-59.49) [4]5.20 (1.09-24.69) [5]54.97 (9.24-326.88)
VacA	20 (40.8)[3,5]	37 (75.5)[3]	9 (52.9)	28 (87.5)[5]	6 (46.2)	[3]0.005 [5]< 0.001	[3]4.89 (1.62-14.73) [5]19.64 (3.75-102.98)
HspB	15 (30.6)	21 (42.9)	5 (29.4)	16 (50.0)	6 (46.2)	NS	
FlagA	13 (26.5)	22 (44.9)	6 (35.3)	16 (50.0)	5 (38.5)	NS	
UreA	10 (20.4)	13 (26.5)	4 (23.5)	9 (28.1)	3 (23.1)	NS	
UreC	13 (26.5)[3,5]	19 (38.8)[3]	4 (23.5)	15 (46.9)[5]	4 (30.8)	[3]0.042 [5]0.018	[3]2.84 (1.04-7.75) [5]4.02 (1.27-12.80)
UreE	12 (24.5)[3,5]	26 (53.1)[3]	8 (47.1)	18 (56.3)[5]	3 (23.1)	[3]0.004 [5]0.003	[3]4.41 (1.59-12.26) [5]6.29 (1.88-21.04)
UreH	8 (16.3)	13 (26.5)	5 (29.4)	8 (25.0)	4 (30.8)	NS	

[1]Adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, and presence of maternal and family risk factors; [2]P values were calculated by χ^2 test; [3]Comparison between controls and all PE group; [4]Comparison between controls and PE-only group; [5]Comparison between controls and PE FGR group. CI: Confidence intervals; NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation; CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; HspB: Heat shock protein B; FlagA: Flagellin A; Ure: Ureases.

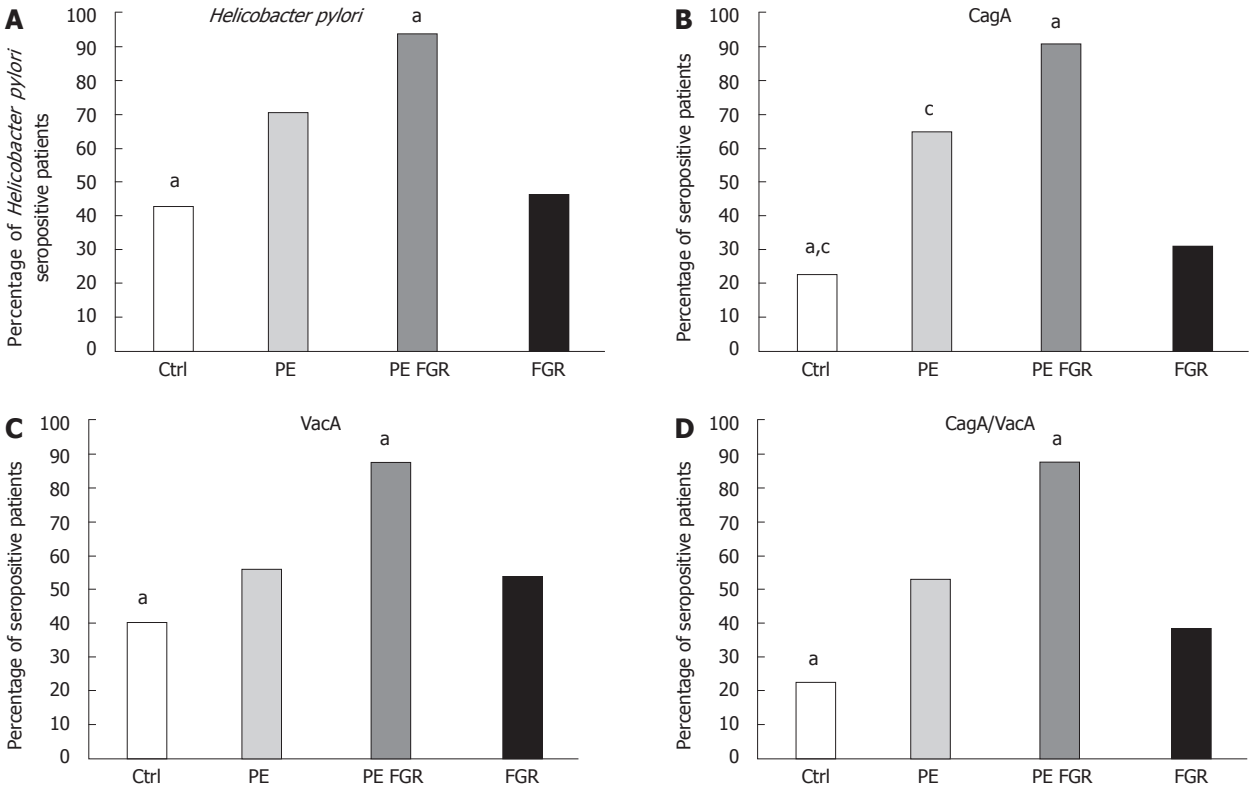

Figure 1 Percentage of *Helicobacter pylori* (A), CagA (B), VacA (C), and CagA/VacA (D) seropositive women in control, PE, PE FGR, and FGR groups. [a]*P* < 0.05 between controls and PE-FGR; [c]*P* < 0.05 between controls and PE without FGR. CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; PE: Pre-eclampsia; FGR: Fetal growth retardation.

Table 5 Cytotoxin-associated antigen A/vacuolating cytotoxin A dual seropositivity *n* (%)

	Controls (*n* = 49)	All PE (*n* = 49)	PE-only (*n* = 17)	PE FGR (*n* = 32)	FGR-only (*n* = 13)	*P* value[1,2]	Odds ratio[1] (95% CI)
CagA+VacA+	11 (22.4)[3,4]	37 (75.5)[3]	9 (52.9)	28 (87.5)[4]	5 (38.5)	[3]< 0.001	12.10 (3.76-38.91)
						[4]0.001	45.44 (7.79-265.18)
CagA-VacA+	9 (18.4)	0 (0)	0 (0)	0 (0)	1 (7.7)	NS	
CagA+VacA-	0 (0.0)	3 (6.1)	2 (11.8)	1 (3.1)	0 (0.0)	NS	
CagA-VacA-	29 (59.2)[3,4]	9 (18.4)[3]	6 (35.3)	3 (9.4)[4]	7 (53.8)	[3]0.001	0.13 (0.04-0.42)
						[4]< 0.001	0.04 (0.01-0.22)

[1]Adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, and presence of maternal and family risk factors; [2]*P* values were calculated by chi-square test (χ^2); [3]Comparison between controls and all PE group; [4]Comparison between controls and PE FGR group. CI: Confidence intervals; NS: Non significant; CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; PE: Pre-eclampsia; FGR: Fetal growth retardation.

patients seropositive for UreC (OR 4.02, 95% CI: 1.27-12.8) and UreE (OR 6.29, 95% CI: 1.88-21.04) (Table 4).

Association among CagA/VacA seropositivity and leukocyte blood count, platelet count, and serum amino-transferases values

Considering seropositivities for CagA and/or VacA antigens, we found that the total leukocyte count was significantly decreased in VacA only seropositive patients relative to seronegative, CagA+/VacA- and CagA+/VacA+ patients ($P = 0.003$; $P = 0.014$ and $P = 0.012$, respectively, Table 6). Moreover, the basophiles percentage, but not total count, was significantly increased in CagA/VacA double seropositive compared to seronegative patients ($P = 0.002$, Table 6). No differences among groups were found for the other investigated parameters (Table 6). Analyzing amino-transferases levels after HELLP cases exclusion, ALT was significantly increased in CagA only seropositive patients relative to the other groups ($P = 0.02$; $P = 0.025$; $P = 0.023$, respectively, Table 6), while no differences were found for AST levels (Table 6).

DISCUSSION

In the present study, we reported a direct association between *H. pylori* virulence and the onset of pre-eclampsia complicated by FGR. Moreover, by investigating seropositivity for *H. pylori* virulence factors, we were able to

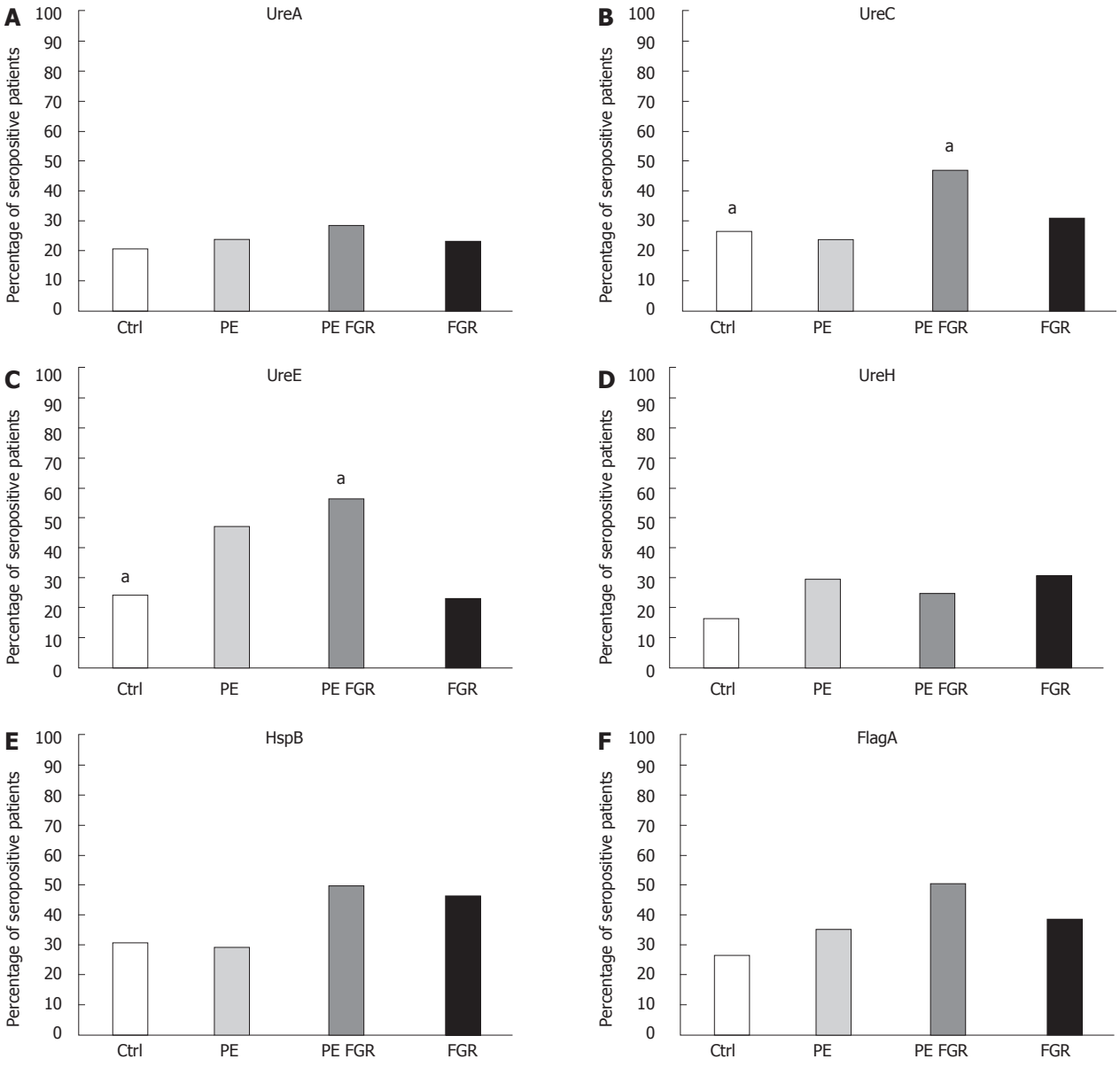

Figure 2 Percentage of Ureases (A-D), HspB (E), and FlagA (F) seropositive women in control, PE, PE FGR, and FGR groups. $^{a}P < 0.05$ *vs* controls. HspB: Heat shock protein B; FlagA: Flagellin A; Ure: Urease; PE: Pre-eclampsia; FGR: Fetal growth retardation.

distinguish pre-eclampsia and FGR without hypertension as different pathologies.

It is accepted that pre-eclamptic pregnancies, complicated or not by FGR, are characterized by severe maternal inflammation[1]. Less is known about "pure" FGR pregnancies, probably because of a biased classification system that considers FGR a secondary disease or a complication of pre-eclampsia. We found elevated maternal leukocytes count, typical sign of inflammation, in all pathological pregnancies relative to controls. However, while leukocytosis in PE patients, as previously reported[29,30], was mainly due to elevated neutrophils levels, a typical marker of bacterial infection[31], in FGR-only mothers, leukocytosis was due to increase in monocytes, eosinophils, and lymphocytes. Moreover, we found significantly higher transaminases levels in the PE group, even after the exclusion of HELLP cases, known to be characterized by elevated hepatic enzymes. The trigger of this exacerbated inflammatory response still remains unknown.

Graham *et al*[21] previously demonstrated a direct association between abnormal total leukocyte count and *H. pylori*-infection in patients with duodenal ulcer disease. They reported a significant fall in total white cell and neutrophils counts in patients successfully treated by *H. pylori* antibiotic therapy[21]. Moreover, they observed higher AST levels in CagA-positive patients, even after antibiotic treatment, thus assuming that AST levels are not directly associated with *H. pylori* infection[21]. Furthermore, we previously reported a correlation between *H. pylori* infec-

Table 6 Hematological values and cytotoxin-associated antigen A/vacuolating cytotoxin A antigens

Variables	Normal values in Italian female population range	CagA-VacA- (n = 45) Median (25th-75th)	CagA-VacA+ (n = 10) Median (25th-75th)	CagA+VacA- (n = 3) Median (25th-75th)	CagA+VacA+ (n = 53) Median (25th-75th)	P value[2]
Total leukocyte count ($1 \times 10^3/\mu L$)	4.00-11.00	12.02 (10.6-13.13)[3]	8.95 (7.75-10.5)[3,5,6]	14.1 (12.4-16.34)[5]	11.27 (9.62-13.64)[6]	[3]0.003; [6]0.012; [5]0.014
Neutrophils ($1 \times 10^3/\mu L$)		10.02 (8.88-11.40)	7.45 (4.87-9.48)	12.75 (11.03-14.48)	8.43 (6.9-10.5)	NS
(%)	45.0-73.0	78.8 (67.05-88.85)	71.9 (68.1-77.3)	83.4 (78.2-88.6)	72.05 (67.4-80)	NS
Lymphocytes ($1 \times 10^3/\mu L$)		1.72 (1.11-2.85)	2.13 (1.68-2.13)	1.61 (0.93-2.28)	2.34 (1.63-3.07)	NS
(%)	19.0-47.0	15.3 (8.9-23.2)	20.6 (17.4-23.5)	10.95 (5.7-16.2)	18.75 (14-23.3)	NS
Monocytes ($1 \times 10^3/\mu L$)		0.57 (0.26-0.89)	0.54 (0.43-0.6)	0.78 (0.68-0.88)	0.6 (0.44-0.84)	NS
(%)	3.0-9.0	4.75 (2.15-6.85)	5.2 (4.9-6)	5.1 (4.8-5.4)	5.81 (4.3-7.4)	NS
Eosinophils ($1 \times 10^3/\mu L$)		0.05 (0.02-0.18)	0.09 (0.03-0.21)	0.06 (0.03-0.08)	0.06 (0.03-0.15)	NS
(%)	0.2-4.4	0.55 (0.1-1.45)	1.2 (0.2-2)	0.4 (0.2-0.6)	0.58 (0.3-1.17)	NS
Basophils ($1 \times 10^3/\mu L$)		0.01 (0-0.02)	0.03 (0.01-0.03)	0.02 (0.02-0.03)	0.03 (0.01-0.04)	NS
(%)	0.1-1.3	0.1 (0-0.2)	0.2 (0.2-0.3)	0.15 (0.1-0.2)	0.2 (0.2-0.3)	0.002
Platelets[1] ($1 \times 10^3/\mu L$)	150-400	222 (169-249)	175 (154-209.5)	214 (210-228)	191 (166-242)	NS
ALT[1] (U/L)	< 34	16 (12.5-26)[4]	11 (9-12)[5]	32 (30-178)[4,5,7]	17 (11.5-24)[7]	[4]0.020; [5]0.025; [7]0.023
AST[1] (U/L)	< 31	18 (15.5-23.5)	13 (12-19)	58 (15-143)	18 (14.5-26)	NS

[1]Hemolysis-elevated liver enzymes-low platelets cases excluded; [2]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test; [3]Comparison between CagA-VacA- and CagA-VacA+ groups; [4]Comparison between CagA-VacA- and CagA+VacA- groups; [5]Comparison between CagA-VacA+ and CagA+VacA- groups; [6]Comparison between CagA-VacA+ and CagA+VacA+ groups; [7]Comparison between CagA+VacA- and CagA+VacA+ groups. CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NS: Non significant.

tion and the onset of pre-eclampsia during pregnancy, suggesting that this Gram-negative bacterium could cause or contribute to the etiopathogenesis of pre-eclampsia[7], by inducing the pro-inflammatory state.

In the present study, we further investigated *H. pylori* and pre-eclampsia association by considering the main markers of *H. pylori* virulence and infection persistence, which are useful for understanding the severity and characteristics of the infection.

H. pylori strains carrying the CagA antigen are known to be among the most virulent and are associated with increased inflammation[13]. VacA is a *H. pylori* toxin crucial to promote and maintain bacterial colonization[14]. Importantly, combined seropositivity for both CagA and VacA directly correlates with elevated morbidity[32-34]. We previously reported a strong association between CagA positive *H. pylori* infection and the onset of PE in Italian women[7]. In the present study, we also found that CagA/VacA dual seropositivity is specifically associated with pre-eclampsia and, in particular, with PE complicated by FGR. In contrast, the absence of both anti-CagA and anti-VacA antibodies is associated with a lower risk of PE. Interestingly, the association with CagA-/VacA+ was found only in controls and normotensive women with FGR pregnancies, while CagA+/VacA- patients belong to the PE groups. Our data suggest that the CagA antigen is associated with a more severe pattern, while VacA alone is not sufficient to cause the severe systemic inflammation typical of PE. The highest leukocyte count and ALT level observed in CagA+/VacA- patients further corroborated this hypothesis, while subjects seropositive only for VacA were characterized by the lowest median leukocyte values (Table 6). CagA/VacA dual seropositivity was the most frequent condition in PE complicated by FGR patients (Table 5), and was associated with intermediate leukocyte and ALT values (Table 6). Therefore, we speculate that CagA, with or without VacA, may contribute to the onset of pre-eclampsia, while VacA seropositivity could attenuate CagA virulence. Our results indicate that severe (CagA positive) and persistent (VacA positive) maternal *H. pylori* infections are strongly associated to pre-eclampsia complicated by feto-placental compromise, as indicated by FGR. Therefore, chronic and severe *H. pylori* infections could contribute not only to the exacerbated maternal inflammatory response leading to pre-eclampsia, but also to the abnormal placentation typical of FGR.

Importantly, FGR without PE does not present significant differences relative to physiological controls for either *H. pylori* (Table 3, Figure 1A) and CagA/VacA dual seropositivity (Figure 1D), suggesting that different etiopathogenetic mechanisms lead to "pure" FGR.

Another key *H. pylori* virulence factor is Urease, an enzyme that modifies environmental pH to allow *H. pylori* colonization[35]. Moreover, it helps to activate pro-inflammatory cytokines production[15]. We determined seropositivity for A, E and H urease subunits and for UreC in pre-eclamptic and/or FGR pregnant women relative to controls. In PE women relative to controls, we found significantly higher seropositivity only for the UreE subunit; the carrier of nickel ions and pivotal for proper enzyme activity[36-38]. The rate of seropositivity for UreC, the enzyme necessary for bacterial cell wall formation[39], was significantly higher in PE pregnancies complicated by FGR, as we previously showed for CagA/VacA dual-seropositivity. These data suggest that UreE and UreC contribute to the onset of both PE and PE-FGR.

Even though pre-eclampsia has been extensively investigated, the only effective therapeutic option remains a timed, programmed delivery. Our data clearly demonstrate a direct correlation between severe and persistent *H. pylori* infection and the onset of PE complicated by FGR, opening up attractive perspectives for the design of new preventive and therapeutic interventions for pre-eclampsia.

Although specific combinations of different antibiotics are effective in eradicating *H. pylori*, antibiotics-resistant strains are already emerging, thus decreasing the efficacy of existing therapies[40]. Pharmacogenomics-based treatments seem to increase the cure rates and new therapeutic approaches targeting *H. pylori* virulence factors are required[40]. In the case of pregnancy-related diseases, it would be preferable to prevent the exacerbated inflammation typical of PE, thus avoiding pharmacologic therapies during pregnancy. Recently, several clinical trials and animal studies have focused on generating *H. pylori* recombinant vaccines[41,42]. They demonstrated the possibility of eliciting an immunological response against *H. pylori* in humans, and to eradicate and protect against the infection in mice[43]. Experimental *H. pylori* vaccines have been created using bacterial urease and designed as oral preparations.

In conclusion, our results define pre-eclampsia complicated by FGR and "pure FGR" as different pathologies. Moreover, we demonstrated a direct role for *H. pylori* CagA/VacA positive strains in the etiopathogenesis of PE-FGR. Our data further emphasize the importance of an accurate classification of the multifactorial and multiform pre-eclamptic disease. It is generally accepted that PE is a syndrome that includes several pathologies with different etiopathogenesis but with similar clinical manifestations. For this reason, PE is usually classified on the basis of symptoms severity (moderate or severe) or of symptoms onset (early- or late-onset PE). We strongly believe that, as demonstrated by the present study, pre-eclampsia should also be classified as placental (with feto-placental involvement) or maternal (without feto-placental compromise)[44], both of which may have early or late onset. This classification will lead to a better management of this devastating pregnancy-related disorder. Further studies are required to identify specific *H. pylori*-related therapeutic targets.

COMMENTS

Background

Pre-eclampsia (PE), a severe hypertensive pregnancy-related syndrome that affects 5%-8% of women worldwide, represents the main cause of feto-maternal mortality and morbidity. Despite being the object of intense investigation, the etiopathogenesis of PE is still poorly understood, and no effective therapeutic interventions are available in clinical practice.

Research frontiers

Several lines of evidence suggest that maternal sub-clinical infections could play a pivotal role in the onset of PE. *Helicobacter pylori* (*H. pylori*) could directly cause or intensify the generalized inflammation and endothelial dysfunction typical of this syndrome.

Innovations and breakthroughs

The data represent a major advance in the understanding of PE etiopathogenesis and add pivotal information for an accurate classification of this multifactorial and multiform syndrome. In fact, the authors clearly demonstrated a direct correlation between severe and persistent *H. pylori* infection and the onset of PE complicated by fetal growth retardation (FGR).

Applications

The findings open up new, attractive perspectives regarding the design of effective preventive and therapeutic interventions for pre-eclampsia associated with *H. pylori* infection.

Terminology

FGR is defined as failure of the fetus to achieve its genetically determined growth potential and is commonly considered a severe complication of PE.

Peer review

The work is a contribution to the understanding of *H. pylori*'s pathogenic role in PE, associated or not with FGR. The association between maternal infection and PE has been evaluated by several researchers and is a good field to study the etiopathogenesis of this critical clinical condition. Authors confirmed that persistent and virulent *H. pylori* infections cause or contribute to PE complicated by FGR.

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BRIEF ARTICLE

Infliximab in pediatric inflammatory bowel disease rapidly decreases fecal calprotectin levels

Anssi Hämäläinen, Taina Sipponen, Kaija-Leena Kolho

Anssi Hämäläinen, Kaija-Leena Kolho, Hospital for Children and Adolescents, Helsinki University Central Hospital, University of Helsinki, Helsinki FIN-00029, Finland
Taina Sipponen, Division of Gastroenterology, Helsinki University Central Hospital, University of Helsinki, Helsinki FIN-00029, Finland
Author contributions: Hämäläinen A performed the research, analyzed the data and contributed to drafting of the article; Sipponen T contributed to study conception and writing of the article; and Kolho KL designed the research, contributed to the interpretation of data and writing of the article.
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Correspondence to: Kaija-Leena Kolho, MD, PhD, Hospital for Children and Adolescents, University of Helsinki, PO Box 281, Helsinki FIN-00029, Finland. kaija-leena.kolho@helsinki.fi
Telephone: +358-9-42774740 Fax: +358-9-47175299
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Abstract

AIM: To study the response to infliximab in pediatric inflammatory bowel disease (IBD), as reflected in fecal calprotectin levels.

METHODS: Thirty-six pediatric patients with IBD [23 Crohn's disease (CD), 13 ulcerative colitis (UC); median age 14 years] were treated with infliximab. Fecal calprotectin was measured at baseline, and 2 and 6 wk after therapy, and compared to blood inflammatory markers. Maintenance medication was unaltered until the third infusion but glucocorticoids were tapered off if the patient was doing well.

RESULTS: At introduction of infliximab, median fecal calprotectin level was 1150 μg/g (range 54-6032 μg/g). By week 2, the fecal calprotectin level had declined to a median 261 μg/g ($P < 0.001$). In 37% of the patients, fecal calprotectin was normal (< 100 μg/g) at 2 wk. By week 6, there was no additional improvement in the fecal calprotectin level (median 345 μg/g). In 22% of the patients, fecal calprotectin levels increased by week 6 to pretreatment levels or above, suggesting no response (or a loss of early response). Thus, in CD, the proportion of non-responsive patients by week 6 seemed lower, because only 9% showed no improvement in their fecal calprotectin level when compared to the respective figure of 46% of the UC patients ($P < 0.05$).

CONCLUSION: When treated with infliximab, fecal calprotectin levels reflecting intestinal inflammation normalized rapidly in one third of pediatric patients suggesting complete mucosal healing.

Key words: Crohn's disease; Ulcerative colitis; Surrogate markers; Pediatrics; Monoclonal antibodies; Infliximab

Peer reviewers: Won Ho Kim, MD, Professor, Department of Internal Medicine, Yonsei Uiversity College of Medicine, 134 Shinchon-dong Seodaemun-ku, Seoul 120-752, South Korea; Dr. Charles B Ferguson, MRCP, Department of Gastroenterology, Belfast City Hospital, 51 Lisburn Road, Belfast BT9 7AB, United Kingdom

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INTRODUCTION

The recent development of easily applicable fecal surrogate markers for intestinal inflammation has provided

new means for objective assessment of disease activity and treatment response in chronic inflammatory bowel disease (IBD), a disease becoming more prevalent among children[1]. This is especially important in pediatric patients with limited possibility for follow-up endoscopy due to invasiveness. The fecal levels of neutrophil-derived markers, such as fecal calprotectin or lactoferrin, reflect the mucosal influx of inflammatory cells in the intestine, thus associating with the presence of active inflammation. In IBD, fecal calprotectin levels relate to the findings in endoscopy but also with the grade of histological inflammation[1-4]. When compared to clinical scores and serum inflammatory markers, fecal calprotectin is the most accurate tool to detect the presence of active mucosal inflammation in the colon[4-6]. The negative predictive value for the presence of active inflammation is high (87%)[4]. In children, it has been shown that the level of fecal calprotectin[2,3,7,8] or lactoferrin[6,9] may guide the need for endoscopy.

The data on fecal markers during therapy of IBD are sparse. We showed recently that during glucocorticoid therapy in pediatric patients, fecal calprotectin levels rarely declined below the limit of a raised value, suggesting ongoing mucosal inflammation. However, in those clinically responding to therapy, fecal calprotectin values fell markedly during the first month of therapy[10]. In children presenting with clinically quiescent IBD, only one third of the patients have fecal calprotectin levels below the upper normal limit, whereas the others have raised values, although not reporting subjective symptoms[11]. In adults, fecal calprotectin values are associated with mucosal healing in Crohn's disease (CD) patients who respond to therapy with tumor necrosis factor (TNF)-α antagonists or other IBD medication[12,13]. In a pilot study by Buderus *et al*[14], the levels of fecal lactoferrin were measured in five children on infliximab therapy, who showed a decline after the first infusion in each case. The pattern of fecal calprotectin levels during introduction of TNF-α antagonist therapy in children has not yet been described.

In pediatric patients, TNF-α antagonists have emerged for therapy of severe IBD that does not respond to conventional treatment[15-17]. Fecal calprotectin provides a non-invasive means to assess the presence of intestinal inflammation, therefore, we conducted a prospective study in pediatric patients treated with TNF-α antagonist infliximab. Our aim was to study the pattern of fecal calprotectin concentrations during the early phase of therapy.

MATERIALS AND METHODS

Study population

We prospectively studied 36 children (median age 14 years, range 5.6-17.6 years; 20 boys 20, 16 girls) diagnosed with IBD according to the Lennard-Jones criteria[18], and consecutively introduced to therapy with infliximab. In two cases, the diagnosis of CD was based on extensive aphthous ulceration visualized by wireless capsule endoscopy. All patients had moderate to severe disease that did not respond to treatment with 5-aminosalicylic acid (5-ASA), immunosuppressants or glucocorticoids. In four cases, infliximab was introduced shortly after a diagnosis of extensive small bowel disease. In three patients, the indication for anti-TNF-α agent was fistulating disease, and in all the others, poor response to maintenance medication or steroid dependency. The study group comprised 23 pediatric patients with CD, and 13 with ulcerative colitis (UC). The background data, disease distribution, and medication of the patients are shown in Table 1. Fourteen patients underwent ileocolonoscopy, seven underwent wireless capsule endoscopy, and seven magnetic resonance imaging enterography within 1 m prior to the introduction of infliximab therapy, confirming active disease.

Table 1 Background data of 36 pediatric patients with inflammatory bowel disease treated with infliximab

Variable	Result
Age median (range)	14 (5.6-17) yr
Sex	20 boys, 16 girls
Diagnosis	
CD	23
Ileitis	8
Ileocolitis	8
Colitis	7
UC	13
Left-sided colitis	4
Pancolitis	9
Maintenance medication at baseline	
5-ASA	10
5-ASA + azathioprine/6-MP	12
Azathioprine/6-MP	7
None	7
Prednisolone/budesonide at baseline	19
Disease duration (median, range)	2.1 (0.4-7.6) yr

CD: Crohn's disease; UC: Ulcerative colitis; 5-ASA: 5-aminosalicylic acid; 6-MP: 6-mercaptopurine.

TNF-α antagonist infliximab (Remicade®) was scheduled at 5 mg/kg at weeks 0, 2 and 6. All infusions were administered at the Hospital for Children and Adolescents, Helsinki University, Finland during February 2008 to December 2010. The maintenance medication was unaltered until week 6, but if the patient improved clinically, glucocorticoids were tapered off. At each visit, the patients provided a stool sample for fecal calprotectin measurement and a blood sample for measurement of inflammatory marker erythrocyte sedimentation rate (ESR), and hemoglobin. Fecal calprotectin was measured in the routine clinical laboratory by a quantitative enzyme immunoassay (PhiCal Test, Calpro AS, Oslo, Norway; NovaTec Immunodiagnostica, Dietzenbach, GmBH, Germany) and the values quoted as normal were < 100 μg/g stool[10,19]. The clinical activity of the disease was assessed by physicians global assessment (PGA) score from 1 to 3[20].

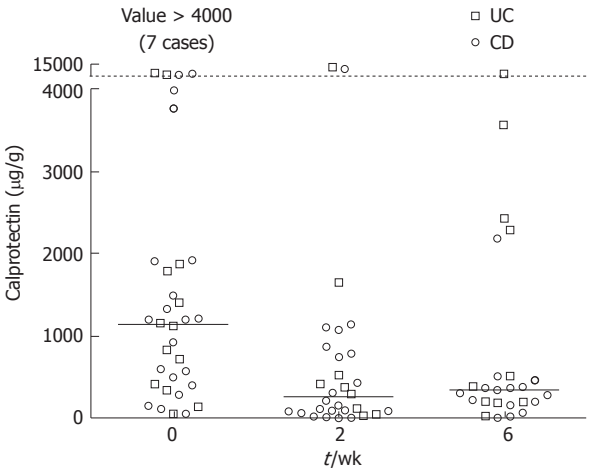

Figure 1 Fecal calprotectin levels at baseline, and 2 and 6 wk after introduction of infliximab therapy in children with Crohn's disease or ulcerative colitis. The decline in levels between baseline and week 2 was statistically significant ($P < 0.001$). CD: Crohn's disease; UC: Ulcerative colitis.

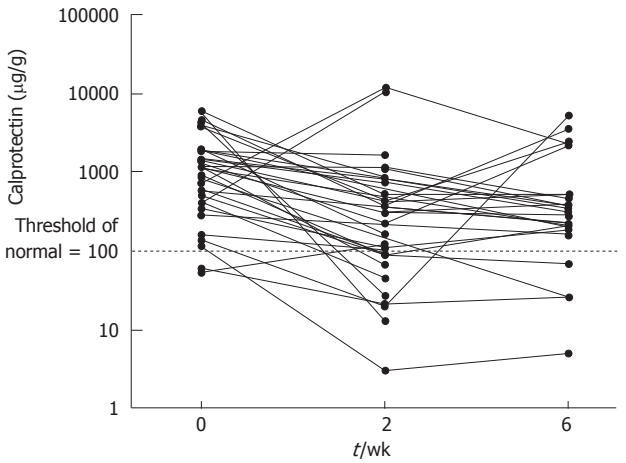

Figure 2 Fecal calprotectin levels at baseline, and 2 and 6 wk after introduction of infliximab therapy showing the individual variation in children with inflammatory bowel disease.

Ethical considerations

The ethics committee of Helsinki University Central Hospital approved the study protocol. The families attending the study signed an informed consent form.

Statistical analysis

Spearman non-parametric correlation test, Kruskal-Wallis test, Mann-Whitney *U* test, and Fisher's exact test were used. The level of statistical significance was $P < 0.05$. The values are presented as median and range.

RESULTS

Fecal calprotectin was high at the introduction of infliximab therapy, with a median value of 1150 μg/g (range: 54-6032 μg/g). Two patients had fecal calprotectin < 100 μg/g (reference limit for a raised value), and their indication for treatment was steroid-dependent colitis. By week 2, the median level of fecal calprotectin level had declined to 261 μg/g (Figure 1; $P < 0.001$, Mann-Whitney *U* test). In 11 of 30 (37%) patients, fecal calprotectin was below the reference limit (100 μg/g) by week 2. By week 6, there was no additional improvement in the median fecal calprotectin level (345 μg/g, range: 5-5253 μg/g, Figure 1). The individual variation of fecal calprotectin levels is shown in Figure 2.

Disease extension or diagnosis did not relate to fecal calprotectin levels or to treatment response (data not shown). Fecal calprotectin decreased in 21 of 22 (95%) of the CD patients, with a raised value during the introduction phase, but in one case, the response was temporary. By week 6, there was no response in two cases when compared to baseline. One patient with CD presented with low fecal calprotectin levels throughout the study period. In this particular case, the indication for treatment was steroid dependency and steroids were tapered off during induction. Thus, the effect of infliximab therapy on fecal calprotectin could not be assessed, and she was not included in the analysis. Of the UC patients with increased fecal calprotectin at baseline, the level decreased in 10 of 12 (83%) patients, but increased to pretreatment levels or above by week 6 in three of 10 (30%) children. Of the two children with no initial decline in fecal calprotectin level, there was no clinical response and the level stayed constantly high (> 1600 μg/g) or increased more than 10-fold within 6 wk. The child with steroid-dependent UC and low fecal calprotectin at the start of this therapy (high disease activity confirmed in colonoscopy 1.5 mo earlier) showed mild elevation in the level (up to 120 μg/g). Thus, by week 6, fecal calprotectin level suggested a response in 20 of 22 (91%) of the CD patients and in seven of 13 (54%) of the UC patients ($P < 0.05$, Fisher's exact two-tailed test), corresponding to a figure of 22% of non-responsive patients in total.

Blood inflammatory marker ESR decreased from a median value of 20 mm/h (range: 2-46 mm/h) at baseline to 9 mm/h (range: 2-34 mm/h, $P < 0.05$, Mann-Whitney *U* test) at 2 wk. At baseline, 19 of 31 patients (61%) had elevated ESR. By week 2, the respective figure was 28% (9/32). Median PGA score was 2 at baseline (range: 1-3), and by week 2 and 6, the score was 1 (range: 1-3, $P < 0.001$); by week 6, the majority of patients (33/36) presented with a score of 1. For hemoglobin levels, there was no significant increase in the median values [118 g/L (range: 95-152 g/L) at baseline and 124 g/L at 2 wk (range: 80-147 g/L)]. Glucocorticoids were tapered off in 10/19 patients during the induction phase.

DISCUSSION

Therapy with TNF-α antagonists has emerged in pediatric patients suffering from moderate to severe CD[21,22], but recently, a therapeutic response has also been reported in severe UC[16,23]. These therapies are effective but at present have high costs and carry a risk for the development of severe adverse effects, which possibly

hampers their clinical use[24,25]. Thus, it is of key importance to target the therapy on those who show a positive response and to discontinue administration of TNF-α antagonists if the patient is a non-responder[25]. In keeping with this, surrogate markers for the presence of intestinal inflammation such as fecal calprotectin[13,26] are promising and non-invasive means for the assessment of disease activity in IBD. There have only been a few studies on fecal calprotectin related to therapeutic response in IBD. Previously, we have assessed the pattern of fecal calprotectin in acute pediatric IBD from the start of glucocorticoid therapy until their discontinuation[10], and in adults during the first 12 wk of TNF-α antagonist therapy[12]. Here, we showed the pattern of fecal calprotectin in pediatric IBD during the induction phase of TNF-α antagonist therapy, demonstrating a rapid decline in fecal calprotectin levels within the first weeks of induction in the majority of pediatric IBD patients, suggesting an early response.

By week 2 after introduction of infliximab, the median level of fecal calprotectin had declined by 77% from baseline. Expectedly, this rapid decrease in fecal calprotectin in children paralleled the pattern seen in adults after introduction of TNF-α antagonist treatment. In a previous study in adults, endoscopy confirmed remission in 30% of CD patients when assessed at 3 mo[12]. In the present study, in one third of the children, the fecal calprotectin levels had declined to normal - suggesting remission - by week 2 after the start of infliximab therapy. Unexpectedly, there were only a few cases that showed normalization of fecal calprotectin by week 6, thus, the 2-wk result equaled the proportion of children with suggested mucosal healing - the target of IBD therapy[27] - and remission during the induction phase. The finding of an excellent therapeutic response in one third of the patients is comparable to our previous findings in children with acute IBD treated with glucocorticoids (showing a normalization of fecal calprotectin in 27% of patients[10]), and in adults treated with TNF-α antagonist therapy[12] (see above). In two thirds of the patients, fecal calprotectin did not normalize, suggesting incomplete mucosal healing during the induction phase of infliximab therapy. It is important to note that the fecal calprotectin level that is considered as a satisfactory therapeutic response remains undecided. Furthermore, the long-term treatment outcome in pediatric patients related to fecal calprotectin levels warrants further studies. In adult patients, mucosal healing predicts a better long-term outcome[28].

Although primary response to TNF-α antagonist therapy is excellent in children, covering 80%-90% of patients with CD, the therapeutic response according to clinical disease activity may deteriorate during the first year of therapy in a considerable proportion of children. It has been estimated that 34%-49% of initial responders need dose escalation or more intense therapy during the first year of infliximab therapy[15,22]. For infliximab therapy in adults, rates of dose intensification ranging from 31% to 36% at 12 mo are comparable to those in pediatric patients[29,30]. Here, the primary response was possibly lost already during the introduction phase in > 10% of the patients, as reflected in the fecal calprotectin levels. In these particular children, PGA did not decrease either. However, clinical activity indices have less correlation to the presence of mucosal inflammation than fecal neutrophil biomarkers, as shown in adult CD patients[31]. Thus, reliance solely on clinical assessment is insufficient, and constantly high fecal calprotectin concentration during therapy warrants endoscopic evaluation also in children. It should be noted that increased fecal calprotectin level does not discriminate between disease relapse and intestinal infection[32].

Blood hemoglobin levels did not significantly alter during the induction phase, but the median ESR decreased during the induction phase by week 2. However, 39% of the patients had normal ESR at baseline, and in these patients, ESR is not applicable for assessment of therapeutic response. In children with IBD, serum C-reactive protein (CRP) is seldom increased, and disappointingly, the measurement of high-sensitivity CRP does not bring additional benefit for the assessment of disease activity[33]. Thus, CRP levels were not measured systematically in the present study. It has also been shown in adult IBD that CRP is a poor marker in mild to moderate disease[34], and performance of fecal calprotectin is significantly better[6].

As in many pediatric studies, one of the major limitations of the present study was the small size of the study group. The majority of the patients had CD but as there were only 13 cases of UC, comparisons related to diagnosis of IBD should be interpreted with caution. Most patients with CD presented with ileocolitis, and the numbers of patients with terminal ileal disease or CD colitis were too small to allow proper comparisons related to therapeutic response to infliximab therapy.

In conclusion, fecal surrogate markers may provide objective and non-invasive means to determine the response to infliximab in individual patients early in treatment. Also, fecal calprotectin is more reliable than clinical activity indices or blood-borne markers of inflammation. It may be possible to identify responding patients by a rapid drop in fecal calprotectin levels, which can be seen already at week 2. By week 6, little improvement is evident and some patients even appear to lose their therapeutic response. Based on our results, fecal calprotectin is a promising marker for evaluating patient response to TNF-α antagonist therapy and may offer a tool for identifying responding patients at an early stage, for more efficient targeting of treatment.

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COMMENTS

Background

In pediatric patients, tumor necrosis factor (TNF)-α antagonists have emerged for therapy of severe inflammatory bowel disease (IBD), in patients who do not respond to conventional treatment. In children, endoscopy is an invasive procedure, which limits its use in follow-up. Fecal calprotectin is a surrogate marker for the presence of intestinal inflammation and thus provides a non-invasive means to assess disease activity in children.

Research frontiers

The recent development of easily applicable fecal surrogate markers for intestinal inflammation has provided new means for objective assessment of disease activity and treatment response in chronic IBD, a disease becoming more prevalent among children. When compared to clinical scores and serum inflammatory markers, fecal calprotectin is the most accurate tool to detect the presence of active mucosal inflammation in the intestine, and it is easily applicable to pediatric clinical practice.

Innovations and breakthroughs

This is believed to be the first pediatric study to follow fecal calprotectin levels during the induction phase of therapy with TNF-α antagonist agent infliximab. The study showed that, in one third of pediatric patients, fecal calprotectin level normalized by week 2. However, in two thirds of the patients, fecal calprotectin levels stayed elevated by week 6, suggesting incomplete mucosal healing.

Applications

Based on the results, fecal calprotectin is a promising marker for objective evaluation of patient response to TNF-α antagonist therapy and may offer a tool for identifying responding patients at an early stage, for more efficient targeting of treatment. The long-term treatment outcome in pediatric patients related to fecal calprotectin levels after induction therapy warrants further study.

Terminology

IBD consists of Crohn's disease, ulcerative colitis and indeterminate colitis, and is a chronic illness that affects the intestines, with a partly TNF-α-driven inflammation that is effectively abated by TNF-α antagonists such as infliximab. Fecal calprotectin is a neutrophil-derived marker of inflammation that is present in the stools, and is a reliable surrogate for endoscopic evaluation of disease activity.

Peer review

The authors prospectively evaluated the therapeutic response in pediatric IBD patients during introduction to infliximab reflected in fecal calprotectin levels. It is a relevant study and the paper is well presented.

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BRIEF ARTICLE

Five methods for detection of *Helicobacter pylori* in the Turkish population

Orhan Cem Aktepe, İhsan Hakkı Çiftçi, Birol Şafak, İhsan Uslan, Fatma Hüsniye Dilek

Orhan Cem Aktepe, İhsan Hakkı Çiftçi, Department of Microbiology, Faculty of Medicine, Afyon Kocatepe University, Ali Çetinkaya Campus, 03200 Afyonkarahisar, Turkey
Birol Şafak, Microbiology Laboratory, Atatürk State Hospital, Gaziosmanpaşa Mah., Soma Cad., 10100 Balıkesir, Turkey
İhsan Uslan, Gastroenterology Department, Kütahya State Hospital, Afyon Cad., 43100 Kütahya, Turkey
Fatma Hüsniye Dilek, Department of Pathology, Faculty of Medicine, Afyon Kocatepe University, Ali Çetinkaya Campus, 03200 Afyonkarahisar, Turkey
Author contributions: Aktepe OC, Çiftçi İH and Dilek FH designed the research; Aktepe OC, Çiftçi İH, Şafak B and Uslan İ performed the research; Aktepe OC contributed to new analytic tools; Çiftçi İH and Dilek FH analyzed the data; and Aktepe OC and Çiftçi İH wrote the paper.
Correspondence to: Orhan Cem Aktepe, Associated Professor, Department of Microbiology, Faculty of Medicine, Afyon Kocatepe University, Ali Çetinkaya Campus, 03200 Afyonkarahisar, Turkey. aktepef@hotmail.com
Telephone: +90-272-2463304 Fax: +90-272-2463300
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Abstract

AIM: To compare culture analysis, *Helicobacter pylori* (*H. pylori*) stool antigen (HpSA) test, polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) for *H. pylori* detection.

METHODS: One hundred and thirty-two consecutive adult dyspeptic patients receiving diagnostic endoscopy at the department of gastroenterology were enrolled in this study. Culture and histological examination were performed on biopsy specimens. PCR and FISH tests were applied to histopathological samples. Stool samples that were simultaneously collected were tested for the *H. pylori* antigen using the HpSA test and bacterial DNA using stool PCR.

RESULTS: *H. pylori* was positively identified by histological examination in 85/132 (64.4%) of the patients, while positive samples were found in 56 (42.4%), 64 (48.5%), 98 (74.2%), 28 (21.2%) and 81 (61.4%) of the patients by culture, HpSA, PCR, stool PCR and FISH methods, respectively. The results of the culture, biopsy PCR, HpSA and FISH tests, with the exception of the stool PCR, were found to correlate with the histological examination as a gold standard.

CONCLUSION: The HpSA test is a rapid, simple, and noninvasive test for monitoring therapy. FISH is an accurate, rapid, cost-effective, and easy-to-use test for *H. pylori* detection.

Key words: *Helicobacter pylori*; Histology; Polymerase chain reaction; *Helicobacter pylori* stool antigen; Fluorescence *in situ* hybridization

Peer reviewer: Dr. Masayoshi Ito, Department of Endoscopy, Yotsuya Medical Cube, Tokyo 102-0084, Japan

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INTRODUCTION

In 1984, Marshall and Warren[1] reported the discovery of a bacterium, which was subsequently named *Helicobacter pylori* (*H. pylori*)[2], whose habitat was the human gastric mucosa. This bacterium has been shown to play a role in gastritis, peptic ulcer disease, and gastric malignancies[3-5]. Colonization of the human gastric mucosa induces chronic gastritis and peptic ulcer disease[3,4]. In addition,

H. pylori plays a role in the etiology of gastric cancer and cancer of the mucosa-associated lymphoid tissue[5].

The accurate detection of *H. pylori* is essential for the management of patients and for the eradication of the bacterium following treatment. Since the discovery of *H. pylori*, several diagnostic methods have become available for determining the presence of *H. pylori* infection. These tests can be assessed by invasive and noninvasive methods[6]. Assessment of *H. pylori* infection is based on noninvasive tests, such as serological methods, C urea breath test, and bacterial DNA sequences or bacterial antigen detection in stool by the *H. pylori* stool antigen (HpSA) test[7]. Under many circumstances, noninvasive testing is preferred. These tests are attractive because of their simplicity and the ability to provide test results within a few minutes after administration, in a physician's office. In contrast, the direct detection and culturing of *H. pylori* for the diagnosis of infection requires gastric biopsy specimens obtained from invasive gastroduodenoscopy[5]. Culture methods require an incubation period of at least 4-7 d. However, it is important to note that *H. pylori* is a fastidious microorganism and is affected by environmental conditions[8,9]. The presence of *H. pylori* or resistance to antimicrobials can be investigated on gastric tissue samples with molecular methods, such as polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH).

The aim of this study was to compare culture analysis, HpSA test, PCR and FISH to histological examination for the detection of *H. pylori*.

MATERIALS AND METHODS

Clinical samples

One hundred and thirty-two consecutive adult dyspeptic patients receiving diagnostic endoscopy at the department of gastroenterology were enrolled in this study. Written informed consent was obtained from all of the patients before endoscopy, and sample collection and approval by the Local Ethical Committee was taken prior to initiation of the project. Patients who underwent partial or complete gastrectomy, those with prior *H. pylori* eradication therapy, or those who were treated with any antibiotics, colloidal bismuth compounds, proton pump inhibitors, or H_2 receptor blockers within the past 4 wk were excluded from the study.

Endoscopy and biopsy sampling

Endoscopy was performed with a PentaxFG-29W (Pentax, Germany) on patients after an overnight fast. Four gastric biopsies (two from the antrum and two from the corpus) were taken from each patient.

Culture

Two gastric biopsy specimens, one from the antrum and one from the corpus, were obtained and placed in Stuart's transport medium. Cooled samples were transported to the laboratory of the Department of Microbiology within 1-2 h after procurement, as previously described[10]. Specimens were inoculated onto brain-heart infusion agar supplemented with sheep blood (10%), vancomycin (10 mg/L), trimethoprim lactate (5 mg/L), cefsoludin (5 mg/L), and amphotericin (5 mg/L). The plates were microaerobically incubated using CampyGen (Oxoid, United Kingdom) at 37 ℃ for up to 7 d. Positive cultures were identified by colony formation and Gram stain morphology as well as positive catalase, oxidase, and urease tests.

Histology

Two gastric biopsy specimens, one from the antrum and one from the corpus, were fixed in 10% formalin in separate containers and were sent to the Pathology Laboratory. Samples were embedded in paraffin wax, cut at 5 μm thickness, and stained with modified giemsa and hematoxylin and eosin. Histological evaluation of the samples for *H. pylori* was performed according to the Modified Sydney system[11]. The pathologist was unaware of the patients' clinical conditions and other test results.

HpSA

Stool samples were tested for *H. pylori* antigen by the monoclonal antigen FemtoLab *H. pylori* Cnx kits (Connex GmbH, Martinsried, Germany) using the manufacturer's protocol. Approximately 0.1 g of stool sample was added to vials that contained 1 mL of sample diluent and then emulsified by vortexing for 15 s. The tip of the vial was snapped off and 50 μL sample and 50 μL conjugate were added to the test well. The strip was rinsed after incubation for exactly (60 ± 5) min at ambient temperature. After washing, 100 μL substrate was added and then incubated for 10 min. Finally, the stop solution was added and the samples were analyzed on a spectrophotometer at a wavelength of 450 nm.

PCR

Gastric biopsies from all of the study subjects were stored at temperatures at or below -70 ℃ until use. Each biopsy was digested with tissue extraction buffer at 55 ℃ for 3 h. Then, 200 μL phenol was added to the tissue lysate to extract genomic DNA. *H. pylori* genomic DNA from stool samples was extracted according to Gramley *et al*[12]. Genomic DNA was subsequently quantified by PCR with 16S rRNA. Amplified fragments were separated on a 1% agarose gel and visualized under ultraviolet light.

Fluorescence in situ hybridization

Formalin-fixed paraffin-embedded gastric biopsies were sectioned and dehydrated. The sections were then air-dried and hybridized using the commercially available test system Seafast® *H. pylori* Combi Kit (Izinta, Hungary) according to the manufacturer's instructions. The oligonucleotide probe Hpy-1, which targets a specific sequence of 16S rRNA from *H. pylori*, was hybridized to the sections. Evaluation was performed with a fluorescent microscope equipped with a filter for green fluores-

Table 1 Statistical analysis according to standard test

Method	Sensitivity	Specificity	PPV	NPV	OR	RR
Culture	0.6118	0.9149	0.9286	0.4342	16.94	2.14
HpSA	0.7222	0.6667	0.8125	0.4545	5.20	1.79
Biopsy PCR	0.8824	0.5106	0.7653	0.2941	7.83	2.60
Stool PCR	0.2118	0.7872	0.6429	0.6442	0.99	1.00
FISH	0.9294	0.9574	0.9753	0.1176	296.25	8.29

PPV: **Positive predictive value; NPV: Negative predictive value; OR:** Odds ratio; **RR: Relative risk; HpSA:** *Helicobacter pylori* stool antigen; **PCR:** Polymerase chain reaction; **FISH: Fluorescence** *in situ* hybridization.

Table 2 Test results compared to standard test

Method	False positive	False negative	*r*	*P* value
Culture	4	33	0.510[1]	< 0.001
HpSA	12	20	0.276[1]	< 0.002
Biopsy PCR	23	10	0.430[1]	< 0.001
Stool PCR	10	67	0.001	> 0.05
FISH	2	6	0.872[1]	< 0.001

[1]Correlation is significant at the 0.01 level. HpSA: *Helicobacter pylori* stool antigen; **PCR: Polymerase chain reaction; FISH: Fluorescence** *in situ* hybridization.

cence (Nikon Eclipse 600, Japan).

Statistical analysis

The χ^2 and Pearson correlation analysis were conducted and the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), *P* value, *r* value, odds ratio (OR) and relative risk (RR) were calculated using standard formulas for data using SPSS v. 10.0 (IBM, United States).

RESULTS

H. pylori was identified by histological examination in 85/132 (64.4%) of the patients, while 47/132 (35.6%) of the patients were classified as *H. pylori* negative. Furthermore, positive results were obtained in 56 (42.4%), 64 (48.5%), 98 (74.2%), 28 (21.2%) and 81 (61.4%) of patients by the culture method, HpSA analysis, PCR, stool PCR and FISH, respectively. Histological examination results were evaluated by the gold standard, and specificity, sensitivity, PPV and NPV were calculated for each test (Table 1). A high number of false-positive results was observed in the biopsy PCR (23/98; 23.4%). However, a higher rate of false-negative results was obtained with the culture method (33/76; 43.4%). The culture method, biopsy PCR, HpSA and FISH tests were found to correlate with the Pearson correlation analysis. Similarly, these tests were statistically comparable to the histological examination based on the *P* value with the χ^2 test. In contrast, the stool PCR test did not correlate or have a significant *P* value. These data are summarized in Table 2.

DISCUSSION

There are currently several different diagnostic tests that exist for detecting *H. pylori* infection. Each test has its own merits and demerits in terms of indication, sensitivity, specificity, cost and time. Several studies have examined the diagnostic performance of invasive and non-invasive methods[6,7,9,12,13]. However, these studies were biased or demonstrated a lack of agreement[13]. One possible reason for the discrepancies in diagnostic performance might be due to the selection of various reference methods. Currently, there is no established method to provide a definitive or standard diagnosis of *H. pylori* infection. The selection of tests or the use of a combination of tests without identifying any one specific test as a reference standard can introduce bias[14].

One limit of histological detection of *H. pylori* in gastric biopsy specimens is interobserver variability in assessment[15,16]. A meta-analysis has reported that histological examination results have an approximate sensitivity of 0.70 and specificity of 0.90[17]. This may be due to the discrepancies in the evaluation of features of *H. pylori* or the observations of the pathologist, because pathology results are based on subjective interpretation of different features and classification. Various studies on the reproducibility of histopathological data have reached a similar conclusion. However, the histological examination of the gastric biopsy specimen is accepted as the gold standard for the diagnosis of *H. pylori*[18]. In this study, histological examination resulted in 64.4% positivity for *H. pylori*, which showed a good correlation with the positive detection rates of other methods, with the exception of stool PCR.

Culturing biopsy specimens cannot be routinely used because it is time consuming and very difficult to maintain strict anaerobic conditions. However, bacterial cultures can surely provide specific results and informative data. Gisbert and Abraria have reported three studies with culture sensitivity of 0.45 and specificity of 0.98 in 2006[17]. Similarly, we found that the culture sensitivity and specificity was 0.61 and 0.91, respectively. In addition, the statistical analysis showed a PPV of 0.93, NPV of 0.43, OR of 16.94, and RR of 2.14 compared to histological examination.

The HpSA test is available and recommended in the Maastricht 2-2000 Consensus Report[19] for the pretreatment diagnosis of *H. pylori* infection and confirmation of a *H. pylori* cure following treatment. In a Japanese study, the HpSA test had a reported sensitivity of 93.9% and specificity of 95.7%, compared to a diagnosis of infection based on histological examination[20]. However, Blanco *et al*[21] have observed that another stool antigen test showed a low sensitivity (75%-79%), in patients with *H. pylori* infection who were tested after erradication therapy. We studied the accuracy of the HpSA test in the Turkish population. The HpSA test had a sensitivity of 0.72, specificity of 0.67, accuracy of 0.77, PPV of 0.81, OR of 5.20 and RR of 1.79. Thus, the HpSA test results had a low but acceptable correlation with the histological examination.

It has been reported that the FISH method is an accurate, inexpensive, rapid test for the detection of *H.*

pylori in paraffin-embedded gastric biopsy samples, with a high sensitivity and specificity[22-24]. In addition, it can be applied to fresh gastric tissue samples and *H. pylori* isolates from culture[25]. In this study, the FISH method had a strong correlation with the histological examination and exhibited a sensitivity of 0.93, specificity of 0.96, accuracy of 0.94, PPV of 0.98, OR of 296.25 and RR of 8.29. Furthermore, the FISH method may be a very useful *H. pylori* diagnostic tool in microbiology in the future.

In gastric tissue, the presence of *H. pylori* and resistance genes can be investigated by PCR. It has a high sensitivity and specificity, and can be used as a follow-up assessment after therapy[26,27]. In this study, biopsy PCR studies had a sensitivity of 0.88, specificity of 0.51, accuracy of 0.75, PPV of 0.77, OR of 7.83 and RR of 2.60. Moreover, we found that the specificity value was particularly low for the biopsy PCR results. However, there was a discrepancy between our study and previous reports in terms of the specificity of *H. pylori* detection[28,29]. Lunet *et al*[28] have reported a difference in *H. pylori* positivity by histology *vs* PCR from different populations, in Mozambique and Portugal of 63.7% *vs* 93.1% and 95.3% *vs* 98.1%, respectively. Two possibilities could explain this conflicting result. First, a low density of *H. pylori* colonization may explain the histological results. Alternatively, the PCR results may be reliable because of the use of a specific primer for the particular population.

The stool PCR results had a very low sensitivity and OR (0.21 and 0.99) and had no significant correlation with the histological examination. Previous studies and our data clearly indicate that there is no clinical value in the determination of *H. pylori* in human feces by PCR because of insufficient sensitivity, specificity, and accuracy[30].

There are a variety of tests available for the diagnosis of *H. pylori* infection. Therefore, it is important that laboratories choose the test or tests that are appropriate for the conditions of the laboratories, patient numbers, costs, and account for the need to prepare their own diagnostic algorithms.

In conclusion, the culture, biopsy PCR, HpSA test, and FISH methods for the detection of *H. pylori* in this study, with the exception of stool PCR, were found to correlate with histological examination as a gold standard. In addition, there was a conflicting result on biopsy PCR data when compared to histological examination. The HpSA test is a rapid, simple, and noninvasive test with acceptable results that can be used for monitoring therapy. The FISH method is an accurate, rapid, cost-effective and easy-to-use test for the detection of *H. pylori*, and also allows for the simultaneous determination of antibiotic resistance in the same gastric tissue. Therefore, histopathological examination as a gold standard and the FISH test may be the preferred methods to use together for the precise detection of *H. pylori*.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) plays a role in gastritis, peptic ulcer disease and also gastric malignancies such as gastric cancer and cancer of the mucosa-associated lymphoid tissue. The accurate detection of *H. pylori* is essential for the management of patients and eradication of the bacteria following treatment.

Research frontiers

Since the discovery of *H. pylori*, several diagnostic methods have been become available for determining the presence of *H. pylori* infection. However, there is no established method to provide a definitive or standard diagnosis of *H. pylori* infection.

Innovations and breakthroughs

The fluorescence *in situ* hybridization (FISH) test is an accurate, rapid, inexpensive and easy-to-use method for the detection of *H. pylori*, and allows determination of antibiotic resistance in the same gastric tissue simultaneously. In this study, FISH correlated well with histological examination. Therefore, histological examination and the FISH test may be preferred together for the precise detection of *H. pylori*.

Applications

This study suggests that, laboratories choose the test or tests that are appropriate for their own conditions, patient numbers and costs, and have to prepare their own diagnostic algorithms.

Terminology

For the detection of *H. pylori*, culture, *H. pylori* stool antigen test, polymerase chain reaction and FISH were used with histological examination.

Peer review

This was an interesting study, although a few problems need to be resolved before publication. The most important point is the reliability of their gold standard. The reasons for the false-positive and false-negative results of each test should be discussed further.

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BRIEF ARTICLE

Spectrum of final pathological diagnosis of gastric adenoma after endoscopic resection

Kwan Woo Nam, Kyu Sang Song, Heon Young Lee, Byung Seok Lee, Jae Kyu Seong, Seok Hyun Kim, Hee Seok Moon, Eaum Seok Lee, Hyun Yong Jeong

Kwan Woo Nam, Gastroenterology Center, Sun Hospital, Daejeon 301-725, South Korea
Kyu Sang Song, Pathologic Department, Chung-Nam National University Hospital, Daejeon 301-721, South Korea
Heon Young Lee, Byung Seok Lee, Jae Kyu Seong, Seok Hyun Kim, Hee Seok Moon, Eaum Seok Lee, Hyun Yong Jeong, Gastroenterology Unit of Internal Medicine Department, Chung-Nam National University Hospital, Daejeon 301-721, South Korea
Author contributions: Nam KW and Jeong HY performed the majority of the research; Song KS provided pathologic advises and, along with Jeong HY, was also involved in editing the manuscript; Moon HS, Lee ES, Lee HY, Lee BS, Seong JK and Kim SH provided the collection of all the human material in addition to collecting the medical record reviews and analysis for this work; Jeong HY designed the study and Nam KW wrote the manuscript.
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Correspondence to: Dr. Hyun Yong Jeong, Gastroenterology Unit of Internal Medicine Department, Chung-Nam National University Hospital, Daejeon 301-721, South Korea. jeonghy@cnuh.co.kr
Telephone: +82-42-2807159 Fax: +82-42-2544553
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Abstract

AIM: To investigate how many discrepancies occur in patients before and after endoscopic treatment of referred adenoma and the reason for these results.

METHODS: We retrospectively reviewed data from 554 cases of 534 patients who were referred from primary care centres for adenoma treatment and treated for endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) at Chungnam National University Hospital, from July 2006 to June 2009. Re-endoscopy was examined in 142 cases and biopsy was performed in 108 cases prior to treatment. Three endoscopists (1, 2 and 3) performed all EMRs or ESDs and three pathologists (1, 2 and 3) diagnosed most of the cases. Transfer notes, medical records and endoscopic pictures of these cases were retrospectively reviewed and analyzed.

RESULTS: Adenocarcinoma was 72 (13.0%) cases in total 554 cases after endoscopic treatment of referred adenoma. When the grade of dysplasia was high (55.0%), biopsy number was more than three (22.7%), size was no smaller than 2.0 cm (23.2%), morphologic type was depressed (35.8%) or yamada type Ⅳ (100%), and color was red (30.9%) or mixed-or-undetermined (25.0%), it had much more malignancy rate than the others ($P < 0.05$). All 18 cases diagnosed as adenocarcinoma in the re-endoscopic forceps biopsy were performed by endoscopist 1. There were different malignancy rates according to the pathologist ($P = 0.027$).

CONCLUSION: High grade dysplasia is the most important factor for predicting malignancy as a final pathologic diagnosis before treating the referred gastric adenoma. This discrepancy can occur mainly through inappropriately selecting a biopsy site where cancer cells do not exist, but it also depends on the pathologist to some extent.

Key words: Discrepancy; Adenoma; High grade dysplasia; Endoscopic mucosal resection; Endoscopic submucosal dissection

Peer reviewer: Barbara Braden, Professor, Department of Gastroenterology, John Radcliffe Hospital, Headley Way, OX3 9DU Oxford, United Kingdom

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INTRODUCTION

Since gastric adenoma can progress to higher grade dysplasia or cancer, as shown in long-term follow up studies, it should be treated by endoscopic resection or surgical resection[1-3]. Endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) have been approved as standard treatments for gastric adenoma[4]. Pathologic results from the mucosectomy specimens taken from endoscopic resection of gastric adenoma can be different from those of an endoscopic forceps biopsy[5,6]. As endoscopy has been examined more commonly and extensively, prevalence of adenoma referred from a primary care center for endoscopic resection has increased. However, there have been no reports on the histologic discrepancy between the endoscopy-based diagnosis of the referred gastric adenoma and the final pathologic diagnosis, and previous studies did not include analysis of other possible factors for discrepancy[5-7]. This study aimed to elucidate and analyze possible factors affecting discrepancy for referred gastric adenoma.

MATERIALS AND METHODS

A total of 1049 patients with gastric adenoma were endoscopically treated by EMR or ESD between July 2006 and June 2009 at Chungnam National University Hospital. Among these, 534 patients were referred from primary care centres, most of them from the Tae-jeon Chungchoeng province in South Korea. Because it was intended for all the referred patients to undergo endoscopic treatment, most patients were treated with EMR or ESD, except for an extreme few who had a tendency toward bleeding, or were untreatable due to size, location, or comorbidity. Endoscopists decided resection methods (EMR or ESD) from clinical information such as age, size, morphology, color, location and pathologic grade, but there were no strict criteria. Transfer notes, medical records, and endoscopic pictures of these cases were retrospectively reviewed and analyzed. One hundred and forty-two cases were examined by re-endoscopy and 108 cases underwent re-biopsy prior to endoscopic resection according to the judgment of the endoscopist. The main reason for pre-evaluations of endoscopic examination and biopsy before the resection was incomplete or confusing referred medical records for determining treatment methods. This is schematically described in Figure 1. Transfer reports were reviewed for information of histologic grade, biopsy number, date of the biopsy, and the name of the referring center. Pathologic reports on 54 patients were written as mild, moderate, or severe grade dysplasia of the adenoma, instead of low or high grade dysplasia. Grading terms of adenoma required unification for statistical analysis. "Mild grade"or "moderate grade" was classified as low grade and "severe grade" or "moderate to severe grade" was classified as high grade. Three endoscopists performed all EMRs or ESDs and three pathologists diagnosed most of the cases. Endoscopic reports and saved pictures of procedures were reviewed for morphologic type, color, size, and location.

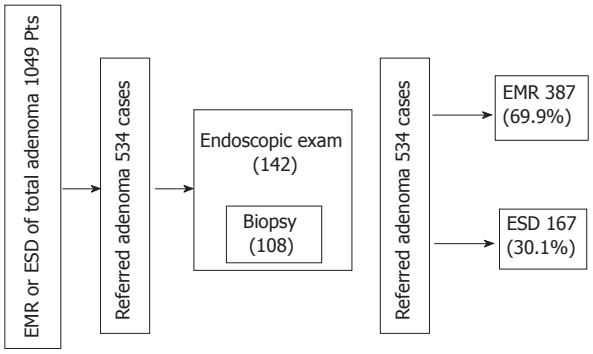

Figure 1 Schematic description of the study design. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; Pts: Patients.

SPSS version 13.0 was used for statistic analysis. The one-way analysis of variance test was used for comparison of continuous variables; for example, age, size, day duration and biopsy number. The χ^2 test was used for other parameters of nominal variables.

RESULTS

Baseline characteristics, endoscopic features and treatment results of referred adenoma

Baseline characteristics and endoscopic features of referred adenomas from primary care centres are shown in Table 1. The mean age of the 554 cases was 66.1 years old. More than 86.4% of cases were located within and under the lower body. Results showed adenomas with no grading record in the transfer note in 92 cases (16.6%), low grade adenomas in 382 cases (69.0%), and high grade adenomas in 80 cases (14.4%). Treatment results of referred adenoma are shown in Table 2. Early gastric cancers were found in 72 cases (13.0%), no adenomatous lesions were found in 56 cases (10.1%), low grade adenomas were found in 356 cases (64.3%), and high grade adenomas were found in 68 cases (12.3%). One case involved mucosa associated lymphatic tissue lymphoma (MALToma) and one complicated case of bleeding were referred. Histologic results of pre-procedure re-endoscopic biopsy were various, from gastritis to adenocarcinoma. In the re-endoscopic biopsy, there were 18 cases (16.7%) of adenocarcinoma and one case of MALToma. The most common complication of EMR and ESD was bleeding (14 cases, 2.5%) which is defined as a case requiring an endoscopic procedure for bleeding control. Perforation (2 cases, 0.4%) and stricture (2 cases, 0.4%) were rare complications of EMR or ESD. There was one case of positive resection margin, in which surgery was

Table 1 The baseline characteristics and endoscopic features of referred adenomas *n* (%)

No. of cases	554
No. of patients	534
Age (yr), mean ± SD	62.1 ± 9.6
Male:female	372:182 (2.04:1)
Histologic grade	
Adenoma (no grading)	92 (16.6)
Low grade	382 (69.0)
High grade	80 (14.4)
No. of referring hospitals	116
No. of Bx, mean ± SD	2.24 ± 1.75
Mean duration between biopsy and procedure	40.7 d
Information of endoscopic photo	449 (81.0)
Size (cm), mean ± SD	1.2 ± 0.8
Morphologic type	
Elevated	275 (49.6)
Flat	206 (37.2)
Depressed	67 (12.1)
Y-Ⅳ	6 (1.1)
Color	
Whitish	332 (59.9)
Reddish	94 (17.0)
Mixed or undetermined	128 (23.1)
Longitudinal location	
Antrum	298 (53.8)
Angle	57 (10.3)
Body	191 (34.4)
High	12 (2.2)
Middle	55 (9.9)
Lower	124 (22.4)
Cardia or fundus	8 (1.5)
Circular location	
Anterior	123 (22.2)
Posterior	119 (21.5)
Lesser curvature	193 (34.8)
Greater curvature	113 (20.4)

Bx: Biopsy; Y-Ⅳ: Yamada type Ⅳ.

Table 2 Treatment results of referred adenomas *n* (%)

Repeat endoscopy	142 (25.6)
Repeat biopsy	108 (19.5)
No. of biopsy, mean ± SD	2.4 ± 1.0
Histologic results of repeat biopsy	
Low grade adenoma	73 (67.6)
High grade adenoma	9 (8.3)
Adenocarcinoma	18 (16.7)
Gastritis	7 (6.5)
Others	1 (0.9) (MALToma)
Endoscopist	
1	462 (83.4)
2	64 (11.6)
3	28 (5.1)
Pathologist	
1	340 (61.4)
2	124 (22.4)
3	83 (15.0)
Others	8 (1.1)
Histologic type; tubulovillous adenoma	10 (1.8)
Type of procedure	
EMR	387 (69.9)
ESD	167 (30.1)
Histologic results of post-procedure	
Low grade adenoma	356 (64.3)
High grade adenoma	68 (12.3)
EGC	72 (13.0)
No adenomatous lesion	56 (10.1)
Others	2 (0.4) (MALToma: 1, transfer by Cx: 1)
Complication	
Bleeding	14 (2.5)
Perforation	2 (0.4)
Stricture	2 (0.4)
Cases with multiple adenoma	
2 adenoma in a patient	12 patients/534 (2.2)
3 adenoma in a patient	4 patients/534 (0.7)

MALToma: Mucosa associated lymphoid tissue lymphoma; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; EGC: Early gastric cancer; Cx: Complication.

performed for completion of treatment. Sixteen patients had multiple adenomas, 12 patients had 2 adenomas and 4 patients had 3 adenomas (Table 2).

Agreement and discrepancy of histologic diagnosis

Comparison of histologic diagnoses between local clinic endoscopic biopsy and repeat endoscopic biopsy and post-procedure specimens are described in Table 3. The rate of discrepancy between primary care center and repeat biopsy was 42.4% (39 cases/92), 38.1% (176 cases/462) between primary care center and post procedure specimens, and 29.6% (32 cases/108) between repeat biopsy and post procedure specimens. The rate of complete agreement was 57.6% (53 cases/108), 61.9% (286 cases/554), and 70.4% (76 cases/108), respectively. In all comparisons, the discrepancy rate of high grade dysplasia was higher than that of other forms of adenomas.

Although the histologic diagnosis of referred adenoma was as low grade dysplasia, it could be high grade (11.0%) or adenocarcinoma (5.8%) in the post procedure. High grade adenoma of the primary care center could also be low grade adenoma (27.5%) or early gastric cancer (55.0%) as a final pathologic diagnosis. Consistent high grade adenoma was only 15.0% between local clinics and post procedure biopsy. All adenocarcinomas of repeat endoscopic biopsies were early gastric cancer in the post procedure, except for one case, which had no adenomatous lesion. When this one case was reviewed with pathologists, the specimen from the repeat endoscopic biopsy was not enough for adenocarcinoma. There were only a few atypical glands, but this could still be suggestive of malignancy (Table 3).

Detection of adenocarcinoma in re-endoscopic repeat biopsy prior to the procedure

Histologic results of re-endoscopic biopsy (108 cases) are shown in (Table 4), according to the endoscopists and pathologists. All of the adenocarcinoma biopsies were performed by endoscopist 1 ($P < 0.001$). Pathologist 1 diagnosed a much larger number of adenocarcinomas than pathologist 2 ($P = 0.048$).

Risk factors for predicting malignancy of referred adenoma

There was no difference between the malignancy group

Table 3 Comparisons of histologic diagnoses *n* (%)

	LG	HG	EGC	NAL	Others	Total	Agreement	Discrepancy
Between local clinic biopsy and repeat biopsy								
Adenoma	11 (68.8)	3 (18.8)	0 (0)	1 (6.3)	1 (6.3)	16 (100)		
LG	51 (83.6)	4 (6.6)	2 (3.3)	4 (6.6)	0 (0)	61 (100)	51 (83.6)	10 (16.4)
HG	11 (35.5)	2 (6.5)	16 (51.6)	2 (6.5)	0 (0)	31 (100)	2 (6.5)	29 (93.5)
Total	73 (67.6)	9 (8.3)	18 (16.7)	7 (6.5)	1 (0.9)	108 (100)	53 (57.6)	39 (42.4)
Between local clinic and post procedure								
Adenoma	60 (65.2)	14 (15.2)	6 (6.5)	11 (12.0)	1 (1.1)	92 (100)		
LG	274 (71.7)	42 (11.0)	22 (5.8)	43 (11.3)	1 (0.3)	382 (100)	274 (71.7)	108 (28.3)
HG	22 (27.5)	12 (15.0)	44 (55.0)	2 (2.5)	0 (0)	80 (100)	12 (15.0)	68 (85.0)
Total	356 (64.3)	68 (12.3)	72 (13.0)	56 (10.1)	2 (0.4)	554 (100)	286 (61.9)	176 (38.1)
Between repeat forcep biopsy and post procedure								
LG	52 (71.2)	1 (15.1)	6 (8.2)	3 (4.1)	1 (1.4)	73 (100)	52 (71.2)	21 (28.8)
HG	3 (33.3)	4 (44.4)	2 (22.2)	0 (0)	0 (0)	9 (100)	4 (44.4)	5 (45.6)
Adenocarcinoma	0 (0)	0 (0)	17 (94.4)	1 (5.6)	0 (0)	18 (100)	17 (94.4)	1 (5.6)
Benign lesion	3 (42.9)	0 (0)	2 (28.6)	2 (28.6)	0 (0)	7 (100)	2 (28.6)	5 (71.4)
Others	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)
Total	58 (53.7)	15 (13.9)	27 (25.0)	6 (5.6)	2 (1.9)	108 (100)	76 (70.4)	32 (29.6)

LG: Low grade; HG: High grade; NAL: No adenomatous lesion; EGC: Early gastric cancer.

Table 4 Results of re-endoscopic forceps biopsy according to endoscopists and pathologists *n* (%)

	LG	HG	Ade	NAL	Others	Total
Endoscopist						
1	34 (54.0)	6 (9.5)	18 (28.6)	4 (6.3)	1 (1.6)	63
2	36 (92.3)	3 (7.7)	0	0	0	39
3	3 (50.0)	0	0	3 (50.0)	0	6
Pathologist						
1	40 (64.5)	4 (6.5)	15 (24.2)	2 (3.2)	1 (1.6)	62
2	20 (66.7)	4 (13.3)	2 (6.7)	4 (13.3)	0	30
3	8 (80.0)	1 (10.0)	1 (10.0)	0	0	10
Others	5 (83.3)	0	0	1 (16.7)	0	6
Deletion of minority						
	Adenocarcinoma		Non-adenocarcinoma			*P* value
Endoscopist						
1	18 (28.6)		45 (71.4)			< 0.001
2	0 (0)		39 (100)			
Pathologist						
1	15 (24.2)		47 (77.8)			0.048
2	2 (6.7)		15 (93.3)			

Ade: Adenocarcinoma; LG: Low grade; HG: High grade; NAL: No adenomatous lesion.

and the non-malignancy group as a final pathologic diagnosis with regard to age, sex, histologic type, duration between local clinic biopsy and procedure, longitudinal and circular location, endoscopist, local clinics, and multiplicity. There was a difference with regard to histologic grade, number of biopsies, size, morphologic type, color, type of procedure, examination of repeat endoscopy, pathologist, and complications (Table 5).

Before the resection, predictive factors for a malignant result were high grade dysplasia (55.0%), a biopsy number of more than three (22.7%), a size of no less than 2.0 cm (23.2%), a morphologic type of depressed (35.8%) or yamada type Ⅳ (100%), and a red (30.9%) or mixed-or-undetermined (25.0%) coloration. There was no statistical significance between less than 1.0 cm and no less than 1.0 cm ($P = 0.124$).

Cases of ESD, repeat endoscopy, or complicated cases had many more malignant results than cases of EMR or direct procedures without re-endoscopy or non-complicated cases. The rate of malignancy was different according to the pathologist ($P = 0.027$). Mean duration from local clinic biopsy to endoscopic treatment did not differ between the malignancy group and the non-malignancy group. There was also no difference between cases (26 cases) with duration of no more than 14 d and cases (33 cases) of duration of more than 90 d. High grade dysplasia showed the highest odds ratio (19.5) with regard to risk factors for malignancy (Table 6).

DISCUSSION

Histologically, gastric adenomas are composed of cells with hyperchromatic, elongated nuclei arranged in a picket-fence pattern with cystic glands and nuclear atypia being occasionally present[8,9]. The malignant potential of adenomas has been demonstrated in long term follow up studies, even in low grade dysplasia, therefore, resection is recommended[3,10]. Since the introduction of EMR in Japan, techniques for endoscopic resection have been continuously advancing; therefore, EMR and ESD are now approved for use in standard treatment of gastric adenoma[4,11,12].

Predictive factors for malignancy

In univariate analysis, risk factors for malignant transformation included location, histologic type (tubulovillous), redness, and high grade dysplasia in the study by Park *et al*[5], and depressed type, high grade dysplasia, redness, ulceration in the study by Jung *et al*[6] in the univariate analysis. In multivariate analysis, only high grade dysplasia had a significant relationship with malignant transformation in the two studies. In our study, predictive factors for

Table 5 Comparison of the non-malignancy group and the malignancy group *n* (%)

	Non-malignancy group	Malignancy group	*P* value
No. of cases	482 (87.0)	72 (13.0)	
No. of patient	462 (86.5)	72 (13.5)	
Age (yr), mean ± SD	61.8 ± 9.8	63.7 ± 9.0	0.132
Sex			
Male	320 (86.0)	52 (14.0)	0.350
Female	162 (89.0)	20 (11.0)	
Histologic grade			
Adenoma	86 (93.5)	6 (6.5)	
Low grade	360 (94.2)	22 (5.8)	< 0.001
High grade	36 (45.0)	44 (55.0)	
Histologic type			
Tubulovillous	7 (70)	3 (30)	0.129
Tubular	475 (83.3)	69 (12.7)	
No. of Bx, mean ± SD	2.1 ± 1.6	2.9 ± 2.2	< 0.001
No. of biopsy			
Undetermined	111 (86.0)	18 (14.0)	
1	47 (92.2)	4 (7.8)	
2	117 (94.4)	7 (5.6)	
3	105 (92.1)	9 (7.9)	
4	47 (72.3)	18 (27.7)	
5	25 (80.6)	6 (19.4)	
6	12 (70.6)	5 (29.4)	
7	1 (33.3)	2 (66.7)	
8	1 (33.3)	2 (66.7)	
3	269 (93.1)	20 (6.9)	< 0.001
4	86 (72.3)	33 (27.7)	
Mean duration between Bx and procedure (d)	40.9	39.3	0.869
Duration between biopsy and procedure			
14 d	24 (92.3)	2 (7.7)	0.658
90 d	29 (87.9)	4 (12.1)	
Size (cm), mean ± SD	1.2 ± 0.8	1.5 ± 1.1	0.003
< 1.0	201 (89.7)	23 (10.3)	
> 1.0, < 2.0	218 (87.9)	30 (12.1)	0.010
> 2.0	63 (76.8)	19 (23.2)	
Morphologic type			
Elevated	252 (91.6)	23 (8.4)	
Flat	187 (90.8)	19 (9.2)	< 0.001
Depressed	43 (64.2)	24 (35.8)	
Y-Ⅳ	0 (0)	6 (100)	
Color			
Whitish	321 (96.7)	11 (3.3)	
Reddish	65 (69.1)	29 (30.9)	< 0.001
Mixed or undetermined	96 (75.0)	32 (25.0)	
Longitudinal location			
Antrum	255 (85.6)	43 (14.4)	
Angle	47 (82.5)	10 (17.5)	0.291
Body	173 (90.6)	18 (9.4)	
Cardia or fundus	7 (87.5)	1 (12.5)	
Circular location			
Anterior	104 (84.6)	19 (15.4)	
Posterior	107 (89.9)	12 (10.1)	0.573
Lesser curvature	171 (88.6)	22 (11.4)	
Greater curvature	95 (84.1)	18 (15.9)	
Type of procedure			
EMR	371 (95.9)	16 (4.1)	< 0.001
ESD	111 (66.5)	56 (33.5)	
Repeat endoscopy			
Yes	110 (77.5)	32 (22.5)	< 0.001
No	372 (90.3)	40 (9.7)	
Endoscopist			
1	397 (85.6)	65 (14.1)	
2	60 (93.8)	4 (6.3)	0.204
3	25 (89.3)	3 (10.7)	
Pathologist			
1	284 (83.5)	56 (16.5)	
2	117 (94.4)	7 (5.6)	0.027
3	74 (89.2)	9 (10.8)	
Others	6 (100)	0 (0)	
Local clinics (> 30 cases)			
1	39 (95.1)	2 (4.9)	
2	37 (90.2)	4 (9.8)	0.224
3	35 (97.2)	1 (2.8)	
4	28 (84.8)	5 (15.2)	
Complication			
Bleeding	9 (64.3)	5 (35.7)	
Perforation	1 (50)	1 (50)	
Stricture	1 (50)	1 (50)	
Total complications	11 (61.1)	7 (38.9)	0.005
No complications	471 (87.9)	65 (12.1)	
Multiplicity			
Patient of single case	449 (86.7)	69 (13.3)	0.464
Patient of multiple cases	13 (81.3)	3 (18.8)	

Bx: Biopsy; Y: Yamada type; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Table 6 Odds ratio of risk factors for malignancy as a final diagnosis

	Odds ratio
High grade dysplasia	19.5
Biopsy number ⩾ 4	5.1
Size ⩾ 2.0 cm	2.4
Depressed or Y-Ⅲ, Y-Ⅳ	7.3
Reddish or undetermined	11.1
ESD	11.7
Repeat endoscopy	2.7
Pathologist 1	2.4
Complications	4.6

ESD: Endoscopic submucosal dissection; Y: Yamada type.

malignancy as a final diagnosis included histologic grade, biopsy number, size, morphologic type and color. High grade dysplasia was the most important risk factor for malignancy, as in previous studies[5,6], with the highest odds ratio (Table 6).

Three out of ten cases (30%) of tubulovillous adenoma were malignancies, compared to only 69/475 cases (12.7%) of tubular adenoma, although this was not statistically significant. Cases with more than three biopsies were more often malignant than cases with fewer biopsies. This might be explained by the assumption that the endoscopist has taken more biopsies when he suspected a malignancy. ESD, re-endoscopy, and complicated groups had more many malignancies than EMR, direct procedure without re-endoscopy, and non-complicated groups, but those are not the cause of malignancy, but the result of strict treatment. Other possible factors affecting malignancy will be discussed below.

Possible causes affecting malignant discrepancy: (1) geographic variety of histology; forceps biopsy can be done only on the adenoma site, when cancer cells are mixed in the same lesion; (2) chronological difference between the time of forceps biopsy and the time of

resection; adenoma can be transformed to malignancy; (3) different criteria of pathologist with regard to malignancy; and (4) different location between forceps biopsy and resection.

Geographic variety of histology

Because relatively small forceps biopsy foci of the polyp cannot represent the entire lesion, there can be a discrepancy between the forceps biopsy and resection specimen of the polyp[13]. Discrepancies before and after endoscopic resection in adenoma are mainly due to the geographic distribution of malignant cells within the adenoma[5,6,14], which means that the discrepancy depends on the location of the initial endoscopic forceps biopsy. It is noteworthy that all of the adenocarcinomas (18 cases) in the re-endoscopic forceps biopsy before the procedure were performed by endoscopist 1, although there was no difference in the discrepancy rate between primary care center and post-treatment according to the endoscopist (Table 4). This may be due to the experience of the endoscopist. An expert endoscopist who can reduce the rate of discrepancy has the ability to determine the location of the cancer cells grossly, approximately to the real histology. A similar two studies on malignant transformation of adenoma presented different discrepancy rates in spite of similar study designs[5,6]. The rate of malignant transformation of adenoma was 6.8% (8/118) in the study by Park *et al*[5] and 55.3% (63/114) in the study by Jung *et al*[6]. These large differences can be understood in the same context.

Chronological difference between the time of forceps biopsy and the time of resection

Gastric adenoma can progress to early gastric cancer, as shown in long term follow-up studies[3,10,15]; even low grade dysplasia has malignant potential. This change can occur over a long period of time. Yamada *et al*[3] reported on one case of 37 low grade dysplasia and one case of 10 high grade dysplasia that progressed to invasive carcinoma over a period of 212 mo and 55 mo, respectively. In our study, duration from the time of initial biopsy to the time of resection was not different between the malignant group and the nonmalignant group. Statistically, the rate of malignancy was also not different between fewer than two weeks (7.7%, 2 cases/33) and fewer than 90 d (12.1%, 4 cases/26) in duration. This means that a treatment delay of roughly three months is not a problem.

Different criteria of pathologist with regard to malignancy

Because criteria between Japanese and Western pathologists are different, international workshops have been steadily and persistently organized in an effort to establish a consensus[16-18]. In 1996, eight pathologists from Japan and Western countries met in Tokyo and individually reviewed a set of 35 gastric biopsy and resection specimens of lesions with potential early neoplasias[16]. There was agreement between Japanese and Western viewpoints in only 11 of the 35 specimens. A different diagnosis can be made for the same specimen, even by an intraobserver in the time interval of three years[19]. Table 5 shows that the malignant discrepancy rate of pathologist 1 is approximately three times greater than that of pathologist 2. The rate of adenocarcinoma diagnosis for re-endoscopic forceps biopsy is also higher for pathologist 1 than pathologist 2 (Table 4). Although the forceps biopsy specimen was not reviewed, it can be assumed that forceps biopsy by the primary care center can be underestimated by the pathologist. However, no difference in the malignant discrepancy rate was observed between primary care centers (Table 5). It is a limitation of our study that the same specimens were not reviewed by pathologists.

Different location between forceps biopsy and resection

Logically, it is possible that either the patient or the sample changed, or that a different mucosectomy site was selected from the diagnostic biopsy site; however, this was not included in the discussion.

COMMENTS

Background

Endoscopic examination is performed more commonly in the primary care center, and gastric adenoma is more frequently referred to tertiary care units.

Research frontiers

There have been so many embarrassing events when previous and post-procedure diagnoses have been different. This research is performed to predict the treatment result and to discover the reasons for these events.

Innovations and breakthroughs

There have been no reports about the discrepancy of referred gastric adenoma and diverse predictive factors, and possible causes are included in this study.

Applications

The results of this study will help endoscopists to predict the results of treatment and to decide the proper treatment option.

Peer review

The authors studied various predictive factors for discrepancy of gastric adenoma and deeply analyzed possible causes of discrepancy.

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BRIEF ARTICLE

Vitamin D supplementation improves sustained virologic response in chronic hepatitis C (genotype 1)-naïve patients

Saif Abu-Mouch, Zvi Fireman, Jacob Jarchovsky, Abdel-Rauf Zeina, Nimer Assy

Saif Abu-Mouch, Liver Unit, Department of Internal Medicine B, Hillel Yaffe Medical Center, Hadera 38100, Israel
Zvi Fireman, Jacob Jarchovsky, Department of Gastroenterology, Hillel Yaffe Medical Center, Hadera 38100, Israel
Abdel-Rauf Zeina, Liver Unit, Hillel Yaffe Medical Center, Hadera 38100, Israel
Nimer Assy, Liver Unit, Ziv Medical Center, Technion Institute, Safed 13100, Israel
Author contributions: Abu-Mouch S and Assy N wrote the paper and contributed equally to this work; Fireman Z and Jarchovsky J participated in study design; Zeina AR participated in the discussion.
Correspondence to: Saif Abu-Mouch, MD, Liver Unit, Department of Internal Medicine B, Hillel Yaffe Medical Center, POB 169, Hadera 38100, Israel. saif@hy.health.gov.il
Telephone: +972-4-3044110 Fax: +972-4-6304408
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Abstract

AIM: To determine whether adding vitamin D, a potent immunomodulator, improves the hepatitis C virus (HCV) response to antiviral therapy.

METHODS: Seventy-two consecutive patients with chronic HCV genotype 1 were randomized into two groups: the treatment group (n = 36, 50% male, mean age 47 ± 11 years) received Peg-α-2b interferon (1.5 μg/kg per week) plus ribavirin (1000-1200 mg/d) together with vitamin D3 (2000 IU/d, target serum level > 32 ng/mL), and the control group (n = 36, 60% male, mean age 49 ± 7 years) received identical therapy without vitamin D. HCV-RNA was assessed by real-time polymerase chain reaction (sensitivity, 10 IU/mL). The sustained virologic response (SVR) was defined as undetectable HCV-RNA at 24 wk post-treatment.

RESULTS: Clinical characteristics were similar in both groups. The treatment group had a higher mean body mass index (27 ± 4 kg/m^2 *vs* 24 ± 3 kg/m^2, P < 0.01), viral load (50% *vs* 42%, P < 0.01), and fibrosis score (> F2: 42% *vs* 19%, P < 0.001) than the controls. At week 4, 16 (44%) treated patients and 6 (17%) controls were HCV-RNA negative (P < 0.001). At week 12, 34 (94%) treated patients and 17 (48%) controls were HCV-RNA negative (P < 0.001). At 24 wk post-treatment (SVR), 31 (86%) treated patients and 15 (42%) controls were HCV-RNA negative (P < 0.001). Viral load, advanced fibrosis and vitamin D supplementation were strongly and independently associated with SVR (multivariate analysis). Adverse events were mild and typical of Peg-α-2b/ribavirin.

CONCLUSION: Adding vitamin D to conventional Peg-α-2b/ribavirin therapy for treatment-naïve patients with chronic HCV genotype 1 infection significantly improves the viral response.

Key words: Hepatitis C; Vitamin D; Sustained viral response; Genotype 1; Fibrosis

Peer reviewer: Sabine Mihm, Professor, Department of Gastroenterology, Georg-August-University, Robert-Koch-Str 40, Göttingen D-37099, Germany

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INTRODUCTION

The current treatment for hepatitis C virus (HCV) infection is pegylated interferon α combined with ribavirin

(Peg/RBV) administered for 24 wk for HCV genotypes 2 or 3, or 48 wk for HCV genotype 1, the most prevalent genotype in Israel, Europe, and North America[1]. The aim of HCV therapy is a sustained virologic response (SVR), defined as an undetectable serum HCV-RNA level at 24 wk after the cessation of therapy. For patients with HCV genotype 1, the rate of SVR ranges between 38% and 46%[2,3]. In subgroups of this population (e.g., Hispanics and African-Americans), the rate of SVR is even lower, reaching only 19%[4]. These differences are not explained by baseline viral load or compliance to treatment. Recent efforts to improve patient outcomes have focused on adding new antiviral therapies specifically targeted to HCV, including inhibitors of either HCV polymerase or protease[5]. However, few studies have addressed the issue of improving the host factors.

Vitamin D is a potent immunomodulator[6,7]. Increased production of 1, 25-dihydroxy vitamin D3 results in the synthesis of cathelicidin, a peptide capable of destroying many viral infectious agents as well as *M. tuberculosis*. Low serum levels of 25-hydroxyvitamin D (< 20 ng/mL) prevent macrophages from initiating this innate immune response, which may explain why African-Americans, who are often vitamin D deficient, are more prone to contracting tuberculosis and viral infections than Caucasians[8]. Moreover, vitamin D improves insulin sensitivity[9], suppresses proinflammatory cytokines, increases anti-inflammatory cytokines, and improves CD4 T cell hyperresponsiveness[10]. Vitamin D deficiency is very common (92%) among patients with chronic liver disease, and at least one-third suffer from severe vitamin D deficiency (< 12 ng/mL)[11]. Israeli subjects from various ethnic backgrounds are at higher risk of vitamin D deficiency[12]. Pettas and co-workers recently showed a low serum vitamin D level to be related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 chronic hepatitis C (CHC)[13]. Its role and relationship to SVR and therapy in CHC are unknown. We reasoned that adding vitamin D to conventional therapy could improve treatment efficacy at weeks 4 [rapid viral response (RVR)] and 12 [early viral response (EVR)] during therapy, and 24 wk after cessation of therapy (SVR).

MATERIALS AND METHODS

Subjects

Study inclusion criteria were age 18-65 years, a chronic HCV genotype 1 infection, no previous treatment for hepatitis C, seronegative for HBV, HDV, and human immunodeficiency virus infections, an absolute neutrophil count of > 1500 per mm^3, a platelet count of > 90 000 per mm^3, and a normal hemoglobin level. Liver biopsies were required within 2 years prior to study entry, and the samples were examined by two pathologists who were unaware of patient identity and treatment regimen. The severity of hepatic inflammation and fibrosis was evaluated by the Ishak score in separate reports for grading and staging[14]. Exclusion criteria were decompensated liver disease (cirrhosis with a Child-Pugh score > 9), another cause of clinically significant liver disease, or the presence of hepatocellular carcinoma.

Study design

This was an intention-to-treat prospective randomized study. The experimental procedures were approved by the institutional review boards of the two participating medical centers. Informed consent was obtained from all participants (Clinical Trial Gov: NCT00804752)

The study included 72 consecutive CHC genotype 1 treatment-naïve patients who were stratified according to ethnic group (i.e., Russian/Jewish/Arab) due to possible differences in vitamin D levels. They were randomly assigned to one of two study groups. The treatment group comprising 36 patients (mean age 47 ± 11 years, 50% male) who received pegylated (peg)-interferon-α-2b (1.5 μg per kg body weight) plus oral ribavirin 1000 mg/d (for body weight < 75 kg) or 1200 mg/d (for body weight > 75 kg) and vitamin D3 (Vitamidyne D, Fischer Pharmaceuticals, Israel) 2000 IU/d, target serum level > 32 ng/mL) for 48 wk. Vitamin D3 was given by oral drops for 4 wk before the initiation of antiviral treatment and after serum levels had reached > 32 ng/mL in all patients in the treatment group. The supplemented vitamin D levels were maintained during the course of therapy with the same dosage as in the lead-in phase. The control group of 36 patients (mean age 49 ± 7 years, 60% male) received peg-interferon-α-2b (1.5 μg/kg body weight) plus ribavirin (1000-1200 mg/d) without vitamin D3 for 48 wk.

Efficacy assessments

Plasma HCV-RNA levels were measured using the COBAS Taq Man HCV assay, version 1.0 (Roche Molecular Systems), with a lower limit of quantification of 35-45 IU/mL and a lower limit of detection of 10 IU/mL. HCV-RNA levels were measured at the time of screening and during the treatment period at weeks 4, 12 and 48. All subjects had at least one follow-up visit at 24 wk after the completion of treatment. Those who had undetectable HCV-RNA levels had another follow-up visit 24 wk later, at which time HCV-RNA levels were measured again. Treatment efficacy was defined as SVR, i.e., undetectable HCV-RNA at 24 wk post-treatment. Clearance of HCV-RNA by real-time polymerase chain reaction (RT-PCR) was assessed at week 4 (RVR), week 12 (complete EVR), and at week 48 of treatment response (early treatment response, ETR). Patients with ETR who tested HCV-RNA positive during follow-up were classified as relapsers. Breakthrough was defined as an increase in the HCV-RNA level of one $\log_{10}$ unit compared with the lowest value. Therapy was discontinued if quantitative HCV-RNA levels at week 12 dropped by < 2 log compared with baseline values (non-responders), and at week 24 if HCV-RNA was still detectable in those patients in whom HCV-RNA dropped > 2 log at week 12[3,15].

Table 1 Baseline demographic, clinical and virologic characteristics of all patients

Baseline demographics	Peg/RBV (n = 36)	Peg/RBV + Vit D (n = 36)	P value
Age (yr)	49 ± 7	47 ± 11	0.123
Males (%)	60	50	0.015
Body mass index (kg/m^2)	24 ± 3	27 ± 4	0.014
HCV genotype: 1a/1b (n)	3/33	3/33	0.138
Baseline HCV-RNA (log IU/mL)	6.2 ± 0.8	6.1 ± 0.7	0.126
High viral load HCV-RNA > 800 000 IU/mL	15 (42%)	18 (50%)	0.033
Baseline ALT (U/L)	56 ± 31	55 ± 28	0.587
Advanced fibrosis (> F2)	7 (19%)	15 (42%)	0.001
Ethnicity (Russian /Jewish/Arab)	28/6/2	29/3/4	0.194

Peg/RBV: Pegylated interferon α and ribavirin; Vit D: Vitamin D; ALT: Alanine aminotransferase; HCV: Hepatitis C virus.

Safety assessments

Biochemical assessments were performed at each visit during the treatment period and at the post-treatment follow-up visit. Data on adverse events were collected and physical examinations were also performed each time. The safety assessment included complete blood count, antinuclear antibody, and thyroid-stimulating hormone levels. Peg-interferon α 2b was reduced to 1.0 μg/kg body weight in patients with a < 750 neutrophil count and withdrawn temporarily in patients with a < 500 neutrophil count. The same dose reduction was applied if platelet levels fell under 50 000 cells/mm^3, with peg-interferon being discontinued when the 25 000 cell/mm^3 threshold was reached. In both treatment arms, the ribavirin dose was tapered by 200 mg/d in patients with a hemoglobin level < 10 g/dL, and discontinued altogether in patients with a level < 8.0 g/dL.

Clinical and laboratory measurements

Vitamin D levels: 25 (OH)-vitamin D3 levels were determined by 125 I-radioimmunoassay (Dia-Sorin, Stillwater, MN, United States)[16]. 25-OH vitamin D is the major circulating form of vitamin D and is used as an indicator of vitamin D status. Vitamin D deficiency was defined as a 25 (OH)-vitamin D serum level < 12 ng/mL, vitamin D insufficiency as 25 (OH)-vitamin D levels of 12-32 ng/mL, and vitamin D sufficiency as levels > 32 ng/mL[12]. Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR)[17]. HOMA-IR was measured at baseline and at 4 wk in both study groups. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Obesity was defined as a BMI exceeding 28 kg/m^2. C-reactive protein was determined by the nephelometric method[18]. Paraoxonase activity was measured according to a method using phenylacetate as a substrate[19]. α tocopherol (vitamin E) was estimated spectrophotometrically[20]. Malondialdehyde concentration was estimated spectrophotometrically using the thiobarbituric acid assay[21]. Calcium, phosphor, vitamin B12, thyroid-stimulating hormone, glucose, insulin, liver enzymes, albumin, bilirubin, prothrombin time, and creatinine were measured by standard biochemical tests.

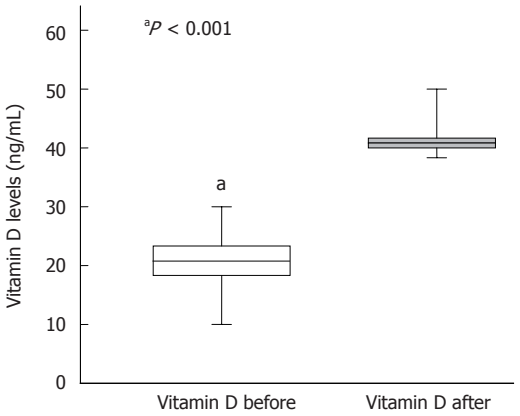

Figure 1 Vitamin D serum levels before and at 4 wk after the beginning of antiviral treatment + vitamin D supplementation (n = 36). Bars represent standard error.

Statistical analysis

Results were expressed as mean ± SD. The difference between the two groups was assessed by the chi-squared test for categorical variables and by the Mann-Whitney rank test for continuous variables. The Spearman correlation was used to express correlations between variables. The primary study endpoint was evidence of the influence of vitamin D on the viral response at weeks 4 and 12 during therapy and at week 24 post-treatment. Logistic regression analysis was performed to detect independent predictors for SVR. The significance level was set at $P < 0.05$. The statistical analyses were carried out with the WINSTAT (Kalmia, CA, United States) software program.

RESULTS

At baseline, 21% of the patients in the treatment group had severe vitamin D deficiency (< 12 ng/mL), 59% had insufficiency, and 20% had sufficient vitamin D levels. The control group baseline tests showed that 27% had vitamin D deficiency, 60% had insufficiency, and 13% had sufficient vitamin D levels. Table 1 shows the clinical and biochemical parameters of the patient populations.

The treatment group had higher BMI levels, viral loads, and fibrosis scores > F2 than the controls (27 ± 4 kg/m^2 *vs* 24 ± 3 kg/m^2, $P = 0.014$; > 800 000 IU/mL, 50% *vs* 42%, $P = 0.033$; 42% *vs* 19%, $P = 0.001$, respectively). There were no differences between the two groups in terms of age, HCV genotype, baseline HCV-RNA, ethnic background, or aminotransferases levels.

Figure 1 depicts the baseline and week 4 vitamin D levels at the beginning of antiviral therapy. Serum vitamin D levels were significantly lower at baseline (20.5 ± 9.0 ng/mL) and increased after 4 wk of vitamin D treatment to a mean level of 37 ± 10 ng/mL. Baseline

Table 2 Viral response, vitamin D levels, biomarkers of inflammation, insulin resistance, and oxidative stress in all patients

Parameter	Peg/RBV (n = 36)	Peg/RBV + Vit D (n = 36)	P value
Viral response			
Relapser	13 (36%)	3 (8%)	0.001
Non-responder	8 (22%)	2 (6%)	0.010
HOMA-IR			
Baseline	4.6 ± 5.7	4.5 ± 1.4	0.123
After 4 wk	5.0 ± 4.0	2.3 ± 1.0[a]	0.001
Basal vitamin	19 ± 6	20.5 ± 9.0	0.177
D-25-OH levels (ng/mL)			
Malondialdehyde (mmol/L)	0.11 ± 0.05	0.13 ± 0.04	0.810
Paraoxonase (mmol/L/min)	0.57 ± 0.1	0.64 ± 0.1	0.120
Vitamin E (μg/mL)	19.7 ± 8.8	21 ± 8.0	0.510
Vitamin B12 pmol/L	316 ± 190	331 ± 170	0.103
CRP mg/dL	0.39 ± 0.3	0.45 ± 0.4	0.100
Triglycerides (mg/dL)	200 ± 80	220 ± 60	0.110

[a]P < 0.001 between HOMA-IR at baseline and HOMA-IR after 4 wk of treatment with vitamin D. HOMA-IR: Homeostasis model assessment of insulin resistance; Peg/RBV: Pegylated interferon α and ribavirin; Vit D: Vitamin D; CRP: C-reactive protein.

vitamin D levels were also lower in the control group (19 ± 6 ng/mL, Table 2).

Figures 2 and 3 show the rates of RVR, EVR, and SVR in the treatment and control groups. At week 4, 16 (44%) patients in the treatment group and 6 (17%) controls were HCV-RNA negative, and at week 12, 34 (94%) and 17 (48%), respectively, were HCV-RNA negative (P < 0.001 for each week). Twenty-four weeks after the cessation of therapy (SVR), 31 (86%) patients in the treatment group and 15 (42%) controls were HCV-RNA negative (P < 0.001). The percentage of relapses and non-responders and the biomarkers of insulin resistance, inflammation, pro-oxidant levels, antioxidant levels, and baseline vitamin D, vitamin E, and vitamin B12 serum levels are shown in Table 2 for both groups.

The rate of viral breakthrough was null. The rates of relapse and non-response were significantly lower in the treatment group compared with the control group [n = 3 (8%) *vs* n = 13 (36%), P < 0.001, and 2 (6%) *vs* 8 (22%), P < 0.01, respectively]. The HOMA-IR index decreased significantly after 4 wk of treatment with vitamin D compared with the control group (from 4.5 ± 1.4 to 2.3 ± 1.0, P < 0.01 *vs* 4.6 ± 5.7 to 5.0 ± 4.0, respectively, P < 0.1). There was no difference between groups for malondialdehyde, paraoxonase, vitamin E, vitamin B12, C-reactive protein, and triglyceride levels.

The adherence to vitamin D treatment was excellent during the entire course, and all patients in the treatment group achieved the target level. Vitamin D levels were maintained during the course of therapy with the same dosage (2000 IU/d) as in the lead-in phase. Predictive factors for SVR in patients treated with Peg/RBV combination therapy are shown in Table 3.

Viral load, advanced fibrosis, baseline vitamin D levels, changes in HOMA-IR, and vitamin D supplementation were significant univariate predictors of SVR. Viral load, vitamin D supplementation, advanced fibrosis and changes in HOMA-IR remained as independent predictors in the multivariate analysis. Thus, vitamin D supplementation emerged as being more responsible for higher SVR than the baseline vitamin D level.

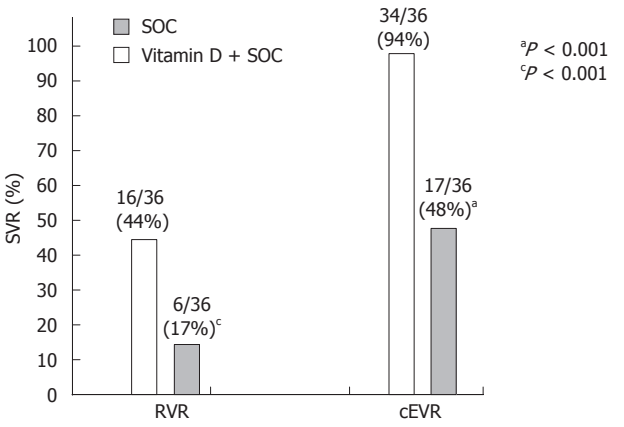

Figure 2 Rate (%) of the rapid viral response and rate of early viral response in the treatment (n = 36) and control (n = 36) groups. RVR was defined as undetectable HCV RNA at 4 wk during treatment. Complete EVR (cEVR) was defined as undetectable HCV RNA at 12 wk during treatment. SOC: Standard of care; RVR: Rapid viral response; EVR: Early viral response; HCV: Hepatitis C virus; SVR: Sustained viral response.

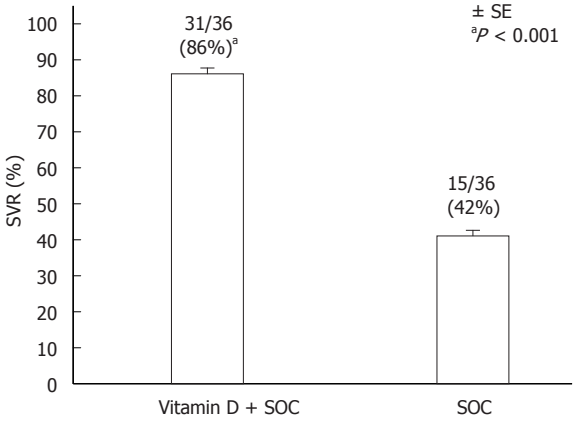

Figure 3 Rate of sustained viral response in the treatment group (Vitamin D + SOC, n = 31/36) and the control group (n = 15/36, SOC) 6 mo after cessation of treatment. SVR was defined as undetectable HCV-RNA at 24 wk post-treatment. Bars represent standard error. SOC: Standard of care; SVR: Sustained viral response; HCV: Hepatitis C virus.

The most common adverse events were mild in nature, similar in both groups, and consistent with typical Peg/RBV-induced systemic symptoms. They included nausea (n = 4), headache (n = 4), insomnia (n = 5), chills (n = 4), myalgia (n = 3), pyrexia (n = 3), pruritus (n = 2), mild neutropenia (n = 3), mild thrombocytopenia (n = 5), and mild anemia (n = 3). There were no serious adverse events. Adherence to Peg/RBV combination therapy was excellent, and there was no difference in dose reduction Peg/RBV combination therapy due to adverse events in either group. No patient discontinued treatment. Changes in laboratory values during the study were consistent with those reported in association with

Table 3 Predictors for sustained virologic response in treatment-naïve hepatitis C virus genotype 1 patients with pegylated interferon α and ribavirin combination therapy

	Odds ratio	95% CI	*P* value
Vitamin D treatment (Yes *vs* No)	2.5	2.0-4.9	< 0.001
Baseline vitamin D (< 20 or > 20 IU/mL)	1.5	1.2-3.8	0.080
Advanced fibrosis (< F2 or > F2)	2.0	1.0-3.6	0.001
High viral load (< 800 000 or > 800 000 IU/mL)	2.8	1.2-4.0	0.001
Baseline CRP (< 0.05 or > 0.5 mg/dL)	1.0	0.5-1.9	0.510
Changes in homeostasis model assessment (%)	1.8	0.5-3.0	0.030

CRP: C-reactive protein; CI: Confidence interval.

the combined use of Peg/RBV[3].

DISCUSSION

The results of this study suggest that the addition of a vitamin D supplement to current standard therapy can significantly improve the rate of RVR, EVR and SVR in treatment-naïve patients with HCV genotype 1 compared the rates with standard therapy alone. The observed SVR in the control group (42%) was consistent with previous reports[2,3]. Overall there was a marked increase in the virologic response at week 4 (44% *vs* 17%), week 12 (94% *vs* 48%), and week 24 after the cessation of therapy (86% *vs* 42%), and a low rate of relapse (8% *vs* 36%) with vitamin D supplementation compared with no supplementation. The rate of relapse in the control group was within the reported 18%-40% range for current standard HCV antiviral therapy[2,22].

There are only two reports examining the association between vitamin D status and outcome of antiviral therapy in patients with chronic HCV viral infection. Petta and co-workers retrospectively analyzed a cohort of 167 patients treated with Peg/RBV for hepatitis C, and detected an association between lower vitamin D serum levels and failure to achieve SVR[23]. Our results provide further support for that data. The second study by Bitetto and co-workers showed that vitamin D supplementation improved the response to antiviral treatment for recurrent HCV in liver transplant recipients[24]. Several differences between those two studies should be noted. Bitetto and co-workers' HCV patients were immunocompromised, and they were supplemented with low-dose vitamin D (800 IU/d) after liver transplantation. In addition, most of their HCV patients (75%) had low vitamin D levels despite treatment. Finally, that study was retrospective and focused on the prevention of osteoporosis, not on the treatment of hepatitis C.

The exact mechanism of action leading to improved RVR, EVR, and SVR in patients receiving vitamin D is unknown. Vitamin D is metabolized by the liver and converted to 1,25-dihydroxy-vitamin D3, which is the active form of the vitamin[6,7]. Individuals with chronic liver disease may have poor conversion from vitamin D3 or any of its other biologically active metabolites[11]. 1,25 vitamin D3 appears to modulate immunity principally *via* regulation of T-cell function[25]. The vitamin D receptor (VDR) is expressed on virtually every type of cell involved in immunity[26]. The immunomodulatory actions of vitamin D are elicited through its direct action on T-cell antigen-presenting cell function[27]. T helper cell type 1 (TH1) actions are intensified when vitamin D is insufficient, as in the majority of our patient population, or when signals through VDR are weak. Regulatory T cell and TH2 cells are diminished, thus favoring an autoimmune TH1 response[28]. This is a pro-inflammatory response which may impair IFN and insulin signaling, thus decreasing the viral response[29,30]. A recent study on 120 patients with chronic HCV genotype 1 infections reported that a TH1 to TH2 ratio of < 15.5 was significantly associated with SVR (odds ratio 9.6)[31]. TH1 and TH2 measurements were not performed in the present study. Persistent HCV infection modulates the balance between immune stimulatory and inhibitory cytokines which can prolong inflammation and lead to fibrosis and chronic liver diseases[32]. More recently, Gutierrez and co-workers showed that vitamin D3 increased VDR protein expression and inhibited viral replication in cell culture[33].

It is well known that people of African and Hispanic descent are less likely to respond to standard therapy[34]. This may be due to a polymorphism of the *interleukin (IL)-28B* gene, polymorphism of VDR or vitamin D deficiency[13,35]. The vast majority of the Russian/Jewish/Arab patients in the present study had vitamin D insufficiency, possibly related to paradoxically low exposure to the sun in this predominantly sunny country and/or to a low supply of vitamin D from their diet.

The impact of diet on liver fibrosis and on response to IFN therapy in patients with HCV chronic hepatitis has been reported before[36]. HCV patients also lack vitamins E and B12[37,38]. A recent study showed that higher levels of vitamin B12 were associated with SVR, but there was no difference in serum levels of those vitamins between the group treated with vitamin D and the controls[39].

Insulin resistance emerged as one of the most important host factors in the prediction of the response in non-diabetic HCV patients treated with Peg/RBV, and is a common factor in the features associated with difficult-to-treat patients[40]. Vitamin D is also known to help prevent type 2 diabetes, and it is possible that low levels of vitamin D lead to insulin resistance[9]. The direct effect of vitamin D may be mediated by binding of its circulating active form to the pancreatic B cell vitamin D receptor[41]. Vitamin D deficiency or insufficiency may alter the balance between the extracellular and intracellular cell calcium pools, which may interfere with normal insulin release[42]. Thus, a lack of either calcium or vitamin D can result in peripheral insulin resistance[41]. Moreover, oxidative stress leeches calcium, and vitamin

D helps absorb calcium[43]. Our current results confirm these findings: the HOMA-IR was higher at baseline in the vitamin D treatment group and improved after 4 wk of therapy compared to the control group. Moreover, the changes in HOMA-IR were strongly associated with SVR (multivariate analysis).

The definition of normal vitamin D serum levels is a subject of debate. In the current study, increasing the vitamin level D to > 32 ng/mL increased the response to antiviral therapy to the same extent in patients with vitamin D deficiency as well as those with vitamin D insufficiency. Multivariate analysis revealed that viral load, advanced fibrosis and vitamin D supplementation remained as independent predictors. Thus, it can be concluded that vitamin D supplementation is responsible for a higher SVR, rather than the baseline vitamin D level. It remains to be determined whether the addition of vitamin D acts by a mechanism other than improvement of insulin resistance or immune function such as the upregulation of toll-like receptors involved in the immune response in HCV-infected patients

Limitations of the present study include the small number of patients, lack of vitamin D level assessment during therapy for the treatment and control groups, and that this prospective and randomized study was not placebo-controlled, thus the patients knew whether or not they received a vitamin D supplement. Another limitation is the lack of data on the TH1 and TH2 immune response. The identification of determinants of the response, such as polymorphisms of the *IL-28B* gene, polymorphism of the VDR and immune function[13,35], may help explain the difference in response rates between patients with different ethnic backgrounds. This was not done in our study since data on IL-28B and on VDR polymorphism were not available at the time the study was designed.

In conclusion, the addition of vitamin D to Peg/RBV combination therapy in treatment-naïve patients who were infected with HCV genotype 1 significantly increased the rates of rapid, early, and sustained viral responses.

COMMENTS

Background

Treating chronic hepatitis C virus (HCV) (genotype1) patients with pegylated interferon and ribavirin, which is considered to be the standard of care, has achieved viral clearance in less than 50% of the patients. Vitamin D is a potent immunomodulator with a beneficial effect against viral and bacterial infections. The vast majority of patients with chronic hepatitis C have low levels of vitamin D. Different new drugs such as protease or polymerase inhibitors are still under investigation and are expensive and have many side effects like rash.

Research frontiers

Vitamin D deficiency is well documented in patients with chronic liver disease. However, treating patients with chronic HCV infection by adding a vitamin D supplement to the standard of care has not been addressed. There are only two reports dealing with the association between vitamin D status and outcome of antiviral therapy for chronic HCV infection.

Innovations and breakthroughs

The current study shows that adding a vitamin D supplement to pegylated interferon and ribavirin significantly increases the rapid, early and late clearance of the virus, in chronic hepatitis C genotype 1 treatment-naïve patients.

Applications

This study emphasizes the importance of vitamin D supplementation when added to standard treatment in all patients with chronic hepatitis C. Further studies are needed to explain the mechanism of vitamin D supplementation for these patients.

Terminology

Hepatitis C is a chronic liver infection that can be complicated by liver failure and liver cancer. Clearance of the virus from the blood is achievable by a combination of pegylated interferon and ribavirin in less than 50% of the patients. Vitamin D has an important role in the treatment of different bacterial and viral infections; this vitamin is synthesized in the skin by absorption of ultraviolet from the sun light. The mechanism of action of this vitamin is unknown, but it may improve the activities of immune cells that are important in the eradication of HCV.

Peer review

This is a well conducted study with a relevant finding, and it is well written.

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BRIEF ARTICLE

Posterior lingual lidocaine: A novel method to improve tolerance in upper gastrointestinal endoscopy

Assaad M Soweid, Shadi R Yaghi, Faek R Jamali, Abdallah A Kobeissy, Michella E Mallat, Rola Hussein, Chakib M Ayoub

Assaad M Soweid, Shadi R Yaghi, Abdallah A Kobeissy, Michella E Mallat, Rola Hussein, Department of Internal Medicine, American University of Beirut Medical Center, 1107 2020 Beirut, Lebanon
Faek R Jamali, Department of Surgery, American University of Beirut Medical Center, 1107 2020 Beirut, Lebanon
Chakib M Ayoub, Department of Anesthesiology, American University of Beirut Medical Center, 1107 2020 Beirut, Lebanon
Author contributions: Soweid AM and Ayoub CM designed the study; Yaghi SR, Mallat ME and Hussein R collected the data; Jamali FR and Kobeissy AA analyzed and interpreted the data, and were also involved in editing the manuscript; Soweid AM and Ayoub CM wrote the paper.
Correspondence to: Dr. Chakib M Ayoub, American University of Beirut Medical Center, PO Box 11-0236, Riad El Solh, 1107 2020 Beirut, Lebanon. ca04@aub.edu.lb
Telephone: +961-1-350000 Fax: +961-1-366098
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Abstract

AIM: To evaluate the effect of posterior lingual lidocaine swab on patient tolerance to esophagogastroduodenoscopy, the ease of performance of the procedure, and to determine if such use will reduce the need for intravenous sedation.

METHODS: Eighty patients undergoing diagnostic esophagogastroduodenoscopy in a tertiary care medical center were randomized to either lidocaine swab or spray. Intravenous meperidine and midazolam were given as needed during the procedure.

RESULTS: Patients in the lidocaine swab group (SWG) tolerated the procedure better than those in the spray group (SPG) with a median tolerability score of 2 (1, 4) compared to 4 (2, 5) ($P < 0.01$). The endoscopists encountered less difficulty performing the procedures in the SWG with lower median difficulty scores of 1 (1, 5) compared to 4 (1, 5) in the SPG ($P < 0.01$). In addition, the need for intravenous sedation was also lower in the SWG compared to the SPG with fewer patients requiring intravenous sedation (13/40 patients *vs* 38/40 patients, respectively, $P < 0.01$). The patients in the SWG were more satisfied with the mode of local anesthesia they received as compared to the SPG. In addition, the endoscopists were happier with the use of lidocaine swab.

CONCLUSION: The use of a posterior lingual lidocaine swab in esophagogastroduodenoscopy improves patient comfort and tolerance and endoscopist satisfaction and decreases the need for intravenous sedation.

Key words: Esophagogastroduodenoscopy; Upper gastrointestinal endoscopy; Local anesthesia; Lidocaine; Sedation

Peer reviewer: Hoon Jai Chun, MD, PhD, AGAF, Professor, Department of Internal Medicine, Institute of Digestive Disease and Nutrition, Korea University College of Medicine, 126-1, Anam-dong 5-ga, Seongbuk-gu, Seoul 136-705, South Korea

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INTRODUCTION

Esophagogastroduodenoscopy (EGD) is an essential and very commonly used procedure for the evaluation of a

multitude of gastrointestinal (GI) symptoms including abdominal pain, hemorrhage, dysphagia, odynophagia, and reflux[1-3]. Although EGD is fairly safe, it carries a low risk of complications including perforation, bleeding, infection, and medication reactions/adverse effects[2,4,5]. Several studies showed that patients with advanced age and those with cardiopulmonary disease may carry an increased risk for the procedure especially when high doses of intravenous (IV) sedatives are used[2,6,7]. Various IV agents such as midazolam, meperidine, propofol, and fentanyl have been used over the past few decades for their anxiolytic, amnestic, and analgesic effects during the procedure[8-10]. However, these agents carry potential serious adverse effects especially in high risk patients. These complications include apnea, hypoxia, vomiting, hypotension, agitation, and allergic reactions[11-16]. In addition, complications of IV sedation contributed to cost increase due to unexpected hospitalizations and work related absenteeism on the procedure day. This has led to search for modes of anesthesia that carry less complication rates and, at the same time, provide satisfaction for both patient and endoscopist[12,15,17-24]. Few studies have used different forms of topical anesthesia including, spray, lollipop, and inhaler with mixed results. Some of these topical agents still carried a risk of retching, vomiting, and apnea[8,19,20,23,25-28]. Conventionally, topical lidocaine spray is used combined with IV analgesics and sedatives before and during the procedure to achieve a high level of patient comfort and endoscopist satisfaction[4,19,26,27,29].

The rationale behind the use of topical anesthesia is to suppress the gag reflex that may account for some of the EGD-related discomfort. The gag reflex is one of the normal reflexes induced by stimulation of the pharynx and velar area. It involves the contraction of pharyngeal constrictors induced by touching one of the five trigger zones that include: base of tongue, uvula, palate, posterior pharyngeal wall, and palatopharyngeal and palatoglossal folds[30]. The gag reflex consists of an afferent and an efferent arches. The afferent receives input from nerve fibers of the glossopharyngeal nerve which are relayed in the nucleus solitaris. The efferent arch is supplied by the nucleus ambiguus through the vagus nerve (Figure 1)[31]. These nuclei are at close proximity to the vomiting and salivating centers, which explains the experience of retching and excessive salivation when the gag reflex is elicited[30]. Both superficial and deep sensory receptors are involved in the physiology of the gag reflex, and this makes a pharyngeal plexus block superior to topical lidocaine spray in suppressing the reflex[32]. When a person eats, central voluntary action on the pharyngeal muscles dominates over the gag reflex and this is why there is no gagging when eating[30]. Therefore, if lidocaine is to be applied specifically to the above-mentioned five trigger areas in the pharynx, then the gag reflex would be markedly attenuated or even ablated during the procedure, which may further increase the patients' tolerance to EGD and in turn decrease IV sedation use. The use of lidocaine in the gel form may be ideal since a dense/sticky form of lidocaine may provide a more reliable local anesthesia compared to the spray.

In this study, the efficacy of posterior lingual lidocaine as a potential anesthetic technique in patients undergoing EGD was compared to that of the conventional lidocaine spray. Our main objective is to evaluate the effect of posterior lingual lidocaine application on patient tolerance to the procedure and the ease of performance of the procedure. Our secondary aim is to determine if such use will reduce the need for IV sedation.

MATERIALS AND METHODS

Patients

Our target population was patients undergoing diagnostic EGD for various indications at the American University of Beirut-Medical Center (AUB-MC). The study was approved by the Institutional Review Board committee at AUB-MC in accordance with Helsinki Declaration. The details regarding the study objectives and risks were fully explained to the patients and those who agreed to participate in the study were recruited and signed the informed consent.

Study design

After signing the informed consent, patients were randomly assigned to one of two study groups: the swab group (SWG) who received 150 mg of lidocaine gel or the spray group (SPG) who received 300 mg lidocaine spray. Lidocaine spray was administered using the same technique in 3 consecutive 30-s intervals, each consisting of 10 sprays (10 mg/dose) of Xylocaine® Pump Spray 10% (AstraZeneca AB, Sodertalje, Sweden). In the swab group, Xylocaine® Jelly 2% (AstraZeneca AB, Sodertalje, Sweden), with a lidocaine concentration of 20 mg/mL was used. A total of 7.5 mL (150 mg) of lidocaine gel was gradually applied to the base of the tongue and the peritonsillar areas. The endoscopist was totally blinded to the randomization. The endoscope used in the procedures was GIF-1T 240 (Olympus Optical, 11 mm diameter, Tokyo, Japan). All the patients had IV lines inserted and their vital signs (blood pressure, heart rate, respiratory rate) and pulse oxymetry were continuously monitored during the procedure. The data that was collected by the research fellow from all enrolled patients before the procedure included the following parameters: age, gender, past medical history, past surgical history, medications, allergies, alcohol use, smoking, illicit drug use, and history of previous endoscopy (including tolerance to it). None of the participants had any severe pulmonary disease (asthma and chronic obstructive pulmonary disease). The research fellow then determined the patients' anxiety level according to a scale from 1 to 5 (1 = no anxiety to 5 = extreme anxiety). After the topical anesthetics were applied, the time for the onset of the topical anesthesia was also noted (from the time the local anesthetic was applied till patients reported numb-

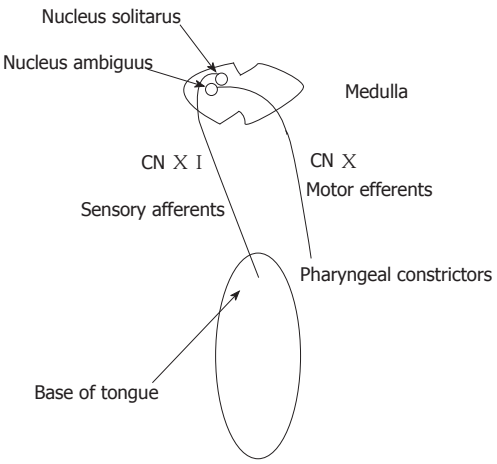

Figure 1 The gag reflex pathway: afferent fibers from the trigger areas in the pharynx and tongue carried by the glossopharyngeal nerve (cranial nerve X I) to the nucleus solitarus which sends the input to the nucleus ambiguus in the medulla oblongata. Efferent fibers from the nucleus ambiguus carried *via* the vagus nerve (CN X) to the pharyngeal constrictors to contract and cause gagging. CN: Cranial nerve.

ness in the oral cavity and the inability to swallow).

In both study groups, the decision to administer IV sedation during the procedure was made by the endoscopist depending on the patient's tolerance and the presence or absence of signs of discomfort, like excessive gag, retching, or restlessness. Sedatives used were midazolam and meperidine. The duration of the procedure was also noted.

Endoscopist's assessments

After the administration of the local anesthetics, the endoscopist rated the gag reflex based on a scale from 1 to 5 (1 = absent to 5 = strong). After the procedure, the endoscopist determined the ease of the procedure based on a scale from 1 to 5 (1 = easy to 5 = difficult). Finally, the amount of IV sedation given was recorded.

Patients' assessments

After the procedure was concluded, patients were monitored in the recovery room. Afterwards, a questionnaire was filled in by the participants to determine tolerance to the procedure based on a scale from 1 to 5 (1 = no difficulties encountered to 5 = very difficult). Also, symptoms during (retching, nausea, vomiting, abdominal pain, dyspnea, cough) and after (sore throat, nausea, vomiting, abdominal pain, dyspnea, cough) the procedure were recorded. Patients were also asked to specify the most uncomfortable phase of the procedure and their willingness to repeat the procedure using the same local anesthetic.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0 (SPSS, Inc., Chicago, IL). The nonparametric Mann-Whitney test was used to compare ordinal variables (such as the gag reflex, procedure evaluation, *etc*.) and data that are not normally distributed (the doses of meperidine and midazolam) between SWG and SPG groups.

The χ^2 test was utilized to compare categorical variables between the 2 groups. Continuous variables were assessed with an independent sample *t* test. A P value < 0.05 was considered to be significant.

Table 1 Patient characteristics

	SWG (n = 40)	SPG (n = 40)	P value
Gender (M/F)	12/28	19/21	0.11
Mean age, yr (SD)	55.8 (18.1)	48.5 (18.2)	0.07
Smoking (yes/no)	15/25	23/17	0.07
Caffeine (yes/no)	37/3	32/8	0.19
Alcohol (yes/no)	12/28	14/26	0.63
Previous EGD (yes/no)	17/23	23/17	0.18

SWG: Swab group; SPG: Spray group; M: Male; F: Female; EGD: Esophagogastroduodenoscopy. P value for difference between groups using χ^2 or Fisher's exact tests.

Table 2 Pre-procedure evaluation, median (min, max)

	SWG (n = 40)	SPG (n = 40)	P value[1]
Anxiety	3 (1, 5)	3 (1, 5)	0.67
Time to onset of anesthesia (s)	80 (30, 300)	50 (20, 120)	< 0.01
Gag reflex	2 (1, 5)	4 (2, 5)	< 0.01

SWG: Swab group; SPG: Spray group. Anxiety rate: 1 = no anxiety to 5 = extreme anxiety; Gag reflex scale: 1 = absent to 5 = strong. [1]P value for difference between groups using nonparametric Mann-Whitney test.

RESULTS

Patient characteristics and demographics

Our study included 80 consecutive patients who underwent an elective EGD at AUB-MC. There were 31 males (38.8%) and 49 females (61.2%) with a mean age of 52.2 ± 18.4 years. There was no statistically significant difference in the patients' characteristics in both groups (Table 1).

Pre-procedure evaluation

Anxiety before the procedure was rated on an ascending scale from 1 to 5 as detailed in the method section. There was no significant difference between the two groups; the median anxiety scores were 3 (1, 5) for subjects in SWG and SPG.

The time interval between the lidocaine administration and the onset of anesthesia was significantly longer in the SWG as compared to the SPG, with median time 80 (30, 300) and 50 (20, 120) s (P < 0.01, respectively).

The SPG had significantly stronger gag reflex than the SWG with respective median scores of 4 (1, 5) and 2 (1, 5) (P < 0.01, Table 2).

IV sedation use

IV sedation was administered more frequently in the SPG than the SWG (95% *vs* 32%, P < 0.01).

Table 3 Use of intravenous sedation, median (min, max)

	SWG (n = 40)	SPG (n = 40)	P value
Use of IV sedation (yes/no)	13/27	38/2	< 0.01
Meperidine dose (mg)	0 (0, 50)	25 (0, 75)	< 0.01[1]
Midazolam dose (mg)	0 (0, 3)	2 (0, 4)	< 0.01[1]

SWG: Swab group; SPG: Spray group; IV: Intravenous. [1]P value for difference between groups using Mann-Whitney test.

Table 4 Procedure evaluation, median (min, max)

	SWG (n = 40)	SPG (n = 40)	P value
Ease of procedure - endoscopist	1 (1, 5)	4 (1, 5)	< 0.01
Procedure tolerance - patient	2 (1, 4)	4 (2, 5)	< 0.01[1]
Patient willingness to repeat procedure (yes/no)	32/8	2/38	< 0.01[1]

SWG: Swab group; SPG: Spray group. Endoscopist difficulty scale: 1= easy to 5 =difficult; Patient tolerance scale: 1 = no difficulties encountered to 5 = very difficult. [1]P value for difference between groups using nonparametric Mann-Whitney test.

The amount of meperidine administered was significantly lower in the SWG compared to the SPG, with median doses of 0 (0, 50) and 25 (0, 75) mg, respectively, $P < 0.01$. Similarly, the dose of midazolam was significantly lower in the SWG as compared to the SPG with median doses of 0 (0, 3) and 2 (0, 4) mg ($P < 0.01$, respectively, Table 3).

Endoscopists' evaluation

The endoscopist's assessment of the degree of procedure difficulty showed that the procedures were significantly easier to perform in the SWG than the SPG, with median difficulty scores of 1 (1, 5) and 4 (1, 5) ($P < 0.01$, respectively, Table 4).

Additionally, the procedure was significantly much easier to perform in subjects who did not receive IV sedation compared to those who received either meperidine or midazolam, with median difficulty scores of 1 (1, 3) and 4 (1, 5) ($P < 0.01$), respectively.

Patients' evaluation

Patients in the SWG tolerated the procedure more with a median tolerability score of 2 (1, 4) as compared to 4 (2, 5) in the SPG ($P < 0.01$, Table 4).

The most difficult part of the procedure was the introduction of the endoscope as reported by 68.8 % of patients. Thirty two (80%) subjects in the SWG expressed their willingness to repeat the procedure under the same local anesthesia, versus only 2 (5%) patients in the SPG ($P < 0.01$, Table 4).

Side effects

The side effects during and after the procedure were similar in both groups except for retching which was significantly lower in the SWG than in the SPG (13/40 *vs* 31/40 patients, respectively, $P < 0.01$, Table 5).

Table 5 Procedure-related symptoms

	SWG (n = 40)	SPG (n = 40)	P value
During the procedure			
Retching (yes/no)	13/27	31/9	< 0.01
Cough (yes/no)	10/30	12/28	0.62
Abdominal pain (yes/no)	1/39	1/39	1
Dyspnea (yes/no)	0/40	4/36	0.12
After the procedure			
Sore throat (yes/no)	5/35	8/32	0.55
Abdominal pain (yes/no)	1/39	5/35	0.2
Nausea/vomiting (yes/no)	0/40	1/39	1

SWG: Swab group; SPG: Spray group. P value for difference between groups using χ^2 or Fisher's exact tests.

Complications

None of the procedures was aborted due to complications, excessive agitation or major patient discomfort.

DISCUSSION

The use of conscious sedation along with lidocaine spray is the standard of care in upper GI endoscopy[4,19,26,27,29]. However, IV sedation may cause potential harm to the patients especially the elderly with co-morbidities. These side effects include hypotension, respiratory depression, and paradoxical agitation[7,11-16]. The potential risks of upper GI endoscopy are mostly related to the use of IV sedation[1,2,11,16,27,33-35]. Studies done by Campo *et al*[6] and Mulcahy *et al*[7] showed that a high level of anxiety, young age, and a strong gag reflex are risk factors for poor tolerance to upper GI endoscopy. On the other hand, a study done by Pereira *et al*[27] showed that patients' anxiety did not contribute to procedure tolerance. Local oropharyngeal anesthesia including lidocaine has been studied in several trials with the results showing that the use of the lidocaine spray or gel with IV sedation increased the tolerability and ease of the procedure and reduced the risk of discomfort during the procedure[23,26,28,29,36]. A single study, however, was done on the lidocaine lollipop which showed excellent efficacy in achieving patient comfort even without the use of IV sedation[28]. The action of local oropharyngeal anesthesia is achieved mainly by inhibiting the gag reflex which is one of the most important factors affecting the tolerability and ease of the procedure[6,28]. So in order to perform the procedure without possibly using IV sedation, an effective local agent that suppresses the gag reflex should be used.

Our study showed that when lidocaine gel is applied to the posterior lingual area, it effectively suppresses the gag reflex, significantly increases the patient tolerability to the procedure, improves endoscopist satisfaction of the procedure, and considerably decreases the need for IV sedation. The level of anxiety and age were similar in both groups; thus, these factors can be eliminated as confounding variables. Therefore, lidocaine gel could be

used as a sole agent in upper GI endoscopy sparing the use of IV sedation with its potential complications. In addition, this may help patients resume their daily activities immediately after the procedure. Although we did not compare the cost of the lidocaine gel and spray, the use of the gel appears to be more cost-effective since potential adverse events related to IV sedation are reduced. The maximal dose of lidocaine used in the spray group was 300 mg. This dose of lidocaine is within the recommended dose of 5 mg/kg and does not exceed the potentially toxic dose of 500 mg[37]. Moreover, higher doses of topical lidocaine had been used in prior studies. Sutherland *et al*[38], for instance, utilized topical doses of 380 mg of lidocaine and concluded that the blood levels were still within therapeutic range. The dose of lidocaine used in the gel group was much lower (150 mg) than that in the spray group. Despite that decrease in the dose, there was more effective suppression of the gag reflex in the gel group, and hence, better tolerance to the EGD.

Sample size was one of the few limitations in this study. Because it was a small sample, subgroup analysis could not be performed. Another limitation that might have affected our results can be attributed to the impairment in judgmental abilities caused by the sedatives used in some cases.

In conclusion, this study presented evidence that the use of lidocaine swab applied to the posterior lingual area was an effective mode of local anesthesia in upper GI endoscopy. This can lead to reduction in the use of IV sedatives (and potentially their complications) and may decrease the overall cost of the procedure. This may be a very promising modality especially in the elderly patients who have comorbidities, and in office-based upper GI endoscopy. However, larger, multicenter studies should be done to confirm and validate the results of our study.

ACKNOWLEDGMENTS

The data of this study has been presented as a poster at the Digestive Disease Week in New Orleans, 2010.

COMMENTS

Background

Esophagogastroduodenoscopy (EGD) has become an essential and very commonly used procedure for the diagnostic and therapeutic evaluation of a multitude of upper gastrointestinal (GI) symptoms and diseases. EGD is considered a safe procedure with a very low risk of complications. Medication administered for local anesthesia and for conscious sedation during the procedure can pose some adverse effects especially in the elderly population. So finding ways to decrease the need for these drugs would decrease the complication rates. The rationale behind the use of topical anesthesia is to decrease the gag reflex that may account for a major part of EGD-related discomfort. Using lidocaine as a topical anesthetic in the gel form may be ideal since a dense/sticky form of lidocaine may provide a more reliable local anesthesia compared to the spray thus increasing the patients' tolerance to EGD and in turn decreasing the need for intravenous (IV) sedation.

Research frontiers

Improving the tolerance and ease of execution of EGD procedures has gained much interest recently. The primary objective of this clinical research approach is to decrease the need for drugs used for conscious sedation to spare patients the side effects and the costs of elevated doses of such agents. Research is currently focusing on increasing the effectiveness of drug administration, improving patients' tolerance, and using/developing ultrathin endoscopes.

Innovations and breakthroughs

This study showed that when lidocaine gel is applied to the posterior lingual area, it effectively suppresses the gag reflex, significantly increases the patient tolerability to the procedure, improves endoscopist satisfaction of the procedure, and considerably decreases the need for IV sedation.

Applications

The authors presented evidence that the use of lidocaine swab applied to the posterior lingual area was an effective mode of local anesthesia in upper GI endoscopy. This can lead to reduction in the use of IV sedatives (and potentially their complications) and may decrease the overall cost of the procedure. This may be a very promising modality especially in the elderly patients with comorbidities, and in office-based upper GI endoscopy.

Terminology

Conscious sedation: Defined as moderate sedation by the American Society of Anesthesiologists. It is the reduction of irritability or agitation by administration of sedative drugs such as midazolam with purposeful preservation of the response to verbal or tactile stimulation. Posterior lingual lidocaine swab: A technique whereby local anesthesia is achieved by the application of lidocaine gel to the base of the tongue and the peritonsillar areas as opposed to application *via* the aerosolized spray form routinely utilized.

Peer review

This article showed that the effectiveness of the posterior lingual lidocaine swab is statistically significant. The study design and analysis ensures the validity of achieved results and nearly eliminated causes of random error. Nonetheless, increasing the patients' number and possibly involving other centers in this study would undeniably increase its power and reliability.

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BRIEF ARTICLE

Statin use and the risk of colorectal cancer: A population-based case-control study

Meng-Hsuan Cheng, Hui-Fen Chiu, Shu-Chen Ho, Shang-Shyue Tsai, Trong-Neng Wu, Chun-Yuh Yang

Meng-Hsuan Cheng, Division of Pulmonary and Critical Medicine, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan, China
Meng-Hsuan Cheng, Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, China
Meng-Hsuan Cheng, Department of Internal Medicine, Ping-Tung Hospital, Department of Health, Executive Yuan, Ping-Tung 90054, Taiwan, China
Hui-Fen Chiu, Institute of Pharmacology, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, China
Shu-Chen Ho, Institute of Occupational Safety and Health, College of Health Sciences, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, China
Shang-Shyue Tsai, Department of Healthcare Administration, I-Shou University, Kaohsiung 80708, Taiwan, China
Trong-Neng Wu, Department of Public Health, China Medical University, Taichung 40402, Taiwan, China
Trong-Neng Wu, Division of Environmental Health and Occupational Medicine, National Health Research Institute, Miaoli 10694, Taiwan, China
Chun-Yuh Yang, Faculty of Public Health, College of Health Sciences, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, China
Chun-Yuh Yang, Division of Environmental Health and Occupational Medicine, National Health Research Institute, Miaoli 10694, Taiwan, China
Author contributions: Cheng MH wrote the manuscript; Ho SC performed the statistical analysis; Chiu HF, Tsai SS and Wu TN provided essential insight into the interpretation of the results; Yang CY contributed to the study design and interpretation of the data and had full access to all the data in the study and took responsibility for the integrity of the data and accuracy of the data analysis.
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Correspondence to: Chun-Yuh Yang, PhD, MPH, Faculty of Public Health, Kaohsiung Medical University, 100 Shih-Chuan 1st RD, Kaohsiung 80708, Taiwan, China. chunyuh@kmu.edu.tw
Telephone: +886-7-3121101 Fax: +886-7-3110811
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Abstract

AIM: To investigate whether the use of statins is associated with colorectal cancer risk.

METHODS: We conducted a population-based case-control study in Taiwan. Data were retrospectively collected from the Taiwan National Health Insurance Research Database. Cases consisted of all patients who were aged 50 years and older and had a first-time diagnosis of colorectal cancer between the period 2005 and 2008. The controls were matched to cases by age, sex, and index date. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using multiple logistic regression.

RESULTS: We examined 1156 colorectal cancer cases and 4624 controls. The unadjusted ORs for any statin prescription was 1.10 (95% CI = 0.94-1.30) and the adjusted OR was 1.09 (95% CI = 0.91-1.30). When statin use was categorized by cumulative dose, the adjusted ORs were 0.99 (95% CI = 0.78-1.27) for the group with cumulative statin use below 105 defined daily doses (DDDs); 1.07 (95% CI = 0.78-1.49) for the group with cumulative statin use between 106 and 298.66 DDDs; and 1.30 (95% CI = 0.96-1.75) for the group with cumulative statin use of 298.66 DDDs or more compared with nonusers.

CONCLUSION: This study does not provide support for a protective effect of statins against colorectal cancer.

Key words: Case-control study; Colorectal cancer; Pharmacoepidemiology; Statins

Peer reviewer: Pascal Gervaz, PhD, Department of Surgery, University Hospital Geneva, 4, Rue Gabrielle Perret Gentile, Geneva 1211, Switzerland

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INTRODUCTION

Statins are inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A reductase which is a key enzyme in the rate-limiting step in cholesterol synthesis[1]. Statins are commonly used as cholesterol-lowering medications and have shown effectiveness in the primary and secondary prevention of heart attack and stroke[2,3]. The extensive evidence in this field has led to widespread use of these drugs.

Rodent studies indicate that statins are carcinogenic[4]. In contrast, several recent studies on human cancer cell lines and animal tumor models indicate that statins may have chemopreventive properties through the arrest of cell cycle progression[5], induction of apoptosis[1,6], suppression of angiogenesis[7,8], and inhibition of tumor growth and metastasis[9]. Results of a meta-analysis and observational studies revealed either no association[10-17] or a decrease in cancer incidence[18-26]. The reasons for the varying results are unclear but may be related to methodological issues, including small sample size and short follow-up periods[27].

Several epidemiologic studies have investigated the association between statin use and risk of colorectal cancer and the results have been inconsistent. Ten studies reported no statistically significant association between statin use and colorectal cancer risk[10,12,15,17,20-21,27-30]. However, three recent case-control studies reported that statin use is associated with a significant reduction in the risk of colorectal cancer[22,31-32].

Since large numbers of people utilize statins on a long-term basis, and because epidemiologic data linking statin use and risk of colorectal cancer are conflicting, we undertook the present study in Taiwan to evaluate the association between statin use and colorectal cancer risk.

MATERIALS AND METHODS

Data source

The National Health Insurance (NHI) program, which provides compulsory universal health insurance, was implemented in Taiwan on March 1, 1995. Under the NHI, 98% of the island's population can receive all forms of health care services including outpatient services, inpatient care, Chinese medicine, dental care, childbirth, physical therapy, preventive health care, home care, and rehabilitation for chronic mental illness. In cooperation with the Bureau of NHI, the National Health Research Institute (NHRI) of Taiwan randomly sampled a representative database of 1 000 000 subjects from the entire NHI enrollees by means of a systematic sampling method for research purposes. There were no statistically significant differences in age, gender, and healthcare costs between the sample group and all enrollees, as reported by the NHRI. This dataset (from January 1996 to December 2008) includes all claim data for these 1 000 000 subjects, and offers a good opportunity to explore the relation between the use of statins and risk of colorectal cancer. These databases have previously been used for epidemiological research, and information on prescription use, diagnoses, and hospitalizations has been shown to be of high quality[33-35].

Because the identification numbers of all individuals in the NHRI databases were encrypted to protect the privacy of the individuals, this study was exempt from full review by the Institution Review Board.

Identification of cases and controls

Cases consisted of all patients who were aged 50 years and older and had a first-time diagnosis of colorectal cancer (International Classification of Diseases, 9th revision, Clinical Modification Code 153-154) over a 4-year period, from January 1, 2005 to December 31, 2008, and who had no previous diagnosis of cancer.

Controls comprised patients who were admitted to hospital for diagnoses that were unrelated to statin use including orthopedic conditions, trauma (excluding wrist and hip fractures), and other conditions (acute infection, hernia, kidney stones, cholecystitis)[12,36]. Wrist and hip fractures were excluded because previous studies have reported a reduced risk of osteoporosis among statin users[37-40]. We identified four control patients per case patient. Control patients were matched to the cases by sex, year of birth, and index date and were without a previous cancer diagnosis. For controls, the index date (date of hospital admission) was within the same month of the index date (date of first-time diagnosis of colorectal cancer) of their matched case.

Exposure to statins

Information on all statin prescriptions was extracted from the NHRI prescription database. We collected the date of prescription, the daily dose, the number of days supplied. The defined daily doses (DDDs) recommended by the WHO[41] were used to quantify use of statins. Cumulative DDDs were estimated as the sum of dispensed DDD of any statins (lovastatin, pravastatin, rosuvastatin, fluvastatin, simvastatin, or atorvastatin) from January 1, 1996 to the index date.

Potential confounders

For all individuals in the study population, we identified variables which might confound the associations between statin use and colorectal cancer, including diabetes mellitus, cholecystectomy, liver disease, colorectal polyps, and inflammatory bowel disease, recorded between January 1, 1996, and the index date. In addition, we also obtained

Table 1 Demographic characteristics of colorectal cancer cases and controls

Variable	Cases (n = 1156)	Controls (n = 4624)	Odds ratio (95% CI)
Age, yr (mean ± SD)	68.34 ± 10.40	69.29 ± 10.40	-
Female (%)	447 (38.67)	1788 (38.67)	-
No. of hospitalizations	0.29 ± 0.93	0.26 ± 0.74	P = 0.23
Diabetes (%)	422 (36.51)	1560 (33.74)	1.13 (0.99-1.29)
Cholecystectomy (%)	21 (1.82)	105 (2.27)	0.80 (0.50-1.28)
Liver disease (%)	422 (36.51)	1861 (40.25)	0.85 (0.75-0.98)
Colorectal polyps (%)	56 (4.84)	76 (1.64)	3.05 (2.14-4.33)
Inflammatory bowel disease (%)	82 (7.09)	315 (6.81)	1.04 (0.81-1.34)
Colonoscopy (%)	153 (13.24)	42 (0.91)	16.64 (11.75-23.57)
FOBT (%)	152 (13.15)	216 (4.67)	3.09 (2.48-3.84)
NSAID (%)	636 (55.02)	2767 (59.84)	0.82 (0.72-0.93)
Use of other lipid-lowering drugs (%)	31 (2.68)	180 (3.89)	0.68 (0.46-1.00)

FOBT: Fecal occult blood testing; NSAID: Non-steroidal anti-inflammatory drug; CI: Confidence interval.

prescription data for other lipid-lowering drugs (including fibrate, niacin, bile-acid binding resins, and miscellaneous medications) and non-steroidal anti-inflammatory drugs (NSAIDs) that could potentially confound the association between statin use and the risk of colorectal cancer. We defined users of the above-mentioned medications as patients with at least one prescription over one year prior to the index date. Furthermore, colonoscopy, fecal occult blood testing (FOBT), and number of hospitalizations one year before the index date were treated as confounders.

Statistical analysis

For comparisons of proportions, chi-square statistics were used. A conditional logistic regression model was used to estimate the relative magnitude in relation to the use of statins. Exposure was defined as patients who received at least one prescription for a statin at any time between January 1, 1996 and the index date. In the analysis, the subjects were categorized into one of four statin exposure categories: nonusers (subjects with no prescription for any statins at any time between January 1, 1996 and the index date), low (the lowest 50th percentile; ≤ 105 DDDs); medium (50th-75th percentile; 106-298.66 DDDs); and high (above the 75th percentile; > 298.66 DDDs) based on the distribution of use among controls. Odd ratios (ORs) and their 95% confidence intervals (CIs) were calculated using patients with no exposure as the reference. Analyses were performed using the SAS statistical package (version 8.02, SAS Institute Inc). All statistical tests were two-sided. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Records from 1156 colorectal cancer cases and 4624 selected matched controls were included in the analyses.

Table 2 Associations between statin use and colorectal cancer risk in a population-based case-control study, Taiwan, 2005-2008

	Cases (n)/ controls (n)	Crude OR (95% CI)	Adjusted OR (95% CI)[1]
Overall			
No statin use	914/3727	1.00	1.00
Any statin use	242/897	1.10 (0.94-1.30)	1.09 (0.91-1.30)
Cumulative use			
0	914/3727	1.00	1.00
1-105 DDD	112/451	1.02 (0.82-1.27)	0.99 (0.78-1.27)
106-298.66 DDD	60/221	1.11 (0.83-1.49)	1.07 (0.78-1.49)
> 298.66 DDD	70/225	1.27 (0.96-1.68)	1.30 (0.96-1.75)

OR: Odds ratio; CI: Confidence interval; DDD: Defined daily dose. [1]Adjusted for matching variable, diabetes, number of hospitalizations, cholecystectomy, liver disease, colorectal polyps, inflammatory bowel disease, colonoscopy, fecal occult blood testing, non-steroidal anti-inflammatory drugs and use of other lipid-lowering drugs.

Table 1 shows the distribution of demographic characteristics and selected medical conditions of the cancer cases and controls. The mean age was 68.34 years for cancer cases and 68.81 years for the controls. Case subjects were more likely to have had preventive services (screening colonoscopy and FOBT). The case group had a significantly higher rate of colorectal polyps than control patients. Use of other lipid-lowering drugs and NSAIDs were not significantly different between cases and controls.

The observed associations between the use of statins and colorectal cancer are shown in Table 2. Ever-use of any statins was associated with a slight but not statistically significant increased colorectal cancer risk (adjusted OR = 1.09, 95% CI = 0.91-1.30). When statin use was categorized by cumulative dose, the adjusted ORs were 0.99 (95% CI = 0.78-1.27) for the group with cumulative statin use below 105 DDDs; 1.07 (95% CI = 0.78-1.49) for the group with cumulative statin use between 106 and 298.66 DDDs; and 1.30 (95% CI = 0.96-1.75) for the group with cumulative statin use of 298.66 DDDs or more compared with nonusers. Overall, we found no association between cumulative statin use and colorectal cancer risk. ORs for cancers of the colon and rectum considered separately were similar (data not shown).

DISCUSSION

In this population-based case-control study, we found that statin drug use was not associated with colorectal cancer risk. Our findings are consistent with ten recent studies which reported no associations between statin use and overall colorectal cancer risk[10,12,15,17,20-21,27-30].

Our results, however, conflict with three recent case-control studies. In a case-control study conducted in Israel, a reduced risk of colorectal cancer was found to be associated with the use of statins for at least 5 years, compared with less than 5 years of use (OR = 0.50, 95% CI = 0.40-0.63)[22]. Another population-based study from Germany showed that statin use was associated

with a 35% (OR = 0.65, 95% CI = 0.43-0.99) colorectal risk reduction occurring within 1-4 years of statin use and no further risk reduction was seen after 5 years or more[31]. Neither study characterized the dose or duration of statins in detail and both studies defined statin use by recall. In a nested case-control study consisting solely of veterans with diabetes, using national databases of the Department of US Veterans Affairs and Medicare-linked files, Hachem *et al*[32] reported an odds ratio 0.91 (95% CI = 0.86-0.96) for colorectal cancer in relation to any statin use. However, there is no clear dose-response or duration-response relationship between filled statin prescriptions and colorectal cancer risk.

Duration of statin use may be important when investigating the chemopreventive effects of statins. We assessed exposure to statins measured as cumulative DDDs. Cumulative DDDs is a time-independent variable in which the daily supplies of each statin prescription dispensed were summed over time from January 1, 1996 to the index date. Because cumulative DDDs and statin duration are highly interrelated, it was not possible to model them together. Similar findings were noted when statin users were stratified by duration (data not shown).

There are at least two differences between our study and the study of Hachem *et al*[32]. First, their study population was limited to mostly male veterans with active access to health care and thus they were more likely to be prescribed a statin than the general population. Statin use was present in 51% of the study population. In our study this number was 19.4%. Second, the above-mentioned study was conducted among patients with diabetes who are known to have a higher likelihood of developing colorectal cancer[42]. Therefore, it is possible that it was easier to show benefit owing to the generally elevated risk in patients with diabetes[32]. Using an epidemiologic study which is restricted to patients with major risk factors means that the results of the restricted study may not necessarily apply to the portion of the population that was excluded. Whether a protective effect only occurs among patients who are already at higher risk of colorectal cancer requires further study. Other studies have reported a possible protective effect of statins in patients with diabetes on lung (adjusted OR = 0.43, 95% CI = 0.38-0.49)[24], pancreatic (adjusted OR = 0.32, 95% CI = 0.23-0.44)[25], and liver cancer (adjusted OR = 0.74, 95% CI = 0.64-0.87)[26].

One of the strengths of our study is the use of a computerized database, which is population-based and is highly representative. Because we included all patients newly diagnosed with colorectal cancer from 2005 to 2008, and because the control subjects in this study were selected from a simple random sampling of the insured general population, we can rule out the possibility of selection bias. Statins were available only on prescription. Because the data on statin use were obtained from an historical database which collects all prescription information before the date of colorectal cancer, recall bias for statin use was thus avoided.

Several limitations of the present study should be noted. First, although we adjusted for several potential confounders in the statistical analysis, a number of possible confounding variables, including family history of colorectal cancer, dietary habits or physical activity, and alcohol and tobacco use, which are associated with colorectal cancer were not included in our database. Second, we were unable to contact the patients directly about their use of statins because of anonymization of their identification number. Using pharmacy records representing dispensing data rather than usage data might have introduced an overestimation of statin use. However, there is no reason to assume that this would be different for cases and controls. Even if the patients did not take all of the statins prescribed, our findings would underestimate the effect of statin use. Third, lovastain and pravastatin (available in 1990), simvastatin (available in 1992), and fluvastatin (available in April, 1996) became available prior to patient enrollment in the database. Prescriptions for these drugs prior to 1996 would not be captured in our analysis. This could have underestimated the cumulative DDDs and may weaken the observed association. In addition, some exposure misclassification was likely caused by the fact that information on prescription was available only from 1996. Such misclassification, however, was likely to be non-differential, which would tend to underestimate rather than overestimate the association. Fourth, we were unable to analyze the risks for users of distinct statins separately due to the relatively small number of cases and the relatively small number of statin users. Fifth, data on the accuracy of discharge diagnoses are not available in Taiwan. Potential inaccurate data in the claims records could lead to possible misclassification. However, there is no reason to assume that this would be different for cases and controls. Lastly, as with any observational study, residual confounding by unmeasured factors which are different between cases and controls is also possible. However, the confounding effect of medical attention could be corrected for by introducing the number of hospitalizations into the conditional logistic regression model.

In summary, the results of this study do not provide support for an association between statin use and colorectal cancer risk. Given the widespread use of statins, it is prudent public health policy to continue monitoring cancer incidence among statin users, particularly as the duration of use is increasing[12].

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COMMENTS

Background

Experimental studies have shown that statins have potential protective effects against cancer. Several epidemiologic studies have investigated the association between statin use and risk of colorectal cancer, and the results are inconsistent.

Research frontiers

This study was undertaken to examine the relationship between statin use and the risk of colorectal cancer.

Applications

Statins are widely used cholesterol-lowering drugs, and the duration of use is increasing. Further and larger studies are needed to determine the long-term effects of statin use on cancer development and to clarify whether statins are truly effective for cancer chemoprevention.

Peer review

This is a nice population-based study which strength is the fact that Taiwan National Health Insurance research program provides extensive data of one million patients. The methodology and statistical analysis is adequate and altogether the authors provide convincing evidence that statin use does not protect against the risk of colorectal cancer.

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BRIEF ARTICLE

IκB kinase-beta inhibitor attenuates hepatic fibrosis in mice

Jue Wei, Min Shi, Wei-Qi Wu, Hui Xu, Ting Wang, Na Wang, Jia-Li Ma, Yu-Gang Wang

Jue Wei, Min Shi, Wei-Qi Wu, Hui Xu, Ting Wang, Na Wang, Jia-Li Ma, Yu-Gang Wang, Department of Gastroenterology, Shanghai Changning Central Hospital, Shanghai 200336, China

Author contributions: Wei J, Wang YG, Shi M and Xu H designed the study; Wei J, Wang YG, Xu H, Shi M, Wu WQ, Wang T and Wang N carried out the study; Wei J, Shi M, Xu H and Wang N contributed new reagents/analytic tools; Wei J, Wang YG and Ma JL analyzed the data; Wei J and Wang YG wrote the paper.

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Correspondence to: Yu-Gang Wang, MD, PhD, Department of Gastroenterology, Shanghai Changning Central Hospital, Shanghai 200336, China. wang_yugang@sina.com
Telephone: +86-21-62909911 Fax: +86-21-62906478

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Abstract

AIM: To investigate the anti-fibrosis effect of IκB kinase-beta inhibitor (IKK2 inhibitor IMD0354) in liver fibrosis.

METHODS: Twenty male C57BL6 mice were divided into four groups. Five high-fat fed mice were injected with lipopolysaccharide (LPS, 10 mg/kg) intraperitoneally and five high-fat fed mice were without LPS injection to build models of liver injury, and the intervention group (five mice) was injected intraperitoneally with IKK2 inhibitor (IMD 30 mg/kg for 14 d), while the remaining five mice received a normal diet as controls. Hepatic function, pathological evaluation and liver interleukin-6 (IL-6) expression were examined. Western blotting and real-time polymerase chain reaction were used to detect the expressions of nuclear factor-κB (NF-κB), alpha-smooth muscle actin (α-SMA), tumor growth factor-beta1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), type Ⅰ and type Ⅲ collagen proteins and mRNA.

RESULTS: A mouse model of liver injury was successfully established, and IMD decreased nuclear translocation of NF-κB p65 in liver cells. In the IMD-treated group, the levels of alanine aminotransferase (103 ± 9.77 μ/L *vs* 62.4 ± 7.90 μ/L, $P < 0.05$) and aminotransferase (295.8 ± 38.56 μ/L *vs* 212 ± 25.10 μ/L, $P < 0.05$) were significantly decreased when compared with the model groups. The histological changes were significantly ameliorated. After treatment, the expressions of IL-6 (681 ± 45.96 *vs* 77 ± 7.79, $P < 0.05$), TGF-β1 (Western blotting 5.65% ± 0.017% *vs* 2.73% ± 0.005%, $P < 0.05$), TNF-α (11.58% ± 0.0063% *vs* 8.86% ± 0.0050%, $P < 0.05$), type Ⅰ collagen (4.49% ± 0.014% *vs* 1.90% ± 0.0006%, $P < 0.05$) and type Ⅲ collagen (3.46% ± 0.008% *vs* 2.29% ± 0.0035%, $P < 0.05$) as well as α-SMA (6.19 ± 0.0036 μ/L *vs* 2.16 ± 0.0023 μ/L, $P < 0.05$) protein and mRNA were downregulated in the IMD group compared to the fibrosis control groups ($P < 0.05$).

CONCLUSION: IKK2 inhibitor IMD markedly improved non-alcoholic fatty liver disease in mice by lowering NF-κB activation, which could become a remedial target for liver fibrosis.

Key words: Liver fibrosis; IKK2 inhibitor; Nuclear factor-kappa B; Tumor growth factor-beta1; Interleukin-6; Alpha-smooth muscle actin; C57BL mouse

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INTRODUCTION

The incidence rate of non-alcoholic fatty liver disease (NAFLD) has increased annually. Simple steatosis in the

early stage may gradually develop into fatty hepatitis[1,2], and subsequently develop towards hepatic fibrosis and liver cirrhosis[3]. In an advanced stage, the incidence rate of liver cancer, multiple organ failure and other fatal complications reaches up to 0.6%-3%[4]. Based on the World Health Organization prognostication, chronic liver disease is the ninth leading cause of death in western countries and this situation will not be improved in the coming decades[5]. Among patients with non-alcoholic steatohepatitis (NASH), there were 10%-25% of patients that developed hepatic fibrosis or even liver cirrhosis[6-9]. The nosogenesis of NASH remains unclear, but the hypothesis of "secondary strike" has been widely accepted[10,11]. Unfortunately, some treatments initially gradually improve liver adipose degeneration, but are unable to achieve long-term control[9,12].

IκB kinase (IKK) is a large protein complex that is 700-900 kDa, including the two kinase subunits IKKα (IKK1) and IKKβ (IKK2) and one regulatory subunit that is either nuclear factor-kappa B (NF-κB) essential modifier or IKKγ. IKK2 is part of the inhibitor of the κB (IκB) IKK complex, which activates NF-κB through phosphorylation of the IκBs, leading to a series of inflammatory reactions[13-16]. Many effective drugs can reduce the inflammatory reaction in the liver by inhibiting the nuclear factor IKK2-NF-κB pathway and reducing insulin resistance in the liver[17]. Mathers *et al*[5] have demonstrated that application of the IKK2 inhibitor reduces fat accumulation in the liver and body weight gain in the mice. It has been reported that antioxidants inhibit the activity of NF-κB and can reduce inflammatory reactions[18] or even change fibrotic tissue[19].

We speculated that specific inhibition of NF-κB activation by an IKK2 inhibitor could effectively suppress the expression of inflammatory factors and even improve hepatic fibrosis. Therefore, in our study, a NASH model was established by a high-fat diet in mice and intraperitoneal injection of lipopolysaccharides (LPS) promoted acute hepatic injury and the expression of inflammatory factors. An IKK2 inhibitor (IMD0354) was used to suppress the NF-κB signaling pathway. Liver function and histological changes were observed and expression levels of interleukin-6 (IL-6), tumor growth factor-beta1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), as well as other pro-inflammatory and pro-fibrosis factors, were determined. We focused on measurement of the fibrosis index, which represented hepatic stellate cells (HSCs), alpha-smooth muscle actin (α-SMA) and the expression levels of collagen I , collagen Ⅲ and mRNA, which showed fibrotic hepatic changes. Accordingly, we investigated potential therapeutic prospects of the IKK2-NF-κB signaling pathway for reversing fibrosis in NAFLD.

MATERIALS AND METHODS

Experimental protocol and animal model

Twenty-four-week-old male C57BL6 mice, weighing approximately 12-16 g, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Science. Mice were housed in a clean grade barrier systems laboratory in the Medical Laboratory Animal Center of Shanghai Jiao Tong University. Animals were randomized into four groups: the control group (n = 5), high-fat (HF) diet group (n = 5), HF + LPS group (n = 5), and HF + LPS + IMD (IKK2 inhibitor) group (n = 5). The mice in the control group were given a normal diet (ND) and the HF group animals were fed with an HF diet for 10 wk. The ND chow was supplied by the Animal Center of the Medical College of Subsidiary Basic Medical of Shanghai Jiao Tong University. The HF diet (50% fat, pork fat 18%, yolk 12%, sugar 8% and basal diet 62%) was supplied by SLAC Precision Equipment Inc. Mice in the intervention group were intraperitoneally injected with 30 mg/kg IKK2 IMD 0354 (Tocris Bioscience, Bristol, United Kingdom) for 14 d, and at the end of 12 wk this was combined with intraperitoneal injection of 10 mg/kg LPS (Sigma-Aldrich, St Louis, MO, United States). Mice were then sacrificed after fasting for 12 h. Subsequently, 1 mL eyeball blood was obtained and all mice were killed by cervical dislocation. The liver tissue was fast fixed and lightly washed in ice-cold phosphate buffered solution (PBS). Then, part of the liver was placed into 10% formalin fixation solution, while the other part of the liver was quickly stored at -70 ℃ for cryopreservation. The blood was sent to the laboratory of Renji Hospital for liver enzyme assays. Some liver tissue was embedded in paraffin for 24 h and was then observed by hematoxylin and eosin (HE) staining, Masson staining and immunohistochemistry (IHC). Other liver tissue was saved under an ultra low temperature for Western blotting and polymerase chain reaction (PCR) procedures.

Biochemical and liver enzymes assays

Serum was collected to analyze alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using automatic biochemical instrumentation at Renji Hospital Lab, Shanghai, China.

Histopathological staining and analysis

For HE and Masson staining, 4-μm liver tissue sections were cut from the same position and embedded in paraffin after being stabilized in 10% formalin. Changes in liver tissues were observed under a light microscope.

Evaluation of inflammation activity: scores of inflammation activity were in accordance with chronic liver disease activity[20], and were divided into four parts; i.e., portal area inflammation (P), lobular inflammation (L), patch necrosis (PN) and bridging necrosis (BN, including lobular necrosis). Every item was recorded as 1, 2, 3 or 4 based on degrees of pathological changes. The scores counting formula was: $P + L + 2 \times (PN + BN)$.

Fatty hepatic fibrosis: fibrosis scores were divided into four stages according to the degree of fibrosis in three areas of the liver; i.e., the liver acinus, the portal vein, and bridging fibrosis, as well as the presence or

absence of liver cirrhosis. S1 indicated perisinusoidal space fibrosis of three areas of the local or extensive liver acinus; S2 indicated the above pathological changes with local or extensive periportal fibrosis; S3 indicated S2 pathological changes with local or extensive bridging fibrosis; and S4 indicated fatty liver cirrhosis, forming fibrous septa that divided lobuli hepatic and central veins to the portal area and formed false lobules.

Immunohistochemical analysis

Liver tissue sections (4 μm) were prepared for IL-6 immunohistochemical study. The glass was treated by polylysine to promote cell attachment. Microwave antigen repairing was carried out with 0.01 mol/L citrate buffer solution (pH 6.0). After blocking with rabbit serum, the sections were incubated overnight at 4 ℃ with monoclonal primary antibody against mouse IL-6 (PPMX, Tokyo, Japan). On the next day, liver sections were taken out and washed with PBS three times and were incubated with the second antibody for 1 h at room temperature. Coloration with freshly prepared diaminobenzidine (DAB) was performed, and then the tissues were counterstained with hematoxylin, dewatered, and then mounted with neutral gum. The second antibody in the ElivisionTM plus Polyer HRP (Mouse/Rabbit) IHC Kit and DAB developer were supplied by Manxin Bio Co®, Fuzhou, China. PBS taking the place of the primary antibody was considered as the negative control. We chose 10 views of each section under light microscopy to obtain the average positive absorbance using ImageProplus2.0.

Enzyme linked immunosorbent assay

Liver (1 g) was placed in PBS with 0.1 mmol/L phenylmethyl sulfonylfluoride (PMSF, Sigma) and was then manually homogenized, centrifuged at 100 000 r/min for 15 min at 4 ℃, and the supernatant was removed. Double antibody enzyme linked immunosorbent assay (ELISA) (ELISA kit TNF-α, BD Bioscience, Franklin Lakes, NJ, United States) was used for detection, and the procedure was strictly based on the guidelines provided by the manufacturer.

Western blotting analysis of NF-κB p65, TGF-β1 and α-SMA

Liver tissue was saved in a refrigerator at -80 ℃ after homogenization, and the tissue protein extract solution was prepared by centrifugation. Based on the operating instructions of the bicinchoninic acid protein quantitation kit (Sunbio, Beijing, China), the concentration of protein was detected. After denaturation, each tissue protein was sampled at 50 μg, and reducibility was performed with sodium dodecyl sulfate polyacrylamide gel electrophoresis cataphoresis in an 8% polyacrylamide gel. Sampling after denaturation was performed and electrophoresis was started. The electrophoresis voltage of the condensed glue and separation gel was 80 V and 120 V, respectively. The electrophoresis terminated after bromophenol blue electrophoresis moved to the bottom of the glue, and a damp-dry transmembrane (polyvinylidene fluoride membrane) was applied with a constant 50 mA current for 90 min. The membrane was sealed at room temperature with 5% defatted milk powder prepared with Tris-buffered saline Tween-20 (TBST) and primary antibodies (NF-κB p65, TGF-β1, α-SMA and β-actin, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, United States) and was incubated overnight in a swing bed at 4 ℃. After the film was washed with TBST buffer solution in the swing bed, the second antibody 1:3000 (rabbit polyclonal antibody, Manxin Bio, Fuzhou, China) was incubated at room temperature for 1 h. After being repeatedly washed in TBST buffer solution, DAB staining was performed. After proper staining, the reaction was terminated by water. In a dark room, a nitrocellulose filter was put into a brightening agent with sufficient contact, and was then exposed to light with an X-ray device. The image was developed and fixed. Simultaneous determination of the expression level of β-actin in the same filter was carried out as an internal control. Separate analyses were performed for each sample and the experiment was repeated three times. We obtained the integrated density value by Microsoft BandScan, and the ratio of the target bands to β-actin substantiated the presence of the proteins NF-κB p65, TGF-β1 and α-SMA.

RNA extraction and analysis of mRNA expression of types Ⅰ and Ⅲ collagen, α-SMA and TGF-β1

Total RNA was isolated from snap-frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, United States) and the ratio between the absorbance values at 260 nm and 280 nm gave an estimate of RNA purity. Real-time (RT)-PCR was performed using a one-step RT-PCR kit from the Shanghai Daweike Biotechnology Company. Two micrograms of the total RNA was chosen and reverse transcription was performed. Its reaction product was placed into a 50-μL PCR reaction system. α-SMA, TGF-β1, types I and Ⅲ collagen, and the specific primer of the internal reference β-actin was used in PCR amplification, and agarose electrophoresis was performed. Electrophoresis results were scanned with a BioSens GelImaging System. For the PCR primer sequences and fragment lengths (Table 1). The real-time survey meter (7500 Sequence Detection System) was obtained from ABI, United States. The PCR conditions were: predegeneration for 2 min at 50 ℃, denaturation for 20 s at 95 ℃, annealing for 45 s at 60 ℃, and extension for 30 s at 72 ℃, with a total of 40 cycles; an internal reference of β-actin was used with predegeneration for 3 min at 94 ℃, denaturation for 20 s at 95 ℃, annealing for 20 s at 60 ℃, extension for 30 s at 72 ℃, with a total of 35 cycles, and extension again for 10 min after the cycles, and then termination at 4 ℃.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed with a one-way analysis of variance using SPSS17.0 software, followed by Scheffe's test, and com-

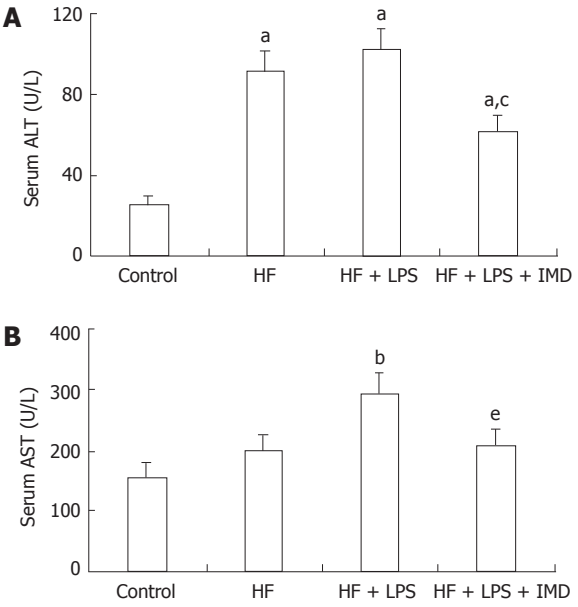

Figure 1 IKK2 inhibitor prevented HF + LPS-induced liver injury, as determined by serum ALT and AST levels. The normal values for ALT and AST were 45 U/L and 160 U/L. Serum ALT (A) and AST (B) were measured in different groups (control group, HF group, LPS-induced HF group and IMD-treated group), data are expressed as mean ± SD. A: $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* the HF and LPS + HF groups; B: $^{b}P < 0.01$ *vs* the control group, $^{e}P < 0.05$ *vs* the LPS + HF group. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

parisons between groups was performed using the Mann-Whitney test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of IKK2 inhibitor (IMD0354) on serum ALT and AST

The levels of ALT and AST for each group are shown in Figure 1. In Figure 1A, the ALT levels of the HF and HF + LPS groups were significantly increased compared to the control group ($P < 0.05$). After treatment with the IKK2 inhibitor (IMD0354), the level of serum ALT in the mice was significantly decreased compared to the HF group ($P < 0.01$), as well as in the HF + LPS group ($P < 0.05$), but was still higher than that of the control group ($P < 0.05$). Figure 1B shows that the change in serum AST was not as significant as that of ALT. The level of serum AST in the HF + LPS group was significantly increased compared to the control group ($P < 0.01$). The level of serum AST when treated with IMD0354 was significantly decreased compared to that of the HF + LPS group ($P < 0.05$).

Effects of IKK2 inhibitor on liver inflammation and fibrosis during liver injury development

HE staining results showed that pathological changes in the mice were in line with the diagnostic gold standard of chronic NASH (Figure 2A). At the same time, typical hepatic fibrosis was observed with Masson staining (Figure 2B). In the control group, the structure of the hepatic lobules was clear without inflammatory cell infiltration in the portal area and without fibrotic tissue hyperplasia. The liver sections of the HF and HF + LPS groups showed that the normal structure of the hepatic lobules was lost, the structure of the blood vessels in the liver was disordered with severe liver cell degeneration, patch necrosis and bridging necrosis, and there were many inflammatory cell infiltrates in the portal area. There was also light to moderate hyperplasia of the broglia fibrils and fibrous septa were formed occasionally. Inflammation and fibrosis scores showed significant differences compared with the control group ($P < 0.05$). In the IMD-treated group, the structure of the hepatic lobules was normal, liver cell degeneration was significantly decreased, and liver cell necrosis and inflammatory cell infiltration were significantly improved. Fibrillar collagen sediment still existed, which was significantly reduced compared with the controls, and its inflammation score was also significantly decreased ($P < 0.05$), but was still higher than that of the control group ($P < 0.05$, Table 1). The results of the fibrosis scores were analyzed by Mann-Whitney statistical methods, and the results showed that there was a significant difference between the HF + LPS + IMD and HF + LPS groups ($Z = -2.35$, $P = 0.018$, $P < 0.05$, Table 1). There were no differences among the other groups.

Table 1 Pathological scores in liver tissues

Groups	$P + L + 2 \times (PN + BN)$	S0	S1	S2	S3	S4	Z
Control	0	5	0	0	0	0	
HF	10.70 ± 2.62^{a}	0	3	2	0	0	
HF + LPS	13.30 ± 3.83^{a}	0	1	3	1	0	
HF + LPS + IMD	$4.60 \pm 3.83^{a,c}$	0	4	1	0	0	-2.35^{e}

The score of inflammation is given by $P + L + 2 \times (PN + BN)$. $^{a}P < 0.01$ *vs* the control group; $^{c}P < 0.01$ *vs* the HF and HF + LPS groups; $^{e}Z = -2.35$, $P = 0.018$, $P < 0.05$ *vs* the HF + LPS group. *P*: Portal area inflammation; L: Lobular inflammation; PN: Patch necrosis; BN: Bridging necrosis; HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

Immunohistochemistry assay for the changes in IL-6 expression in the livers of mice

Previous studies have demonstrated that many cell factors, such as TNF-α and IL-6, play important roles in the NF-κB dependent signaling pathway[21]. Some evidence has indicated that TNF-α, as well as IL-6, participated in the formation of hepatic fibrosis and had a positive correlation with the level of serum hyaluronic acid, laminin type Ⅳ, for example, suggesting that TNF-α, as well as IL-6, not only mediated inflammatory reactions but also participated in the formation of hepatic fibrosis during the promotion of extracellular matrix (ECM) synthesis. In this study, the average absorbance values (*A*) of liver cell positive immunity of mice in each group were analyzed by Image-pro plus 6.0 and statistical analysis was performed, showing that there was a small amount of IL-6 expression in the control and HF groups, and the expression of IL-6 was significantly increased in the HF

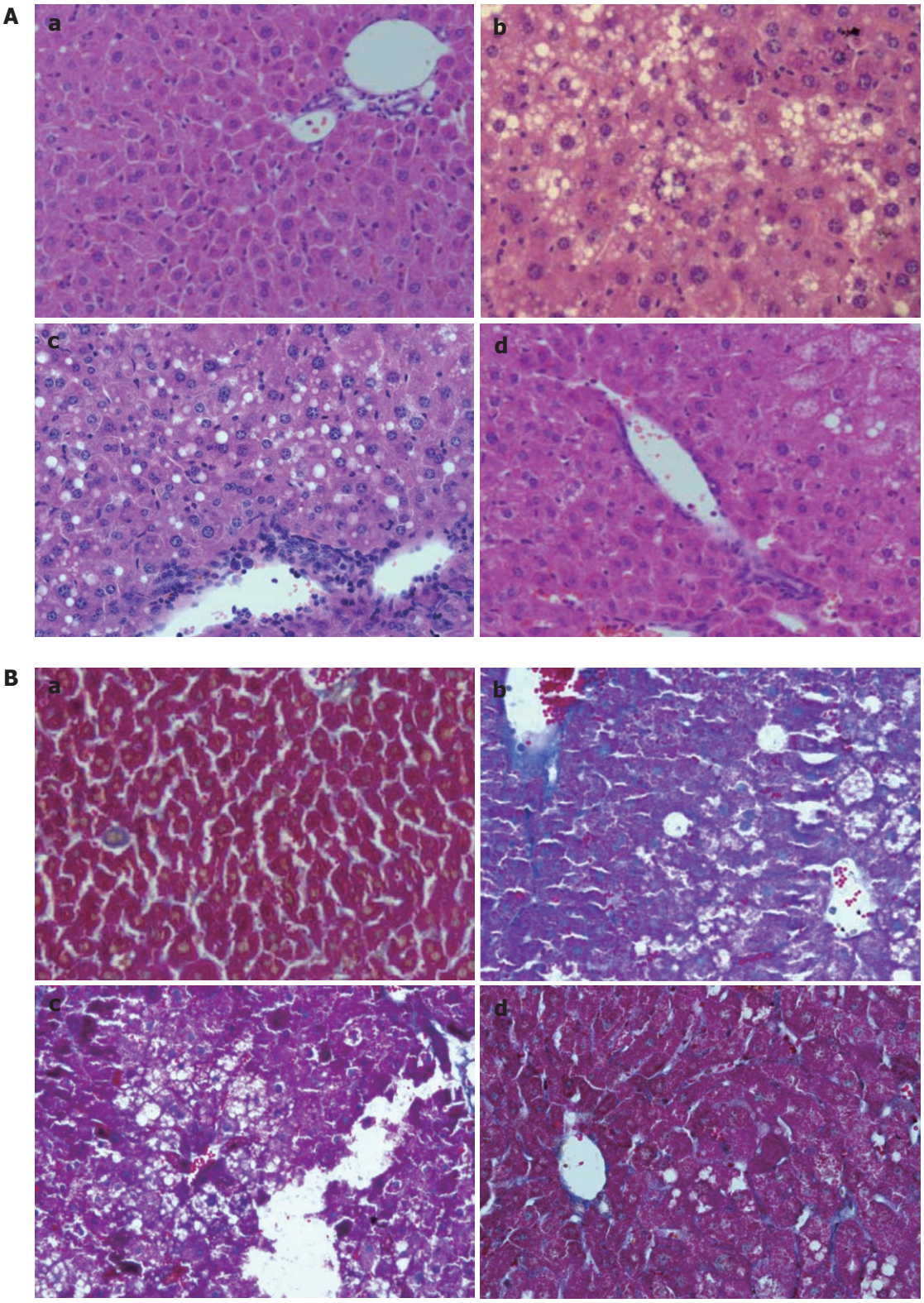

Figure 2 Hematoxylin and eosin stain and Masson staining in sections of (a) control group; (b) HF group; (c) HF + LPS group; and (d) HF + LPS + IMD group. A: Hematoxylin and eosin stain. Macrovesicular steatosis, lobular inflammation and balloon degeneration of hepatocytes were observed in liver sections of HF-treated mice and HF + LPS + treated mice with a significantly large amount of inflammatory cell infiltration surrounding the centrilobular veins of the liver. Significant amelioration was observed in the group treated with IMD (d); B: Masson staining. A thin lining of collagen was observed in the HF group, HF + LPS group and HF + LPS + IMD group. With LPS treatment, there was an increase in the amount of collagen accumulated along the central vein with the presence of collagen in the pericellular area. Treatment with IMD reduced LPS-induced collagen accumulation. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

group after being activated by LPS ($P = 0.013$, $P < 0.05$). IL-6 expression was mainly concentrated in the liver cell cytoplasm around the central veins and portal area, appearing as brown and grainy, and was also expressed

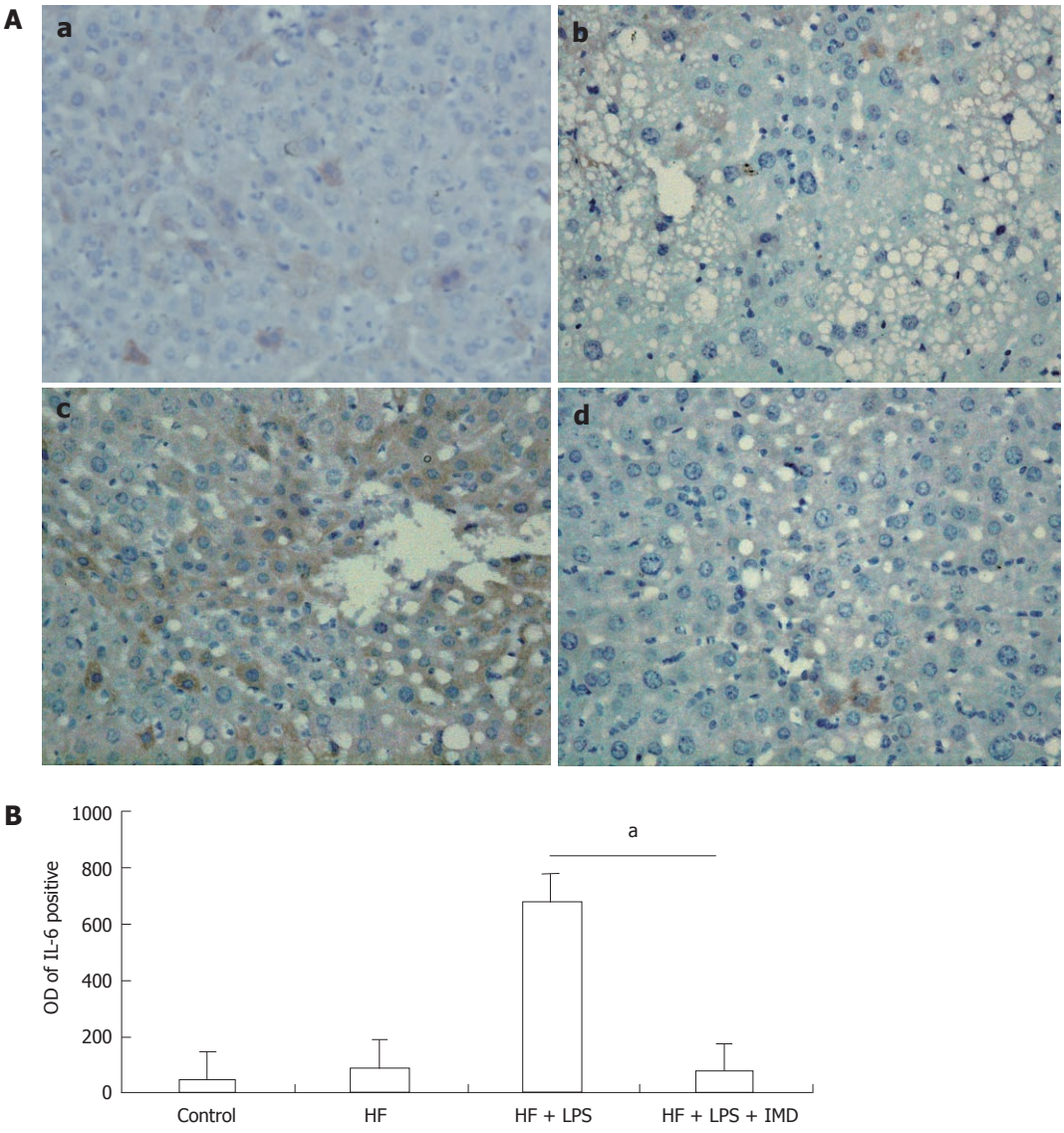

Figure 3 Interleukin-6 expression was assessed by immunohistochemistry. A: Positive staining was observed in hepatocytes in the control group (a), HF group (b), LPS-induced HF group (c) and IMD-treated group (d). B: The optical density (OD) of interleukin-6 (IL-6)-positive areas was measured with ImageProplu6.0 ($^{a}P < 0.05$). HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

in the sinus hepaticus and parts of monocytes. In the HF + LPS group treated with the intervention of IMD, the expression of IL-6 was significantly decreased ($P = 0.012$, $P < 0.05$). There was no significant difference when compared with the HF group ($P = 0.70$, $P > 0.05$), and the expression of IL-6 was higher than the control group (Figure 3).

IKK2 inhibitor (IMD) inhibited an LPS-induced increase in the pro-inflammatory cytokine levels of TNF-α in mice livers

Recent research has shown that the IKK2-NF-κB signal pathway participate in insulin resistance, and cell factors of TNF-α and IL-6 play important roles dependent on NF-κB signals[21], especially in the pathological process of hepatic fibrosis[17,22]. Therefore, TNF-α was the key pro inflammatory factor, which was likely to induce the formation and development of hepatic fibrosis. The protein levels of TNF-α in mouse livers were detected by ELISA. With the development of liver injury, an increased expression of TNF-α was shown in the liver[23]. The levels of TNF-α in mouse livers of the HF and HF + LPS groups were significantly increased compared to the control group ($P < 0.05$). After intervention with the IKK2 inhibitor, the level of TNF-α was significantly reduced compared to the non-intervention group ($P < 0.05$, Figure 4).

IKK2 inhibitor (IMD) decreased nuclear translocation of NF-κB p65 in mice livers in response to LPS and HF exposure

When combined with IκBα in the cytoplasm, NF-κB was inactive. IKK2, as the major subunit of promotion, activated NF-κB and its subunit with phosphorylation, while IMD could inhibit the activation of NF-κB p65 and its nuclear transcription[24]. Therefore, expressions

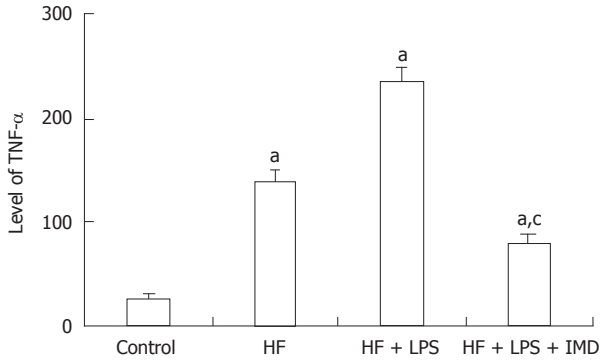

Figure 4 The levels of nuclear factor-κB-dependent pro inflammatory cytokines and tumor necrosis factor-alpha were measured in livers obtained from the control group, HF group, HF + LPS group and HF + LPS + IMD group. $^{a}P < 0.05$, compared with the control group. $^{c}P < 0.05$, significant compared with both the HF and LPS + HF exposed groups. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor; TNF-α: Tumor necrosis factor-alpha.

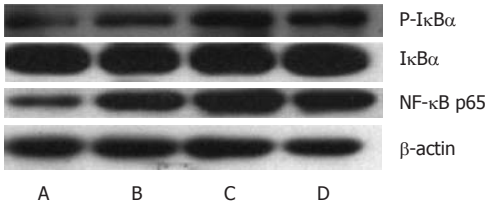

Figure 5 IKK2 inhibitor decreased lipopolysaccharide-induced nuclear translocation of nuclear factor-κB p65 and P-IκBα in livers. Nuclear levels of the p65 subunit of nuclear factor-κB (NF-κB) were measured by Western blotting in different groups (A: Control; B: HF group; C: HF + LPS group; D: HF + LPS + IMD group). β-actin was used as a loading control. Administration of IMD at 30 mg/kg doses decreased the DNA binding activity of NF-κB, which was induced by HF and LPS in mice livers. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

of NF-κB p65 and P-IκBα in the livers in each group was detected to determine the inhibitory action of IMD. Western blotting showed that the expression of NF-κB p65 in the HF group was increased compared to that of the normal diet group. In the HF group, LPS promoted the expression of NF-κB p65, and P-IκBα increased simultaneously, while intervention by the IKK2 inhibitor reduced the pro-inflammatory role of LPS and significantly reduced the expression of NF-κB p65 and its subunit (Figure 5).

IKK2 inhibitor (IMD) decreased protein levels of TGF-β1 and α-SMA related to fibrosis in livers

TGF-β1 is an important inflammatory factor that stimulates accumulation in the ECM and tissue fibrosis. Our results showed that TGF-β1 expression in the liver was increased under the condition of the HF diet and stimulation of LPS. While α-SMA was a marker of HSC activation, its variation tendency was similar to TGF-β1. In the mouse liver model group, the expression of α-SMA was significantly increased compared to the control group. After application of the IKK2 inhibitor, the protein expression of TGF-β1 in livers decreased, and the expression of α-SMA was reduced accordingly.

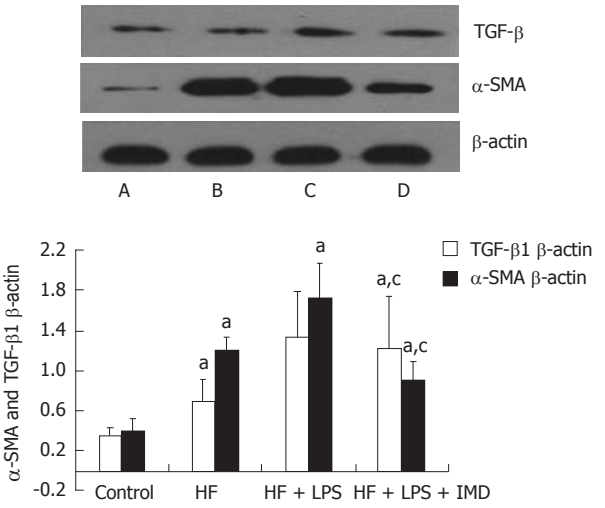

Figure 6 Western blotting analysis of tumor growth factor-beta1, alpha-smooth muscle actin proteins were measured that were involved in IKK2-nuclear factor-κB pathways in the liver in different groups (A: Control; B: HF group; C: HF + LPS group; D: HF + LPS + IMD group). β-actin was used as a loading control. The levels of tumor growth factor-beta1 (TGF-β1) and alpha-smooth muscle actin (α-SMA) measured in livers were increased in the HF and HF + LPS groups. IKK2 inhibitor significantly inhibited LPS and HF-induced expression of TGF-β1 and α-SMA in mouse livers. The ratio of TGF-β1 and α-SMA/β-actin in the liver was increased in other groups, compared with the control group, $^{a}P < 0.05$. IKK2 inhibitor normalized TGF-β1 and α-SMA significantly compared with the HF or LPS + HF groups. $^{c}P < 0.05$. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

Table 2 Oligonucleotide sequences used in real-time polymerase chain reaction

mRNA	Sequence	Length (bp)
Type Ⅰ (Ⅰ α) collagen	F: ACAGTGGTGAACCTGGTGCT	151
	R: CTCCTTTGGCACCAGTGTCT	
Type Ⅲ collagen	F: GGAGCCCCTGGACTAATAG	193
	R: ATCCATCTTTGCCATCTTCG	
α–SMA	F: TGCTGTCCCTCTATGCCTCT	185
	R: GAAGGAATAGCCACGTCAG	
TGF-β1	F: CTTGCCCTCTACAACCAACA	189
	R: CTTGCGACCCACGTAGTAGA	
β-actin	F: TGTGTCCGTCGTGGATCTGA	126
	R: CTTGCGACCCACGTAGTAGA	

α–SMA: Alpha-smooth muscle actin; TGF-β1: Tumor growth factor-beta1.

Correspondingly, activation of HSCs and the formation of collagen decreased, leading to effectively preventing hepatic fibrosis (Figure 6).

IKK2 inhibitor IMD inhibited α-SMA, TGF-β1, types Ⅰ (α Ⅰ) and Ⅲ collagen and mRNA expression in LPS-stimulated mice

The formula described previously was used to measure mRNA relative expression (amount = $2^{-\Delta ct} \times 100\%$), and the relative expression levels of α-SMA, type Ⅰ (α Ⅰ) collagen, type Ⅲ collagen and TGF-β1 mRNA were obtained. RT-PCR was performed with the mouse primers shown in Table 2. The results showed that the expression of TGF-β1 mRNA in the HF group and the HF + LPS group was higher than the control group ($P < 0.05$),

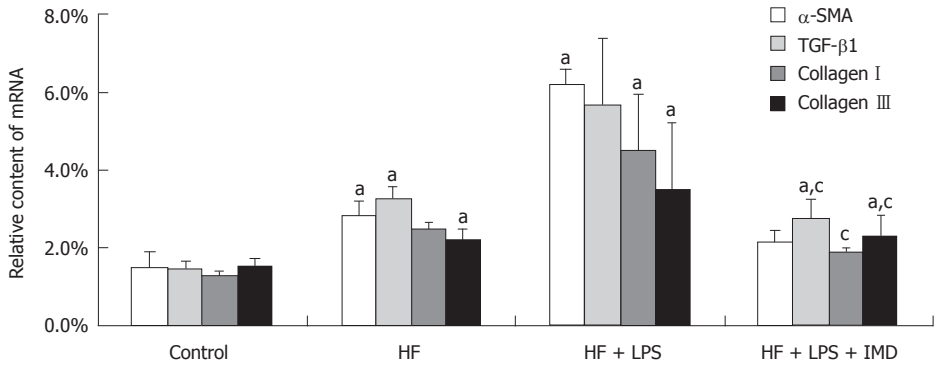

Figure 7 The IKK2 inhibitor inhibited LPS and HF-induced increases in pro inflammatory cytokine levels in mouse livers. The level of tumor growth factor-beta1 (TGF-β1) was measured in the livers of mice in the control, HF, HF + LPS and HF + LPS + IMD groups. Also, expression of the fibrosis index, such as alpha-smooth muscle actin (α-SMA), type Ⅰ collagen and type Ⅲ collagen, were detected in the four groups by real-time polymerase chain reaction. The level of TGF-β1 measured in livers was increased in the HF and HF + LPS groups, compared with the control group, $^{a}P < 0.05$. The mRNA content of type Ⅰ collagen in the HF group and type Ⅲ collagen in the HF + LPS group were significantly higher, $^{a}P < 0.05$, compared with the control group. Intraperitoneally administered IKK2 inhibitor normalized the expression of TGF-β1, as well as the contents of α-SMA, type Ⅰ and type Ⅲ collagen mRNA, compared with the HF or LPS + HF groups. $^{a}P < 0.05$, compared with the control group. $^{c}P < 0.05$, compared with both HF group and LPS + HF exposed group. **HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.**

which was significantly decreased after intervention of IMD. In addition, the fibrosis indexes of α-SMA, type Ⅰ collagen and type Ⅲ collagen in the model group were also increased. In addition, the contents of type Ⅰ collagen in the HF diet group and type Ⅲ collagen in the HF + LPS group were significantly increased, independently, compared to the control group ($P < 0.01$, $P < 0.05$, respectively). After intervention with IMD, the levels of α-SMA, type Ⅰ (α Ⅰ) collagen and Ⅲ collagen were significantly decreased compared to the HF + LPS group ($P < 0.05$). However, there was no significant difference in α-SMA and type Ⅲ collagen with intervention of IMD and the normal group ($P > 0.05$, Figure 7).

DISCUSSION

NAFLD includes simple liver steatosis, NASH and liver cirrhosis, while NASH has become a central issue of chronic liver disease with worldwide attention[7]. Currently, the pathogenesis of NAFLD is not clear, and the hypothesis of "secondary strike" has been widely accepted. It is well known that insulin resistance is involved in the process, and inflammatory reaction, lipid peroxidation, and oxidative stress also play important roles[25-27]. The best measure for preventing the progression of hepatic fibrosis is to prevent or reverse the initial cascade reactions of fibrosis[28]. Therefore, inhibition of the generation of inflammatory factors effectively reduces HSC activation, decreases accumulation in the ECM, and fundamentally reverses fibrosis[29]. The expression of NF-κB is significantly increased in NASH patients, and TNF-α, IL-6, TGF-β, and other inflammatory factors also showed high expression levels[23]. Further research has found that activation of NF-κB is the key step in regulating gene expression of various kinds of pro inflammatory factors in NASH patients[23]. TNF-α is the key pro inflammatory factor and it induces the formation and development of hepatic fibrosis. TGF-β1 mediates the synthesis of different kinds of collagen with time dependence. However, inhibition of TGF-β1 significantly reduces the synthesis of collagen and sedimentation of the ECM[30].

It has been reported that blockage of IKKβ (IKK2) significantly reduces the incidence rate of liver steatosis and improves NASH pathologically[31]. Is it possible that IKKβ (IKK2)-NF-κB is also a key in improving and even reversing hepatic fibrosis? Various macromolecular protein-joining enzymes, including IKKβ (IKK2), IKK or NF-κB inhibitors, have become new types of anti-inflammatory agents; therefore, many researchers have tried to inhibit NF-κB-mediated proinflammatory responses based on these agents. The IKK2 inhibitor played a specific anti-inflammatory role through inhibition of the major subunit IKKβ, which served as a promotor in the IKK protein kinase complex center. In our study, the IKK2 inhibitor (IMD 0354) was used in liver injury in mice. We detected its inhibitory effect on liver NF-κB-dependent inflammatory factors, changes in liver function, histological changes and, at the same time, the expression of TGF-β1 involving fibrosis and relevant fibrosis indexes, such as α-SMA, type Ⅰ collagen and type Ⅲ collagen. It is warranted to investigate the potential therapeutic effect of IMD on hepatic fibrosis.

In our study, during HF-diet-induced chronic non-alcoholic hepatic injury in mice, the serology index of ALT was doubly higher than that of the control group. As for pathological changes, moderate to severe steatosis was observed, inflammatory infiltration was found in the lobules, local inflammatory infiltration was detected in the portal area, Masson staining showed fibrous tissue hyperplasia, Western blotting and RT-PCR results demonstrated that TGF-β1 expression increased, α-SMA content was raised, and there was sedimentation of types Ⅰ and Ⅲ collagen. Therefore, a hepatic injury model with typical inflammation and fibrosis pathological manifestation was successfully established by an HF diet and intraperitoneal injection of LPS, stimulating activation of NF-κB and promoting an inflammatory reaction[32-34].

When chronic hepatic injury occurs, different initial causative agents trigger the activation of HSCs, activation of the Janus kinase-signal transducers and activators of transcription signal transduction pathway[35], promotion of α-SMA expression in sinus hepaticus cells, further proliferation and activation, and synthesis and secretion of the ECM and collagen, which finally enhances the occurrence of hepatic fibrosis. Thus, expression of α-SMA has been considered as one of the dominant features of HSC activation, and has become an important evaluation index for hepatic fibrosis. In our study, when the mice were stimulated with LPS and an HF diet, the expression of α-SMA increased in the liver. At the same time, sedimentation of types I and Ⅲ collagen also occurred, suggesting that HSC was triggered and activated, and then started the process of hepatic fibrosis. However, the results for the group treated with IMD showed that expression of α-SMA, as well as sedimentation of types I and Ⅲ collagen, were significantly reduced, suggesting that inhibition of inflammatory factor expression also effectively suppressed HSC activation, and accordingly blocked the occurrence of hepatic fibrosis from the source.

Fatty tissue was the principle source of cell factors, liver steatosis promoted macrophage infiltration, and activation of HSC promoted the cascading release of many kinds of cell factors with an intensive pro inflammatory role, which further made the pathological changes and insulin resistance more serious[36,37]. We found that inflammatory cell and inflammatory factor expression after stimulation with LPS significantly increased compared to that in the HF group. Liver steatosis in the mice treated with intraperitoneal injection of the IKK2 inhibitor was significantly improved, and inflammatory factors released from the hepatic cell fat were correspondingly reduced compared to that of the HF and LPS groups. In addition, it was shown that the IKK2 inhibitor could significantly reduce pro inflammatory stimulation by LPS, and even reverse the fibrosis process in a mouse hepatic injury model. Western blots demonstrated that NF-κB p65 activation was significantly inhibited, and NF-κB-dependent pro inflammatory factors, such as IL-6, were simultaneously suppressed. Separation of NF-κB and its suppressor factor IκBα resulted in continuous activation of intercellular adhesion molecule-1 and other cell factors, and finally the up regulation of IL-6. The increase in IL-6 stimulated HSC proliferation, induced production of multiple acute-phase proteins, and promoted ECM sedimentation by facilitating matrix degeneration or interaction with its adhesion receptor, leading to significant hepatic fibrosis[38,39]. Similarly, the high expression of IL-6 significantly promoted liver apoptosis. In our study, it was found that after intervention with the IKK2 inhibitor, NF-κB p65 activation was significantly inhibited, IL-6 expression in the livers of LPS model mice was significantly decreased with the improvement of serology indexes and histological changes, and blocking IL-6 expression significantly improved hepatic injury, which could demonstrate that the IKK2 inhibitor could improve hepatic fibrosis by inhibition of the inflammatory factor IL-6.

It is known that TNF-α promotes insulin resistance and the development of liver inflammation, which is related to multiple cell factors, and induces the synthesis of IL-1, IL-6 and C-reactive protein, including caspase 3 and growth arrest and DNA-damage-inducible beta. Adiponectin inhibits expression of TNF-α, as well as other inflammatory factors, with positive feedback[40,41]. A peroxisome proliferator-activated receptor antagonist blocked TNF-α mediated insulin resistance, and it also had intensive anti-inflammatory action[42,43], which made the inflammation signal transduction pathway become a multiple cross and participated in the process of hepatic fibrosis[44]. The level of TNF-α could better reflect the regulatory condition of the inflammatory reaction in hepatic fibrosis. Hepatocellular carcinoma (HCC) invariably develops within a setting of chronic inflammation caused by metabolic liver disease or autoimmunity. Mechanisms that link these two processes are not completely understood, but transcription factors of the NF-κB family have been suggested to be involved. Cytokines such as IL-6 are clearly pivotal players, and high levels of serum IL-6 correlate positively with tumor size and with poor prognosis in HCC patients[45]. Our results showed that the levels of IL-6 and TNF-α in mouse livers in which hepatic fibrosis existed increased, while the IKK2 inhibitor reversed such an imbalance, and α-SMA expression in the liver and sedimentation of collagen I and collagen Ⅲ decreased, illustrating that TNF-α improved liver pathological changes in hepatic fibrosis in mice, relieved inflammatory cell infiltration, and reduced fiber hyperplasia by the IKK2-NF-κB-dependent signaling pathway.

During the process of HSCs activation, TGF-β1 plays a major role as a fibroblast growth factor[46], and the major stimulating factor promoting HSCs to accumulate ECM[47]. TGF-β1 receptors on the surface of HSC complete the signaling pathway combined with Smads[48], which continuously stimulates HSC activation, and finally transcribes target gene expression in the HSC nucleolus, mainly including type I collagen, which plays a key regulatory role in ECM metabolism and function. However, there is evidence demonstrating that NF-κB does not directly activate HSCs[39]. In our experiment, when injury and hepatic fibrosis occurred, protein and gene expression of TGF-β1 increased. While inhibiting the activation of NF-κB certainly reduced the activation of HSCs, TGF-β1 expression was decreased, and the expressions of various kinds of fibrosis factors, such as α-SMA and type I and type Ⅲ collagen, decreased. Therefore, we suggest that indirect correlation or other pathways exist between NF-κB and HSCs, which indirectly reduce TGF-β1 expression, decrease continuous activation of HSCs, and then improve the fibrosis process.

In brief, our *in vivo* experiment demonstrated that an IKK2 inhibitor could significantly decrease the expres-

sion of various inflammatory factors in mouse livers after exposure to LPS, which could play an anti-fibrosis role in inhibiting inflammation, reducing collagen content in liver tissues, and decreasing the expression of hepatic fibrosis correlation factors. IMD inhibited the phosphorylation activation of IκBα and NF-κB stimulated by LPS. Meanwhile, the expression of NF-κB-dependent inflammatory cytokines were suppressed, illustrating that inflammatory factors inducing hepatic injury were effectively reduced by inhibition of the NF-κB signaling pathway. In addition, we found that various fibrosis markers, such as TGF-β1 and α-SMA, as well as type I collagen and type Ⅲ collagen, were decreased in the liver, demonstrating that HSC activation was decreased and ECM accumulation was reduced. Therefore, it was presumed that the mechanism of the IKK2 inhibitor in anti hepatic fibrosis might be relevant, with the inhibition of cell factors promoting HSC activation, indirect inhibition of HSC proliferation, suppression of continuous activation of HSCs by TGF-β1, and then secretory volume and expression levels of α-SMA being reduced. Finally, the extent of hepatic fibrosis was decreased. The major pathological changes of chronic fatty liver disease are insulin resistance and inflammatory reactions[49,50]. In summary, the NF-κB signaling pathway participates with the IKK2 inhibitor playing an influential role in NASH and hepatic fibrosis. Inhibiting the production of a variety of inflammatory factors could effectively reduce HSC activation, decrease accumulation in the ECM, and then reduce fibrosis formation.

COMMENTS

Background

The nuclear factor-κB (NF-κB) signaling pathway improves insulin resistance and fat accumulation in the development of nonalcoholic fatty liver disease (NAFLD). Inhibition of NF-κB activation by an IKK2 inhibitor could effectively suppress the expression of many kinds of inflammatory factors, and even improve hepatic fibrosis. Therefore, the authors of this study investigated the potential therapeutic prospects of the IKK2-NF-κB signaling pathway in the reversion of fibrosis in NAFLD.

Research frontiers

The study is believed to be the first to evaluate the role of IKK2-NF-κB signals in NAFLD. The potential effect of an IKK2 inhibitor is likely to block inflammation through inhibiting NF-κB activation. The IKK2 inhibitor may play an important role in the occurrence and development of NAFLD.

Innovations and breakthroughs

This study explored one of the possible mechanisms of inflammation, which could produce a potentially facilitative effect in the occurrence and development of NAFLD.

Applications

This study provides an experimental basis for future studies on the role of IKK2 in NAFLD. Control of the expression level of IKK2 in the liver may become a new possibility for therapy of NAFLD.

Terminology

In the present study, the authors tested the effect of IKK2 in NAFLD in mice, and found a facilitative effect on the occurrence and development of NAFLD.

Peer review

The paper should be accepted with some minor revisions. IκB kinase-beta inhibitor attenuates hepatic fibrosis in mice liver injury and its potential mechanisms.

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BRIEF ARTICLE

Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer

Shuang Lin, Hong-Gang Jiang, Zhi-Heng Chen, Shu-Yang Zhou, Xiao-Sun Liu, Ji-Ren Yu

Shuang Lin, Hong-Gang Jiang, Zhi-Heng Chen, Department of Oncological Surgery, First Affiliated Hospital of Jiaxing University, Jiaxing 314000, Zhejiang Province, China
Shu-Yang Zhou, Xiao-Sun Liu, Ji-Ren Yu, Department of Gastroenterological Surgery, First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
Author contributions: Lin S wrote the paper and performed the research; Yu JR and Jiang HG designed the research; Liu XS and Chen ZH collected the data; Chen ZH and Zhou SY performed a literature search and retrieved the data; Yu JR was consulted and analyzed the data.
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Correspondence to: Ji-Ren Yu, MD, Department of Gastroenterological Surgery, First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China. yujiren0909@hotmail.com
Telephone: +86-571-87236147 Fax: +86-571-87072577
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Abstract

AIM: To conduct a meta-analysis to determine the relative merits of robotic surgery (RS) and laparoscopic surgery (LS) for rectal cancer.

METHODS: A literature search was performed to identify comparative studies reporting perioperative outcomes for RS and LS for rectal cancer. Pooled odds ratios and weighted mean differences (WMDs) with 95% confidence intervals (95% CIs) were calculated using either the fixed effects model or random effects model.

RESULTS: Eight studies matched the selection criteria and reported on 661 subjects, of whom 268 underwent RS and 393 underwent LS for rectal cancer. Compared the perioperative outcomes of RS with LS, reports of RS indicated favorable outcomes considering conversion (WMD: 0.25; 95% CI: 0.11-0.58; P = 0.001). Meanwhile, operative time (WMD: 27.92, 95% CI: -13.43 to 69.27; P = 0.19); blood loss (WMD: -32.35, 95% CI: -86.19 to 21.50; P = 0.24); days to passing flatus (WMD: -0.18, 95% CI: -0.96 to 0.60; P = 0.65); length of stay (WMD: -0.04; 95% CI: -2.28 to 2.20; P = 0.97); complications (WMD: 1.05; 95% CI: 0.71-1.55; P = 0.82) and pathological details, including lymph nodes harvested (WMD: 0.41, 95% CI: -0.67 to 1.50; P = 0.46), distal resection margin (WMD: -0.35, 95% CI: -1.27 to 0.58; P = 0.46), and positive circumferential resection margin (WMD: 0.54, 95% CI: 0.12-2.39; P = 0.42) were similar between RS and LS.

CONCLUSION: RS for rectal cancer is superior to LS in terms of conversion. RS may be an alternative treatment for rectal cancer. Further studies are required.

Key words: Robotic surgery; Laparoscopic surgery; Rectal cancer; Da Vinci robotic system; Meta-analysis

Peer reviewers: Keiji Hirata, MD, Surgery 1, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan; Dr. Kok Sun Ho, Department of Colorectal Surgery, Singapore General Hospital, Outram Road, Singapore 169608, Singapore; Yik-Hong Ho, Professor, Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

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INTRODUCTION

Over the past 30 years, laparoscopic surgery (LS) has revolutionized general surgical practice, above all affecting

surgery of the gastrointestinal (GI) tract[1,2]. However, with regard to rectal cancer, there are several technical drawbacks to LS, including limited range of motion of instruments in a narrow pelvic cavity, related loss of dexterity, and an inadequate visual field associated with unstable camera view and assistant's traction, which are not under the surgeon's control[3]. Technical advantages of the da Vinci robotic system could overcome the limitations of LS for rectal cancer, by giving the surgeon a 3D view, better ergonomics, enhanced dexterity, precision and control due to the 3D optical system and EndoWrist® Instruments.

Although surgical robots have been successfully applied to a number of disciplines, most notably urological and cardiac procedures[4,5], robotic rectal surgery remains in its infancy. Most studies have been limited by small sample size and a single institution design. To overcome these limitations, a meta-analysis of studies comparing robotic surgery (RS) and LS for rectal cancer should be performed. The aim of this meta-analysis was to determine the relative merits of RS and LS for rectal cancer.

MATERIALS AND METHODS

Study selection

The Pubmed, Embase, Cochrane Library, Ovid, and Web of Science databases were searched systematically for all articles published before June 2011 to compare RS and LS for rectal cancer. The terms used for the search were: "robotic" and "rectal cancer". Only studies in the English language were considered for inclusion. Reference lists of all retrieved articles were manually searched for additional studies. Two reviewers independently extracted the data from each study. All relevant text, tables and figures were reviewed for data extraction. Discrepancies between the two reviewers were resolved by discussion and consensus.

Criteria for inclusion and exclusion

For inclusion in the meta-analysis, a study had to fulfill the following criteria: (1) compare the outcomes of RS and LS, regardless of other diseases; (2) report on at least one of the outcome measures mentioned below; and (3) if dual (or multiple) studies were reported by the same institution and/or authors, either the one of higher quality or the most recent publication was included in the analysis.

Abstracts, letters, editorials and expert opinions, reviews without original data, case reports and studies lacking control groups were excluded. The following studies or data were also excluded: (1) the outcomes and parameters of patients were not clearly reported (e.g., with no clearly reported outcomes of SD; (2) it was impossible to extract the appropriate data from the published results; and (3) there was overlap between authors or centers in the published literature.

Outcomes of interest

The following outcomes were used to compare the two operative techniques: (1) intraoperative data, which included operative time, blood loss and conversion; (2) postoperative data, which included complication, days to passing flatus, and length of stay; and (3) pathological details, which included lymph nodes harvested, distal resection margin (DRM), and positive circumferential resection margin (PCRM) which was defined as a circumferential resection margin (CRM) of $\leqslant$ 1 mm.

Data extraction

Two reviewers independently extracted the following parameters from each study: (1) first author and year of publication; (2) study population characteristics; (3) number of subjects operated on with each technique; and (4) intraoperative data, postoperative data, and pathological details.

Statistical analysis

The meta-analysis was performed using the Review Manager (RevMan) software, version 4.2.2. We analyzed dichotomous variables using estimation of odds ratios with a 95% confidence interval (95% CI) and continuous variables using weighted mean difference (WMD) with a 95% CI. Pooled effect was calculated using either the fixed effects model or random effects model. Heterogeneity was evaluated by χ^2 and I^2. We considered heterogeneity to be present if the I^2 statistic was > 50%. $P < 0.05$ was considered significant.

RESULTS

Selection of trials

The initial search strategy retrieved 154 publications, after screening all titles, abstracts, and full-text. A total of eight studies met our entry criteria and were retrieved for more detailed evaluation. The characteristics of these eight studies are summarized in Table 1[6-13]. Eight studies [six non-randomized controlled trials (NRCTs), two randomized controlled trials (RCTs)] included a total of 661 patients: 268 in the RS group and 393 in the LS group. Two studies were conducted in United States[7,13], three in Korea[6,8,12], two in Italy[10,11], and one in Romania[9]. The sample size of each study varied from six to 123 patients. In the included studies, six were considered as level of evidence 3, and the remaining 2 as level of evidence 2 (according to the grading of the Centre of Evidence-Based Medicine, Oxford, United Kingdom; http://www.cebm.net/index.aspx?o=5653).

In these studies, patients in the two groups were matched for operation time[6,9,11,12], blood loss[9,11], conversion[6-13], complications[6-13], days to passing flatus[6,12], length of stay[6,11,12], lymph nodes harvested[6,9,11,12], DRM[6,11,12], and PCRM[6,7,10].

Meta-analysis of intraoperative data

In four studies, operative time showed that there was no significant difference between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 27.92, 95% CI:

Table 1 Characteristics of included studies

Study	Country	Group	No. of patients	Mean age (yr)	Gender (M/F)	Level of evidence
Park	Korea	RS	52	57.3 (± 12.3)	28/24	3
et al[6]		LS	123	65.1 (± 10.3)	70/53	
Baek	United States	RS	41	63.6 (48-87)	25/16	3
et al[7]		LS	41	63.7 (42-88)	25/16	
Kwak	Korea	RS	59	60 (53-68)	39/20	3
et al[8]		LS	59	59 (53-69)	42/17	
Popescu	Romania	RS	38	53 (± 11.27)	23/15	3
et al[9]		LS	84	60 (± 12.27)	51/33	
Bianchi	Italy	RS	25	69 (33-83)	18/7	2
et al[10]		LS	25	62 (42-77)	17/8	
Patriti	Italy	RS	29	68 ± 10	NA	3
et al[11]		LS	37	69 ± 10	NA	
Baik	Korea	RS	18	57.3 ± 6.3	14/4	2
et al[12]		LS	18	62.0 ± 9.0	14/4	
Pigazzi	United States	RS	6	60 (42-78)	4/2	3
et al[13]		LS	6	70 (57-88)	2/4	

NA: Not available; RS: Robotic surgery; LS: Laparoscopic surgery.

-13.43 to 69.27; $P = 0.19$) (Figure 1A).

In two studies, blood loss did not differ significantly between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -32.35, 95% CI: -86.19 to 21.50; $P = 0.24$) (Figure 1B).

In all eight studies, conversion was found to be significantly lower in the RS group than in the LS group. Moreover, analysis of the pooled data revealed that conversion for RS was significantly lower by 0.25-fold (WMD: 0.25; 95% CI: 0.11-0.58; $P = 0.001$) (Figure 1C).

Meta-analysis of postoperative outcomes

In two studies, number of days to passing flatus was significantly lower in the RS group *vs* the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -0.18, 95% CI: -0.96 to 0.60; $P = 0.65$) (Figure 1D).

In three studies, length of stay was found to be no different in the RS group and the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -0.04; 95% CI: -2.28 to 2.20; $P = 0.97$) (Figure 1E).

In all eight studies, complications were found to be no different in the RS group and the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 1.05; 95% CI: 0.71-1.55; $P = 0.82$) (Figure 1F).

Meta-analysis of pathological details

In the four studies, lymph nodes harvested showed that there was no significant difference between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 0.41, 95% CI: -0.67 to 1.50; $P = 0.46$) (Figure 1G).

In three studies, DRM was found to be significantly lower in the RS group than the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -0.35, 95% CI: -1.27 to 0.58; $P = 0.46$) (Figure 1H).

In three studies, PCRM showed that there was no significant difference between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 0.54, 95% CI: 0.12-2.39; $P = 0.42$) (Figure 1I).

Heterogeneity

A significant heterogeneity was recognized in the following two factors: operative time, blood loss, days to passing flatus, length of stay and DRM.

DISCUSSION

Meta-analysis could be used to evaluate the existing literature in both qualitative and quantitative ways by comparing and integrating the results of different studies and taking into account variations in characteristics that could influence the overall estimate of the outcome of interest[14]. Although meta-analysis has traditionally been applied and best confined to RCTs, meta-analytical techniques using NRCTs might be a good method in some clinical settings in which either the number or the sample size of RCTs was insufficient[15]. To the best of our knowledge, this was the first comprehensive meta-analysis comparing RS versus LS for rectal cancer.

RS is often perceived as being more time-consuming, because of the additional set-up time required[16]. It usually requires two steps for rectal cancer[17,18]. After dissection of the left colon and sigmoid colon and division and ligation of the inferior mesenteric vessels, the da Vinci system must be moved for the next step. However, moving the da Vinci system is a time-consuming and difficult procedure because the robotic devices are heavy and bulky. This meta-analysis revealed that there was no significant difference in operative time between RS and LS. This finding could be attributable to the shortened learning curve, and it has been suggested that the intuitive controls of robotic systems, more comparable with open surgery, could shorten the learning curve, even in the hands of relatively inexperienced laparoscopic surgeons[19]. As we overcame the learning curve with experience and prevented collisions by properly positioning the robotic ports, the operation time decreased. There was no significant difference in blood loss when comparing RS and LS.

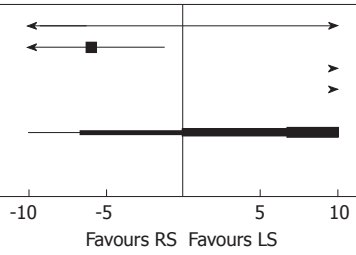
Conversion to open surgery and complications are critical in minimally invasive rectal cancer surgery, because converted patients have higher complication rates[20] and, in one series at least, worse oncological outcomes[21]. Conversion rate was significantly lower in the RS group than in the LS group. Lower conversion with RS might have been due to superior exposure and visualization of the operating field in the pelvis, thanks to the ability of the fixed fourth arm to grip and maneuver organs; the ability of the surgeon to move the 3D camera as required; and the greater ease of dissection afforded by the

A

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 01 Intraoperative data
Outcome: 01 Operative time

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	217.10 (51.60)	18	204.30 (51.90)		22.58	12.80 (-21.01, 46.61)
Patriti *et al*[11] 2009	29	202.00 (12.00)	37	208.00 (7.00)		26.49	-6.00 (-10.92, -1.08)
Popescu *et al*[9] 2010	38	212.00 (47.23)	84	182.00 (37.23)		25.45	30.00 (13.00, 47.00)
Park *et al*[6] 2011	52	232.60 (52.40)	123	158.10 (49.20)		25.49	74.50 (57.81, 91.19)
Total (95% CI)	137		262			100	27.92 (-13.43, 69.27)

Test for heterogeneity: χ^2 = 93.41, df = 3 (P < 0.00001), I^2 = 96.8%
Test for overall effect: Z = 1.32 (P = 0.19)

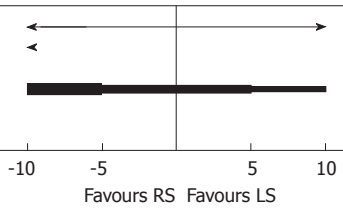


B

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 01 Intraoperative data
Outcome: 02 Blood loss

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Patriti *et al*[11] 2009	29	137.40 (156.00)	37	127.00 (169.00)		29.23	10.40 (-68.27, 89.07)
Popescu *et al*[9] 2010	38	100.00 (50.00)	84	150.00 (50.00)		70.77	-50.00 (-69.16, -30.84)
Total (95% CI)	67		121			100	-32.35 (-86.19, 21.50)

Test for heterogeneity: χ^2 = 2.14, df = 1 (P = 0.14), I^2 = 53.2%
Test for overall effect: Z = 1.18 (P = 0.24)

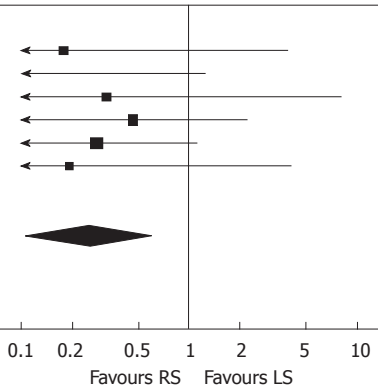


C

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 01 Intraoperative data
Outcome: 03 Conversion

Study or sub-category	RS *n/N*	LS *n/N*	OR (fixed) 95% CI	Weight %	OR (fixed) 95% CI
Pigazzi *et al*[13] 2006	0/6	0/6			Not estimable
Baik *et al*[12] 2008	0/18	2/18		9.17	0.18 (0.01, 3.99)
Patriti *et al*[11] 2009	0/29	7/37		24.51	0.07 (0.00, 1.26)
Bianchi *et al*[10] 2010	0/25	1/25		5.54	0.32 (0.01, 8.25)
Popescu *et al*[9] 2010	2/38	9/84		20.01	0.46 (0.10, 2.25)
Baek *et al*[7] 2011	3/41	9/41		31.42	0.28 (0.07, 1.13)
Kwak *et al*[8] 2011	0/59	2/59		9.34	0.19 (0.01, 4.11)
Park *et al*[6] 2011	0/52	0/123			Not estimable
Total (95% CI)				100	0.25 (0.11, 0.58)

Total events: 5 (RS), 30 (LS)
Test for heterogeneity: χ^2 = 1.46, df = 5 (P = 0.92), I^2 = 0%
Test for overall effect: Z = 3.20 (P = 0.001)

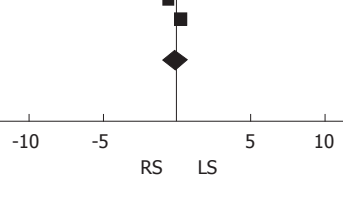


D

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 02 Postoperative data
Outcome: 01 Days to passing flatus

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD(random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	1.80 (0.40)	18	2.40 (1.30)		47.61	-0.60 (-1.23, 0.03)
Park *et al*[6] 2011	52	3.20 (1.80)	123	3.00 (1.10)		52.39	0.20 (-0.33, 0.73)
Total (95% CI)	70		141			100	-0.18 (-0.96, 0.60)

Test for heterogeneity: χ^2 = 3.66, df = 1 (P = 0.06), I^2 = 72.7%
Test for overall effect: Z = 0.45 (P = 0.65)



E

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 02 Postoperative data
Outcome: 02 Length of stay

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	6.90 (1.30)	18	8.70 (1.30)		41.56	-1.80 (-2.65, -0.95)
Patriti *et al*[11] 2009	29	11.90 (7.50)	37	9.60 (6.90)		21.11	2.30 (-1.22, 5.82)
Park *et al*[6] 2011	52	10.40 (4.70)	123	9.80 (3.80)		37.34	0.60 (-0.84, 2.04)
Total (95% CI)	99		178			100	-0.04 (-2.28, 2.20)

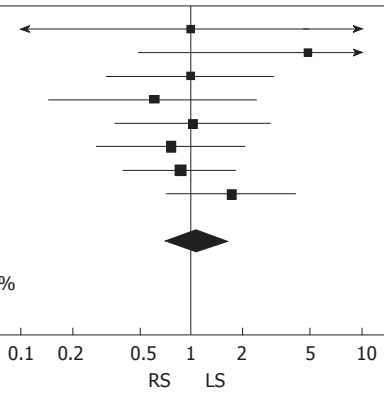
Test for heterogeneity: χ^2 = 11.49, df = 2 (P = 0.003), I^2 = 82.6%
Test for overall effect: Z = 0.03 (P = 0.97)

F

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 03 Intraoperative data
Outcome: 03 Complications

Study or sub-category	RS *n/N*	LS *n/N*	OR (fixed) 95% CI	Weight %	OR (fixed) 95% CI
Pigazzi *et al*[13] 2006	1/6	1/6		1.7	1.00 (0.05, 20.83)
Baik *et al*[12] 2008	4/18	1/18		1.58	4.86 (0.49, 48.57)
Patriti *et al*[11] 2009	7/29	9/37		12.21	0.99 (0.32, 3.08)
Bianchi *et al*[10] 2010	4/25	6/25		10.25	0.60 (0.15, 2.47)
Popescu *et al*[9] 2010	6/38	13/84		13.87	1.02 (0.36, 2.94)
Baek *et al*[7] 2011	9/41	11/41		17.47	0.77 (0.28, 2.11)
Kwak *et al*[8] 2011	18/59	20/59		28.27	0.86 (0.40, 1.85)
Park *et al*[6] 2011	10/52	15/123		14.65	1.71 (0.71, 4.12)
Total (95% CI)	268	393		100	1.05 (0.71, 1.55)

Total events: 59 (RS), 76 (LS)
Test for heterogeneity: χ^2 = 4.15, df = 7 (P = 0.76), I^2 = 0%
Test for overall effect: Z = 0.22 (P = 0.82)



G

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 03 Pathologic details
Outcome: 01 Lymph nodes harvested

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI
Baik *et al*[12] 2008	18	20.00 (9.10)	18	17.40 (10.60)		2.84	2.60 (-3.85, 9.05)
Patriti *et al*[11] 2009	29	10.30 (4.00)	37	11.20 (5.00)		25.05	-0.90 (-3.07, 1.27)
Popescu *et al*[9] 2010	38	11.37 (3.80)	84	11.07 (3.20)		61.25	0.30 (-1.09, 1.69)
Park *et al*[6] 2011	52	19.40 (10.20)	123	15.90 (10.10)		10.86	3.50 (0.20, 6.80)
Total (95% CI)	137		262			100	0.41 (-0.67, 1.50)

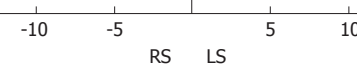
Test for heterogeneity: χ^2 = 5.24, df = 3 (P = 0.16), I^2 = 42.7%
Test for overall effect: Z = 0.74 (P = 0.46)

H

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 03 Pathologic details
Outcome: 02 Distal resection margin

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	4.00 (1.10)	18	3.70 (1.10)		41.56	0.30 (-0.42, 1.02)
Patriti *et al*[11] 2009	29	2.10 (0.90)	37	4.50 (7.20)		12.24	-2.40 (-4.74, -0.06)
Park *et al*[6] 2011	52	2.80 (1.90)	123	3.20 (2.10)		45.14	-0.40 (-1.04, 0.24)
Total (95% CI)	99		178			100	-0.35 (-1.27, 0.58)

Test for heterogeneity: χ^2 = 5.62, df = 2 (P = 0.06), I^2 = 64.4%
Test for overall effect: Z = 0.73 (P = 0.46)



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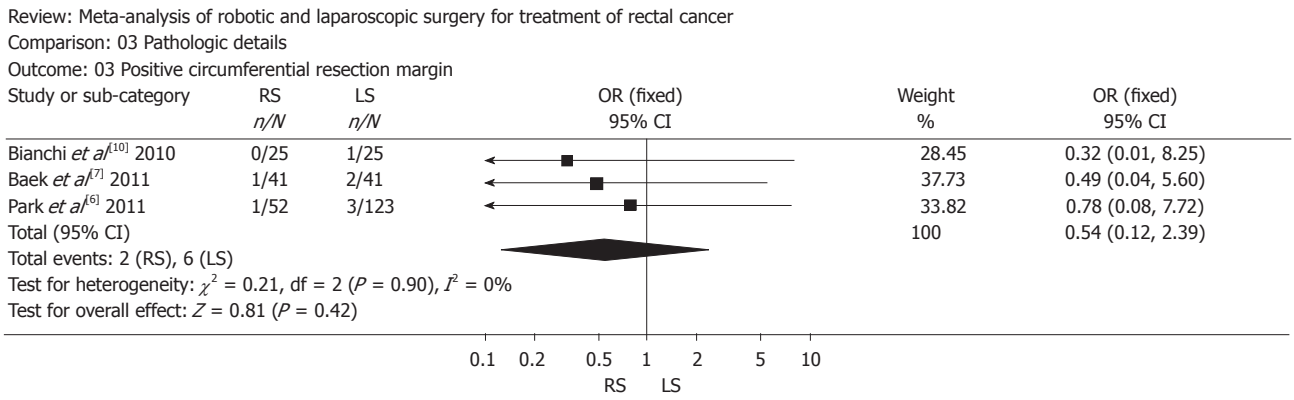

Figure 1 Forest plot displaying the results of the meta-analysis on operative time (A), blood loss (B), conversion (C), days to passing flatus (D), length of stay (E), complications (F), lymph nodes harvested (G), distal resection margin (H) and positive circumferential resection margin (I). RS: Robotic surgery; LS: Laparoscopic surgery; OR: Odds ratio; WMD: Weighted mean difference.

highly maneuverable EndoWrist instruments attached to the robotic arms.

Number of days to passing flatus was lower in the RS group than the LS group, meanwhile, length of stay was found to be no different between the two groups. However, analysis of the pooled data did not reveal any significant difference in this regard. These findings implied that the time required for patients to resume daily activities might not be shorter after RS than LS. There was no significant difference in complications when comparing RS and LS. On the contrary, it has been postulated that these characteristics of RS could make patients recover faster and reduce complications, because with the da Vinci surgical system, robotic arms are used for retraction and dissection during the total mesorectal excision procedure, and their use reduces unnecessary procedures and minimizes iatrogenic tissue injury during retraction. These findings are difficult to explain, and more advanced studies are needed before such conclusions can be drawn.

We postulated that specimen quality could be used as an indicator to predict long-term clinical oncological results. No significant differences were proved between RS and LS in the pathological details, including harvested lymph nodes, DRM and PCRM. The number of harvested lymph nodes, DRM, and PCRM did not differ significantly between the two groups in our meta-analysis. This demonstrated that RS could be performed safely and with a high success rate following oncological principles compared with LS. However, long-term follow-up evaluation is necessary to evaluate the exact oncological outcomes of RS for rectal cancer.

The cost of RS equipment is very high and likely to be a serious impediment to uptake in the foreseeable future[22]. However, it is important to perform a cost-effectiveness analysis between RS and LS. Only one trial has reported that the average total hospitalization costs were higher in the RS group ($83 915) than in the LS group ($62 601), and these differences were not statistically significant[7]. To the best of our knowledge, total hospitalization costs may be due to the greater expense and consumption of operating room resources such as space and the availability of skilled technical staff, and differ significantly between hospitals[23]. Therefore, insufficient data and great heterogeneity precluded a meta-analysis of cost-effectiveness.

Significant heterogeneity in those articles was observed in the operative time, blood loss, days to passing flatus, length of stay and DRM, which may be explained by the difference in skill, extension of lymph node dissection, and duration of learning curve. Regarding the heterogeneity between the articles, random-effect models were used in this meta-analysis.

The results of the present meta-analysis should be interpreted with caution because of several limitations. First, some data came from NRCTs, and the overall level of clinical evidence was low. It has been reported that NRCTs can either exaggerate or underestimate the magnitude of measured effects in a study of intervention regardless of quality scores[24]. However, Abrahama *et al*[25] have found that meta-analysis of well-designed NRCTs of surgical procedures was probably as accurate as that of RCTs. In fact, six studies included in the present study were NRCTs. Second, there was heterogeneity between the two groups, because it was impossible to match patient characteristics in all studies. We applied a random-effect model to take into consideration between-study variation, and it might have been expected to exert a limited influence. Finally, authors might be more likely to report positive results, and studies with significant outcomes were more likely to be published, so potential publication bias might have been present.

In conclusion, the results of this meta-analysis of 661 patients show that RS is superior to LS for rectal cancer in terms of conversion. Therefore, RS may be an alternative treatment for rectal cancer. Further studies are required to better define its role.

COMMENTS

Background

The da Vinci robotic system was introduced as the next advance in minimally

invasive surgery to overcome the technical limitations of laparoscopy, but robotic rectal surgery is controversial because of a lack of well-powered trials.

Research frontiers

Meta-analysis was used to evaluate the relative merits of robotic surgery (RS) and laparoscopic surgery (LS) for rectal cancer in this study.

Innovations and breakthroughs

The meta-analysis reported that RS had favorable outcomes considering conversion, compared with LS for rectal cancer. Meanwhile, operative time, blood loss, days to passing flatus, length of stay, complications and pathological details, including lymph nodes harvested, distal resection margin, and positive circumferential resection margin were similar between RS and LS. This is believed to be the first comprehensive meta-analysis comparing RS and LS for rectal cancer.

Applications

The results of this meta-analysis show that RS is superior to LS in terms of conversion. Therefore, RS may be an alternative treatment for rectal cancer.

Peer review

This paper addressed superiority of RS for rectal cancer, especially due to superior exposure and visualization of the intrapelvic field. This advantage means that surgeons complete the operation without conversion. This paper should be of interest to colorectal surgeons worldwide.

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BRIEF ARTICLE

Therapeutic effects of combined oxaliplatin and S-1 in older patients with advanced gastric cardiac adenocarcinoma

She-Gan Gao, Rui-Nuo Jia, Xiao-Shan Feng, Xuan-Hu Xie, Tan-You Shan, Li-Xian Pan, Na-Sha Song, Yu-Feng Wang, Kai-Li Ding, Li-Dong Wang

She-Gan Gao, Rui-Nuo Jia, Xiao-Shan Feng, Xuan-Hu Xie, Tan-You Shan, Li-Xian Pan, Na-Sha Song, Yu-Feng Wang, Kai-Li Ding, Department of Oncology, First Affiliated Hospital, Cancer Institute, Henan University of Science and Technology, Luoyang 471003, Henan Province, China
Li-Dong Wang, Henan Key Laboratory for Esophageal Cancer, Laboratory for Cancer Research, Basic Medical College, Zhengzhou University, Zhengzhou 450052, Henan Province, China
Author contributions: Gao SG and Feng XS designed the study; Jia RN, Xie XH, Shan TY, Pan LX, Song NS, Wang YF and Ding KL performed the research; Gao SG and Jia RN carried out the analyses and wrote the article; Feng XS, Gao SG and Wang LD approved the final version of the manuscript.
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Correspondence to: Dr. Xiao-Shan Feng, First Affiliated Hospital, Cancer Institute, Henan University of Science and Technology, Luoyang 471003, Henan Province, China. samfeng137@hotmail.com
Telephone: +86-379-64820811 Fax: +86-379-64820811
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Abstract

AIM: To evaluate the effects and safety of combination chemotherapy with oxaliplatin (L-OHP) and S-1 (SOX regimen) in older patients with advanced gastric cardiac adenocarcinoma (GCA).

METHODS: Seventy patients with advanced GCA were classified according to age into an older group (≥ 75 years) and a control group (< 75 years). The SOX regimen was administered to the two groups as follows: S-1 (40 mg/m^2 po bid) on days 1 to 14 followed by a 7-d off period, plus L-OHP (65 mg/m^2 iv) for 2 h on days 1 and 8 of a 21-d cycle. This regimen was repeated for four to six cycles. Response and swallow statuses were evaluated after two cycles (6 wk). Effects and toxicity were evaluated four weeks after chemotherapy was completed.

RESULTS: The response rate was 65.6% (21/32) in the older group and 68.4% (26/38) in the control group (χ^2 = 0.062 and P = 0.804). Improvement in swallowing was 78.1% (25/32) in the older group and 76.3% (29/38) in the control group (χ^2 = 0.032 and P = 0.857). Efficacy was 68.8% (22/32) in the older group and 65.8% (25/38) in the control group (χ^2 = 0.069 and P = 0.793). Toxicities were reversible and similar in both groups (P > 0.05).

CONCLUSION: The SOX regimen is an effective, safe and well-tolerated regimen for older patients with advanced GCA.

Key words: Gastric cardiac adenocarcinoma; Oxaliplatin; S-1; Treatment effect

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City 999075, Cuba

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INTRODUCTION

With constant improvement in the quality of life in modern

society, people's life span has been prolonged. However, the incidence of elderly patients with gastric cardiac adenocarcinoma (GCA) is gradually increasing, and the majority of these patients have advanced disease when they are diagnosed. Thus, these patients have few opportunities for surgery[1]. The only available treatment choice for advanced GCA patients is systemic chemotherapy[2-4]. Although chemotherapy for advanced gastrointestinal cancer has been proven to be superior to best supportive care (BSC) in terms of survival and quality of life[5-7], there has been evidence supporting more serious adverse events observed among older patients than younger patients[8]. For these reasons, most older patients with metastasis are usually offered BSC and not chemotherapy[9]. However, patients who are 75 years old can still have a considerable number of years to survive (perhaps more than 10 years)[10]. Therefore, it is important to find a highly effective and minimally toxic chemotherapy regimen for elderly patients with advanced GCA.

In the last decade, 5-fluorouracil (5-FU) has been considered a cornerstone of therapy for advanced gastrointestinal cancer. Therefore, combining 5-FU with oxaliplatin (L-OHP) is logical because there is considerable evidence of preclinical synergy between the two agents[11]. S-1 is an orally active prodrug of 5-FU which is a fourth generation oral fluoropyrimidine[12]. Recent clinical studies have reported that S-1 in combination with L-OHP has a high response rate ranging from 53% to 59% and an excellent toxicity profile in the treatment of advanced gastric cancer[13-15]. In these studies, however, there were only a few patients of 75 years of age or older. Furthermore, few studies on the outcome of the S-1 and oxaliplatin (SOX) regimen in patients with GCA have been reported. Therefore, we designed this study to determine the response rate and toxicity profile of SOX regimen in GCA patients over the age of 75 years.

MATERIALS AND METHODS

Patients

GCA was confirmed in 70 patients by pathologic diagnosis in the First Affiliated Hospital, Henan University of Science and Technology from March 2008 to October 2010. All patients were treated with chemotherapy for the first time in this study, and they were experiencing symptoms such as difficulty in drinking, difficulty in eating, vomiting mucus, anemia, and emaciation. The degree of cardia stenosis was assessed using the Stooler Classification System[16] and the barium meal examination. The results of the barium meal examination are shown in Table 1. There were 54 cases of grade Ⅲ, 14 cases of grade Ⅳ, and 2 cases of grade Ⅴ dysphagia. All patients were classified as stage Ⅲ or Ⅳ according to the TNM staging, and they had Karnofsky Performance Status (KPS) scores greater than or equal to 60 points, predicted life spans greater than three months, no contraindications to chemotherapy, and no previous treatment with chemotherapy. Their routine blood examinations, electrocardiograms (ECGs), liver function, and kidney function were also normal. All patients were examined with a computed tomography (CT) before and after chemotherapy, and they were evaluated by the same physician.

Table 1 Degree of cardia stenosis

Clinical classifications	Diet conditions	Cardia diameters in the barium meal exam (mm)
Ⅰ	Ordinary diet	8-10
Ⅱ	Semi-liquid diet	6-8
Ⅲ	Liquid diet	4-6
Ⅳ	No drinking	2-4
Ⅴ	Saliva refluxing	0-2

According to the most recent World Health Organization (WHO) definition of aged people, people who are 65 to 74 years old are categorized as "young aged", and people who are 75 to 90 years old are classified as "older people". All the patients were divided into two groups as follows: patients older than 75 years were classified in the older group, and the remaining patients were classified in the control group. Of the 32 participants in the older group (ranging in age from 75 to 89 years old), 24 patients were male and 8 patients were female, with a median age of 79.5 years. Of the 38 participants in the control group (ranging in age from 55 to 74 years old), 29 patients were male and 9 patients were female with a median age of 64 years (Table 2).

Methods

The following chemotherapy program was used: L-OHP (65 mg/m^2 iv) was administered for 2 h on days 1 and 8; S-1 was orally administered at a dose of 40 mg/m^2 bid for 14 d (from the evening on day 1 until the morning on day 15); and a 7-d rest period followed the L-OHP and S-1 treatments in the 3-wk schedule. Treatment was repeated for four to six cycles. In every cycle, both omeprazole (40 mg iv bid) and tropisetron (5 mg iv qd) were administered before chemotherapy. Furthermore, large doses of oral vitamin B tablets were used to reduce side effects, and low doses of megestrol enhanced appetite and nutrition. Moreover, reconstituted cell colony-stimulating factor was given if needed. Participants were advised to avoid cold food, drinks and water. Blood, urine and stool routine examinations were carried out weekly, and ECG, liver function and kidney function were also checked weekly. Furthermore, a KPS score was determined weekly.

If patients had dysphagia to an extent greater than grade Ⅳ due to cardia stenosis, the stenosis was dilated with a conical Savary-Gilliard silica gel dilator one week before chemotherapy followed by insertion of a gastric canal. High protein and high vitamin liquid nasal feeds were then started. If the patient could swallow food after two chemotherapy cycles, the gastric canal was removed.

The sensitivity of the tumor to chemotherapy and improvement of dysphagia were evaluated after two cycles (6 wk). The effects and toxicity were evaluated at

Table 2 Patient characteristics at baseline, case (%)

Characteristics	Older group (n = 32)	Control group (n = 38)
Demography		
Male/female	24 (75)/8 (25)	29 (76.3)/9 (23.7)
Median age, yr (range)	79.5 (75-89)	64 (55-74)
Karnofsky performance status		
Median	80%	80%
100%	1 (3.1)	2 (5.3)
90%	10 (31.2)	13 (34.2)
80%	17 (53.2)	18 (47.4)
60%-70%	4 (12.5)	5 (13.1)
Weight loss > 5%	11 (34.4)	13 (34.2)
Cardia stenosis status		
Ⅰ-Ⅱ	0	0
Ⅲ	25 (78.1)	29 (76.3)
Ⅳ	6 (18.8)	8 (21.1)
Ⅴ	1 (3.1)	1 (2.6)
Histological grade		
G1-2	17 (53.1)	19 (50)
G3	12 (37.5)	14 (36.8)
Others (grade not stated)	3 (9.4)	5 (13.2)
Extent of disease		
Metastatic	10 (31.3)	12 (31.6)
Locally advanced	22 (68.7)	26 (68.4)
Metastatic site		
Lymph nodes	10 (31.3)	12 (31.6)
Liver	3 (9.4)	3 (7.9)
Peritoneum	1 (3.1)	2 (5.3)
Lung	0	1 (2.6)
Others	0	0
No. of metastatic sites		
1	6 (18.8)	6 (15.8)
⩾ 2	4 (12.5)	6 (15.8)

four weeks with a repeat CT and barium meal examination after the chemotherapy was completed.

Evaluation criteria

Evaluation criteria for chemotherapy sensitivity: The evaluation criteria for chemotherapy sensitivity we used were proposed in 1998 by the European Association of Cancer Research and Treatment, United States National Cancer Institute, and National Cancer Institute of Canada. These evaluation criteria are called the Response Evaluation Criteria In Solid Tumors[17]. Participants had a repeat CT scan with contrast two weeks after the completion of chemotherapy to evaluate the therapeutic effects of the chemotherapy according to the maximum diameters of each tumor. A complete response (CR) was defined as the complete disappearance of all lesions after treatment. A partial response (PR) was defined as a decrease greater than or equal to 30% in the maximum diameters of all tumors after treatment. Progressive disease (PD) was defined as an increase greater than 20% in the maximum diameters of tumors or the emergence of more than one new lesion after treatment. When the tumor diameters were between the diameters found in the PR and PD classifications (< 30% decrease or ⩽ 20% increase) after treatment, the effect was classified as stable disease.

Evaluation criteria for improvement of dysphagia: The evaluation criteria for symptom improvement were based on diet intake and the increase/decrease in cardia diameter. The symptoms were assessed using the barium meal examination[18] with the following classifications: CR, post-treatment cardia diameter two times greater than or equal to the pre-treatment cardia diameter with the patient capable of eating ordinary food; PR, post-treatment cardia diameter one time greater than the pre-treatment cardia diameter with the patient capable of eating semi-liquid food; no change (NC), an increase in the cardia diameter by less than 6 mm with the patient capable of eating only liquid food; and PD, a decrease in the cardia diameter with the patient unable to eat liquid food.

Evaluation criteria for short-term effects: Participants had CT scans in the first and fourth week after the chemotherapy session ended. The area of each tumor (referring to the product of the two longest vertical diameters) was measured before and after chemotherapy. The following evaluation criteria were used[19]: CR, complete disappearance of visible lesions for more than one month; PR, a decrease greater than 50% in the tumor for more than one month; NC, a decrease less than 50% or an increase less than 25% in the tumor for more than one month; and PD, one or more lesions increased by greater than 25% or the emergence of a new lump.

Evaluation criteria for side effects: Toxicities were divided into degrees from 0 to Ⅳ according to the WHO criteria for acute and subacute toxic reactions of antineoplastic agents[19].

Statistical analysis

SPSS 10.0 statistical software (SPSS Company, Chicago, Illinois, United States) was used to perform the χ^2 test to evaluate the data. P values less than 0.05 were considered statistically significant.

RESULTS

Chemotherapy sensitivity

A repeat CT of the epigastrium two weeks after starting chemotherapy with the SOX program measured changes in the diameter of the largest tumor and evaluated the sensitivity to chemotherapy of the older group and control group (Table 3).

Symptom (dysphagia) improvement

After two cycles of chemotherapy with the SOX program, an upper gastrointestinal barium meal examination was repeated. Changes in cardia diameters were measured and calculated, and patients were asked about their diets. Symptom improvement was evaluated and compared between groups (Table 4).

Short-term therapeutic effects

After one week and four weeks of chemotherapy with

Table 3 Comparisons of chemotherapy sensitivity, case (%)

Clinical groups	CR	PR	SD	PD	CR + PR
Older group (n = 32)	3 (9.4)	18 (56.2)	11 (34.4)	0	21 (65.6)[a]
Control group (n = 38)	4 (10.5)	22 (57.9)	12 (31.6)	0	26 (68.4)

[a]χ^2 = 0.062, P = 0.804 *vs* control group. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 4 Comparisons of symptom (dysphagia) improvement, case (%)

Clinical groups	CR	PR	NC	PD	CR + PR
Older group (n = 32)	5 (15.6)	20 (62.5)	7 (21.9)	0 (0)	25 (78.1)[a]
Control group (n = 38)	5 (13.2)	24 (63.2)	9 (23.7)	0 (0)	29 (76.3)

[a]χ^2 = 0.032, P = 0.857 *vs* control group. CR: Complete response; PR: Partial response; NC: No change; PD: Progressive disease.

Table 5 Comparisons of short-term chemotherapy effects, case (%)

Clinical groups	CR	PR	SD	PD	CR+PR
Older group (n = 32)	5 (15.6)	17 (53.2)	8 (25)	2 (6.2)	22 (68.8)[a]
Control group (n = 38)	5 (13.2)	20 (52.6)	10 (26.3)	3 (7.9)	25 (65.8)

[a]χ^2 = 0.069, P = 0.793 *vs* control group. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

the SOX program, abdominal CTs were repeated. The maximum diameters of the tumors were measured, and the short-term therapeutic effects in both groups were evaluated (Table 5).

Side effects

The most frequent toxic therapy effects were hematological effects in both groups [grade 3 toxicity found in 13 patients (6 in the older group and 7 in the younger group)]. No grade 4 toxicity was reported. The L-OHP-related peripheral neuropathy appeared to be mild and reversible in the majority of cases. No severe cardiac toxicity or death was recorded among these patients during the study. Details of the side effects are shown in Table 6.

DISCUSSION

The health of the elderly varies from the health of younger patients. Older people are prone to having multiple organ dysfunctions, lower immunity, lower resistance to disease, and lower resistance to senile diseases, leading to reduced tolerance to chemotherapy and increased sensitivity to side effects of these drugs. Generally, caution is required when administering chemotherapy to older patients because they may not be able to tolerate a routine dose or may experience serious side effects. However, a suboptimal dose may not achieve the desired therapeutic effect. Therefore, many experts avoid treating elderly patients with chemotherapy[20].

Table 6 Comparisons of chemotherapy side effects, case (%)

Side effect	Older group (n = 32)			Control group (n = 38)			P[1] value
	I -IV	III	IV	I -IV	III	IV	
Leukopenia	25 (78.1)	3 (9.3)	0	28 (73.7)	4 (10.5)	0	0.666
Anemia	24 (75.0)	2 (6.2)	0	27 (71.1)	2 (5.3)	0	0.900
Thrombocytopenia	23 (71.9)	1 (3.1)	0	27 (71.1)	1 (2.6)	0	0.940
Fever	2 (6.3)	0	0	3 (7.9)	0	0	1.000
Oral mucositis	13 (40.6)	0	0	14 (36.8)	0	0	0.746
Nausea/vomiting	10 (31.3)	0	0	11 (28.9)	0	0	0.834
Diarrhea	14 (43.8)	0	0	16 (42.1)	0	0	0.890
Fatigue	21 (65.6)	2 (6.3)	0	23 (60.5)	1 (2.6)	0	0.660
Sensory neuropathy	18 (56.3)	0	0	21 (55.3)	0	0	0.934
Liver function (ALT/AST)	4 (12.5)	0	0	3 (7.9)	0	0	0.810
Renal function (BUN/Cr)	1 (3.1)	0	0	0	0	0	-
Hand-foot syndrome	0	0	0	0	0	0	-
Myocardial ischemia	0	0	0	0	0	0	-
Anaphylaxis	0	0	0	0	0	0	-

[1]P value for grade I -IV between older group and control group. ALT: Alanine transaminase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatine.

There is evidence[9,21], however, that older patients with advanced gastroesophageal carcinoma may benefit from chemotherapy. Tougeron *et al*[9] reported that palliative treatment is superior to BSC (6.7 ± 2.1 mo *vs* 1.8 ± 0.4 mo) in older patients (> 70 years of age) with advanced esophageal cancer. The effect of S-1 and cisplatin combination therapy in an 80-year-old patient with gastric carcinoma has been reported in a case study, and the histopathological examination of this patient revealed CR of the disease with no cancer cells[21]. Nevertheless, data regarding GCA is limited.

In this study, the SOX program was used to treat elderly people with advanced GCA to achieve the following goals: (1) to enhance the efficacy of treatment by using a new drug; (2) to reduce toxicity and improve tolerance; and (3) to create an opportunity for treatment in elderly patients with poor health.

S-1 is an effective derivative that combines tegafur with the following two modulators of 5-FU metabolism in a 1:0.4:1 molar ratio: 5-chloro-2,4-dihydroxypyridine (CDHP), a reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), and potassium oxonate[12]. Tegafur, an oral prodrug of 5-FU, is gradually converted to 5-FU and is rapidly metabolized by DPD in the liver. The maximum concentration (Cmax) and area under the concentration-time curve (AUC) of 5-FU in plasma during S-1 treatment have been found to be higher than the steady state concentration and AUC of 5-FU in plasma during protracted intravenous infusion of 5-FU at a dose of 250 mg/m^2 per day[22]. Potassium oxonate is an orotate phosphoribosyl transferase inhibitor, which is primarily distributed to the gastrointestinal tract. This component of S-1 decreases incorporation of 5-fluorouridine triphosphate into RNA in the gastrointestinal mucosa, and it reduces the incidence of diarrhea. F-b-alanine (FBAL) is the main metabolite of 5-FU. FBAL and fluorocitrate are thought to cause the neurotoxic and

cardiotoxic effects of 5-FU by inhibiting the tricarboxylic acid cycle[22]. The CDHP component of S-1 inhibits DPD, which is the rate-limiting enzyme in the catabolic pathway of 5-FU. Consequently, the plasma FBAL concentration after oral administration of S-1 is significantly lower than the concentration after continuous infusion of 5-FU[12]. Therefore, the use of S-1 may decrease the incidence of neurotoxicity and cardiotoxicity. Ajani *et al*[23] reported significant safety advantages in the S-1/cisplatin treatment as compared with the infusional fluorouracil/cisplatin treatment for advanced gastric or gastroesophageal adenocarcinoma. They reported the following frequencies resulting from the two treatments: grade 3/4 neutropenia (32.3% and 63.6%, respectively), stomatitis (1.3% and 13.6%, respectively), and hypokalemia (3.6% and 10.8%, respectively).

L-OHP[24-25] is a third generation platinum anticancer drug developed to improve tolerability and ease of administration when compared to cisplatin. The rate at which L-OHP combines with DNA in the body is more than 10 times faster than cisplatin. L-OHP adheres more strongly to DNA, and it has a stronger cytotoxic effect than cisplatin and carboplatin. In addition, the unique diaminocyclohexane group in oxaliplatin avoids some of the resistance mechanisms developed against cisplatin, such as the mismatch repair defect and bypass replication mechanism. A phase Ⅲ trial[26] for metastatic gastroesophageal adenocarcinoma has been conducted, with a treatment of fluorouracil and leucovorin combined with either oxaliplatin [fluorouracil, leucovorin and oxaliplatin (FLO)] or cisplatin [fluorouracil, leucovorin and cisplatin (FLP)] every two weeks. The results of this trial demonstrated that serious adverse events associated with FLO are significantly less than the events associated with FLP (9% and 19%, respectively) and that the median progression-free survival (PFS) improves with FLO when compared to FLP (5.8 mo and 3.9 mo, respectively). This trial also demonstrated that treatment with FLO results in significantly superior response rates (41.3% and 16.7%, respectively), improved median PFS (6.0 mo and 3.1 mo, respectively) and improved overall survival (13.9 mo and 7.2 mo, respectively) when compared to treatment with FLP in patients older than 65 years.

Studies have shown that L-OHP and S-1 are highly active against cancer and that they have a favorable toxicity profile. Furthermore, studies have also shown that L-OHP and S-1 are expected to replace cisplatin and fluorouracil, respectively, as a first-line treatment for advanced gastric cancer[13-15]. Moreover, the SOX program may be considered for treatment of older people because of the greater efficacy and low toxicity of this regimen when compared to cisplatin and fluorouracil.

The SOX regimen in this study resulted in no significant differences between the older and control groups with regard to chemotherapy sensitivity (65.6% and 68.4%, respectively, $P = 0.804$), symptom improvement (78.1% and 76.3%, respectively, $P = 0.857$), and short-term therapeutic effects (68.8% and 65.8%, respectively, $P = 0.793$). More severe side effects caused by the SOX regimen were not detected among the elderly patients when compared to the younger patients, and these side effects did not have a significant effect on treatment administration or quality of life. Therefore, these results suggest that there are treatment options available for elderly patients with cardia obstruction who cannot eat and that there is still an opportunity for these patients to survive if they can get adequate nutrition through nasal feeds.

In summary, the SOX regimen for advanced GCA has high efficacy and mild toxicity, and it can increase the survival and life span of patients with GCA. Moreover, the SOX regimen is a safe chemotherapy program for elderly patients in poor health. Therefore, it is not necessary to entirely avoid chemotherapy in elderly patients with advanced GCA because of their age. Instead, treatment recommendations should consider physiological age and standard KPS score. It is also reasonable to initiate chemotherapy if the patient can obtain sufficient nutrition (e.g., through nasal feeding). However, chemotherapy should not be administered to patients with KPS scores less than 60 points.

In this study, the therapeutic effects of the SOX regimen in both groups were higher than those reported in previous studies of patients with gastric cancer[13-15], which may have been due to the fact that this combination therapy was the first time any of the patients in this study were treated with chemotherapy, resulting in a higher sensitivity and minimal resistance to treatment. Other studies have included patients who had relapsed or failed treatment. Moreover, most of the patients in this study were classified as having stage Ⅲb GCA with only locally advanced cancer. In this study, there were only a few extensive cases of metastasized cancer. Additionally, the SOX program may be more effective at treating GCA than other types of gastric cancer.

COMMENTS

Background

The morbidity of gastric cardiac adenocarcinoma (GCA) in elderly people is gradually increasing, and most elderly GCA patients suffer from advanced carcinoma. Therefore, the opportunity for surgery is low, and only systemic chemotherapy is available for these patients. However, many experts disagree with treating elderly patients with chemotherapy because more serious adverse events have been observed in older patients than in younger patients.

Research frontiers

In the last decade, 5-fluorouracil (5-FU) has been considered a cornerstone for treating advanced gastrointestinal cancers. S-1 is a new orally active prodrug of 5-FU, and clinical studies with S-1/L-OHP (SOX regimen) have reported a high response rate ranging from 53% to 59% and an excellent toxicity profile in the treatment of advanced gastric cancer. In these clinical studies, however, there were only a few patients who were 75 years of age or older. Moreover, only a few studies on the outcome of the SOX regimen in patients with GCA have been reported.

Innovations and breakthroughs

This is the first study to evaluate the effects and safety of the SOX regimen in older patients with GCA. The study showed that the SOX regimen is a safe chemotherapy program for elderly patients with advanced GCA and that this regimen provides a treatment option for elderly patients with GCA.

Applications

The SOX regimen may be an ideal strategy in the future for treatment of older patients with advanced GCA.

Peer review

It is a very interesting topic for the readers.

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CASE REPORT

Enterovenous fistulization: A rare complication of Crohn's disease

Jeong Woo Lim, Kyung-Jo Kim, Byong Duk Ye, Jeong-Sik Byeon, Seung-Jae Myung, Suk-Kyun Yang, Jin Ho Kim

Jeong Woo Lim, Kyung-Jo Kim, Byong Duk Ye, Jeong-Sik Byeon, Seung-Jae Myung, Suk-Kyun Yang, Jin Ho Kim, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 138-736, South Korea

Author contributions: Lim JW and Kim KJ designed the case report; Ye BD, Byeon JS, Myung SJ, Yang SK and Kim JH provided clinical advice; Lim JW wrote the paper; and Kim KJ revised the paper.

Correspondence to: Kyung-Jo Kim, MD, Clinical Associate Professor, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2 dong, Songpa-gu, Seoul 138-736, South Korea. capsulendos@gmail.com
Telephone: +82-2-30103196 Fax: +82-2-4760824
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Abstract

The presence of hepatic portal venous gas (HPVG) is associated with numerous diseases, and has been regarded as a serious, even catastrophic condition. However, anecdotal reports mention that some patients with inflammatory bowel disease (IBD), who developed HPVG after diagnostic examinations of the colon, were successfully managed with antibiotic therapy and have followed benign courses. In contrast, among IBD patients, the development of HPVG is rarely caused by enterovenous fistula. We describe a 32-year-old man with Crohn's ileocolitis who presented with hypotension and fever associated with HPVG, as well as superior mesenteric vein thrombosis, possibly caused by enterovenous fistula, who was successfully managed by surgery. We also review the literature concerning portal venous gas associated with Crohn's disease.

Key words: Crohn's disease; Enterovenous fistula; Portal venous gas

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INTRODUCTION

Hepatic portal venous gas (HPVG), as first described in 1955[1], has been reported in many illnesses, ranging from benign conditions to potentially lethal diseases that require urgent surgical intervention[2]. Overall mortality depends on the underlying disease[3].

Among the various diseases, HPVG associated with Crohn's disease (CD) has rarely been described. With regard to the treatment of patients with CD complicated with HPVG, some anecdotal reports suggested that conservative treatment with antibiotics might be effective. However, early recognition and urgent operative intervention at the time of presentation are still important in patients with complicated CD. We report a 32-year-old patient with CD who presented with fever and hypotension associated with HPVG and mesenteric vein thrombosis who was successfully managed surgically. To our knowledge, this is the first report of HPVG and mesenteric vein thrombosis possibly caused by enterovenous fistula complicated by CD in South Korea. The literature concerning PVG associated with CD is also reviewed.

CASE REPORT

A 32-year-old man was referred for evaluation of mesenteric and portal venous gas detected by abdominal

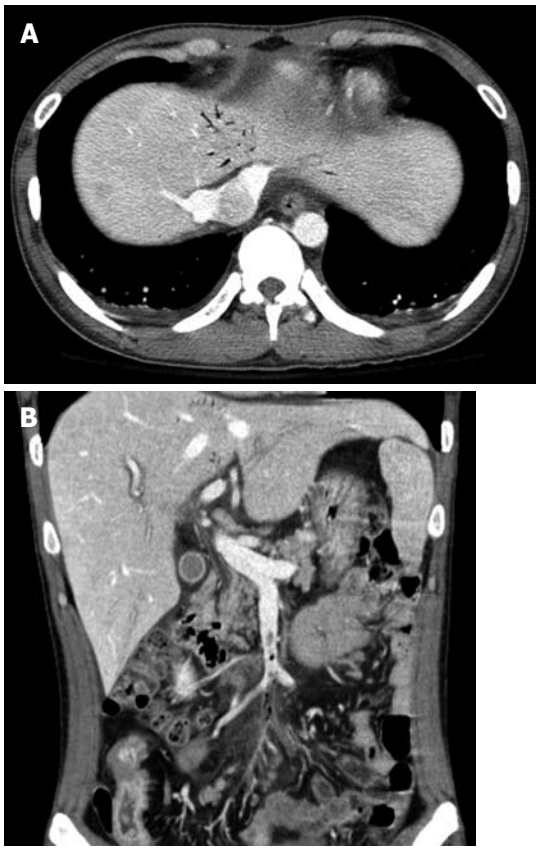

Figure 1 Computed tomography of the abdomen showing evidence of (A) portal venous gas, and (B) superior mesenteric venous gas and thrombus.

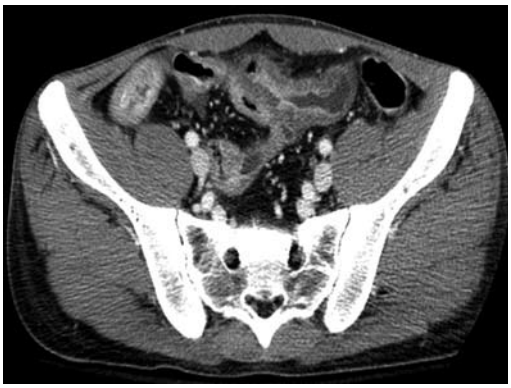

Figure 2 Computed tomography of the abdomen showing evidence of prominent wall thickening with perienteric infiltration in the mid-ileum.

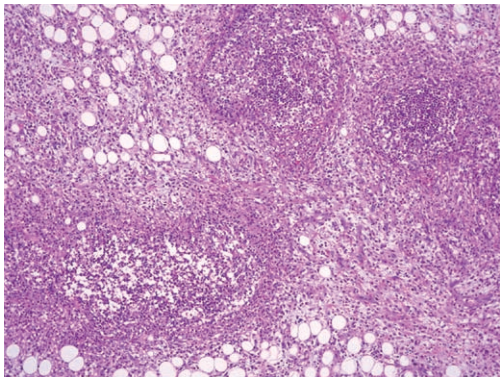

Figure 3 Photography of the pathology, showing the accumulation of many neutrophils in the intravascular lumen (hematoxylin and eosion stain, × 100).

computerized tomography (CT). The patient had complained of lower abdominal pain, nausea, vomiting, and fever. He was diagnosed with CD complicated by perianal fistula 12 years prior to admission, and was treated with azathioprine (25 mg/d) and 5-aminosalicylate (ASA; 3.2 g/d). He had been in his usual state of health on azathioprine and ASA until abdominal pain developed 21 d before admission at which time budesonide (9 mg/d) was added to his regimen of azathioprine and ASA. He continued on all three medications for three weeks until developing fever 2 d prior to admission. At that time, an abdominal CT showed mesenteric and portal venous gas. He was then referred to this hospital.

At admission, his body temperature was 38.5 ℃, blood pressure was 82/47 mmHg, and pulse rate was 105 beats per minute. There was moderate tenderness on the lower abdomen, but no abdominal distension or signs of peritoneal irritation. His white blood cell count was 4600/mm^3 (normal range 4000-10 000/mm^3) with 87% segmented forms. Protein was 4.9 g/dL (normal range 3.5-5.2 g/dL), albumin was 2.1 g/dL (normal range 6.0-8.5 g/dL), C-reactive protein was 21.19 mg/dL (normal range < 0.3 mg/dL), prothrombin time was 1.41 international normalized ratio (INR) (normal range 0.84-1.21 INR), and activated partial thromboplastin time was 37.3 s (normal range 26.3-39.4 s). Plain abdominal films were unremarkable. Abdominopelvic CT revealed the presence of air bubbles in the hepatic portal vein, superior mesenteric vein (SMV), and its contributaries. Intravenous thrombi were also noted (Figure 1). With regard to the bowel, multiple segments of the small bowel were thickened in the mid to the distal ileum and distal colon, especially in a 20 cm segment of the mid-ileum, near the mesenteric venous gas (Figure 2). The abdominopelvic CT findings were compatible with CD complicated with thrombophlebitis of the SMV. Hence, an explorative laparotomy was performed. At operation, a small amount of serous ascites was noted throughout the peritoneal cavity with multiple strictures and areas of inflammation which were also noted along the mid to distal ileum. Moreover, a short segment of mid-ileum showed phlegmonous change. Ileocecal resection was performed. Pathologic examination disclosed transmural inflammation with lymphoid aggregates, multiple microgranulomas and a fistulous tract. In addition, many neutrophils had accumulated in the intravascular space, suggesting the presence of an enterovenous fistula (Figure 3).

After surgery, the patient had an uneventful recovery and was discharged 9 d post surgery. The patient has been on 3 g of mesalazine and 25 mg of 6-mercaptopurine for his colonic disease and has been well for 4 mo after surgery.

DISCUSSION

HPVG has been reported in association with numerous conditions in adults, including intestinal ischemia or necrosis[4], intra-abdominal abscess[5], diverticulitis[6], pneumatosis intestinalis[7], and blunt trauma[8]. When HPVG occurred in association with necrotic bowel, the overall mortality rate rose to about 75%[9]. Thus, in the past, HPVG has been considered as an indicator for urgent surgical intervention with a poor prognosis. However, the development of highly advanced imaging techniques enables earlier detection of potentially severe pathologies, such as bowel ischemia, and allows prompt diagnosis and treatment, which results in significantly reduced mortality rates[10]. HPVG is not always a surgical condition, and its treatment should be based on the underlying disease and the patient's current clinical condition. HPVG in patients with inflammatory bowel disease can be caused by mucosal damage alone, or can occur in combination with bowel distension, sepsis, invasion by gas-producing bacteria, or after colonoscopy, upper gastrointestinal barium examination, barium enema or blunt abdominal trauma. Not all of these conditions require surgical intervention, especially in the absence of peritoneal signs or free gas in the peritoneal space[2].

The prognosis is related to the pathology itself and is not influenced by the presence of HPVG. Among 182 case studies reported by Kinoshita *et al*[2], patients with ulcerative colitis or CD comprised 4% of the total. To our knowledge, 21 cases of PVG associated with CD were reported in the English literature[11-13].

The formation of HPVG in patients with CD can be explained by the following hypothesis; first, elevated intracolonic pressure can permit bowel gas or gas-forming bacteria to gain access to the portal venous circulation. Elevated intracolonic pressure caused by blunt abdominal trauma, or by diagnostic procedures such as colonoscopy or barium enema occurred in 2 and 6 out of 21 patients, respectively, that had relatively benign clinical courses and rarely needed surgical intervention; only one of the eight required surgery. Second, the enterovenous fistula, which is an extremely rare complication of CD, can directly transfer bowel gas to the portal venous system. To date, HPVG associated with enterovenous fistula has been reported in 2 patients[14,15]. Two CD patients with enterovenous fistula required surgery, and one died due to sepsis. Third, HPVG can be the result of mucosal injury and sepsis associated with bowel inflammation and portal pyemia. Mucosal damage secondary to bowel inflammation provides an entry for intraluminal gas into the portal venous system. In addition, sepsis alone without necrotic bowel can be the cause of HPVG[9]. The remaining 11 patients included in that study, who had no identifiable predisposing factors, may have developed HPVG by this mechanism.

In our patient, the second mechanism is the most plausible explanation for HPVG based on surgical pathology and CT findings. Although there was no definite communication between bowel and vessel shown on the abdominal CT scan, a thrombosed vessel along with a thickened bowel suggests possible communication. The fistula between the small bowel and small branches of the superior mesenteric vein was not easily identified on CT because of its small size. Furthermore, histopathology showed many neutrophils accumulated in the intravascular space, an occluded small mesenteric vein caused by inflammatory thrombus, and no evidence of bowel ischemia. Based on the clinical features, histopathology, and imaging findings, mesenteric venous thrombosis was not a plausible diagnosis.

In eleven patients without a predisposing condition, eight (73%) presented with signs of intra-abdominal catastrophe or systemic toxicity and required surgery. One of eight died of disseminated cytomegalovirus infection, and the remaining three out these eleven patients were conservatively managed.

Two cases were reported with a combination of PVG and thrombophlebitis of the portal vein or SMV. These patients presented with septic shock and needed surgical treatment[11,16]. Although PVG itself is not a prognostic indicator, PVG combined with thrombophlebitis of the portal or mesenteric veins can be regarded as an indicator of poor prognosis.

Although the incidence of CD in Asians is still much lower than in Western patients, recent studies have reported that the incidence of CD in Asian populations is gradually increasing[17,18]. As the number of patients with CD increases, the proportion of complicated CD patients also increases. Although PVG accompanied by mesenteric venous thrombosis occurs very rarely in patients with CD, it complicates treatment decisions for the gastroenterologist. Thus, we should exercise caution regarding the clinical management in cases of CD with HPVG. HPVG associated with CD does not always mandate surgical intervention, especially in the absence of peritoneal signs or free intraperitoneal gas. Those patients found to have significant intestinal pathology, as in this case, as seen from imaging studies, or all other symptomatic patients who do not improve after medical treatment, should undergo urgent laparotomy[10]. The clinical course seems to be associated with predisposing factors, such as in this case, where hypotension and fever necessitated urgent surgery.

In conclusion, the finding of PVG is not always an indication for surgical intervention in CD, but PVG caused by enterovenous fistula requires urgent surgical treatment.

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Samir Ahboucha, PhD, Assistant Professor, Équipe Neurosciences, Pharmacologie et Environnement, Université Cadi Ayyad, Faculté des Sciences Semlalia, Avenue My Abdellah, BP 2390, Marrakech 40000, Morocco

Edward J Ciaccio, PhD, Research Scientist, Department of Medicine, HP 804, Columbia University, 180 Fort Washington Avenue, New York, NY 10032, United States

Seng-Kee Chuah, MD, Division of Hepatogastroenterology, Chang Kaohsiung Gang Memorial Hospital, 123, Ta-Pei Road, Niaosung Hsiang, Kaohsiung 833, Taiwan, China

Dr. Charles B Ferguson, MRCP, Department of Gastroenterology, Belfast City Hospital, 51 Lisburn Road, Belfast BT9 7AB, United Kingdom

Gianpiero Gravante, MD, BsC, MBBS, Department of Hepatobiliary and Pancreatic Surgery, Leicester General Hospital, Flat 38, Room 8, Hospital Close, Leicester, LE5 4WU, United Kingdom

Chiun Hsu, MD, PhD, Clinical Associate Professor, Department of Oncology, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei 100, Taiwan, China

Kevin Cheng-Wen Hsiao, MD, Assistant Professor, Colon and rectal surgery, Tri-Service General Hospital, No. 325, Sec 2, Cheng-Kung Rd, Nei-Hu District, Taipei 114, Taiwan, China

Shiu-Ming Kuo, MD, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214, United States

Dr. Giovanni Latella, MD, Professor, Department of Internal Medicine, GI Unit, University of L'Aquila, L'Aquila 67100, Italy

Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Atsushi Masamune, MD, PhD, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Tamir Miloh, MD, Associate Professor, Director of Pediatric Liver/Liver Transplantation Program, Division of Gastroenterology, Phoenix Children's Hospital, 1919 E Thomas Rd, Main Building, Second Floor, Phoenix, AZ 85016, United States

Nobuhiro Ohkohchi, MD, PhD, Professor, Vice-President of University Hospital, Department of Surgery and Organ Transplantation, Advanced Biomedical Applications, Graduate School of Comprehensive Human Science, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575, Japan

Eamonn M Quigley, Professor, Department of Medicine National University of Ireland, Cork University Hospital Clinical Sciences Building, Wilton, Cork, Ireland

Xiaofa Qin, MD, PhD, Department of Surgery, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103, United States

Ala Sharara, MD, FACP, Associate Professor of Medicine, Head, Division of Gastroenterology, Director, Endoscopy Unit, American University of Beirut Medical Center, Associate Consulting Professor, Duke University Medical Center, PO Box 11-0236, Riad El Solh 110 72020, Beirut, Lebanon

Francis Seow-Choen, MBBS, FRCSEd, FAMS, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre No. 09-10, 228510, Singapore

Luca Stocchi, MD, Desk A 30, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States

Phil Sutton, Associate Professor, Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne, Melbourne, VIC 3010, Australia

Shinji Tanaka, MD, PhD, Professor and Director, Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

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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 the 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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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

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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]

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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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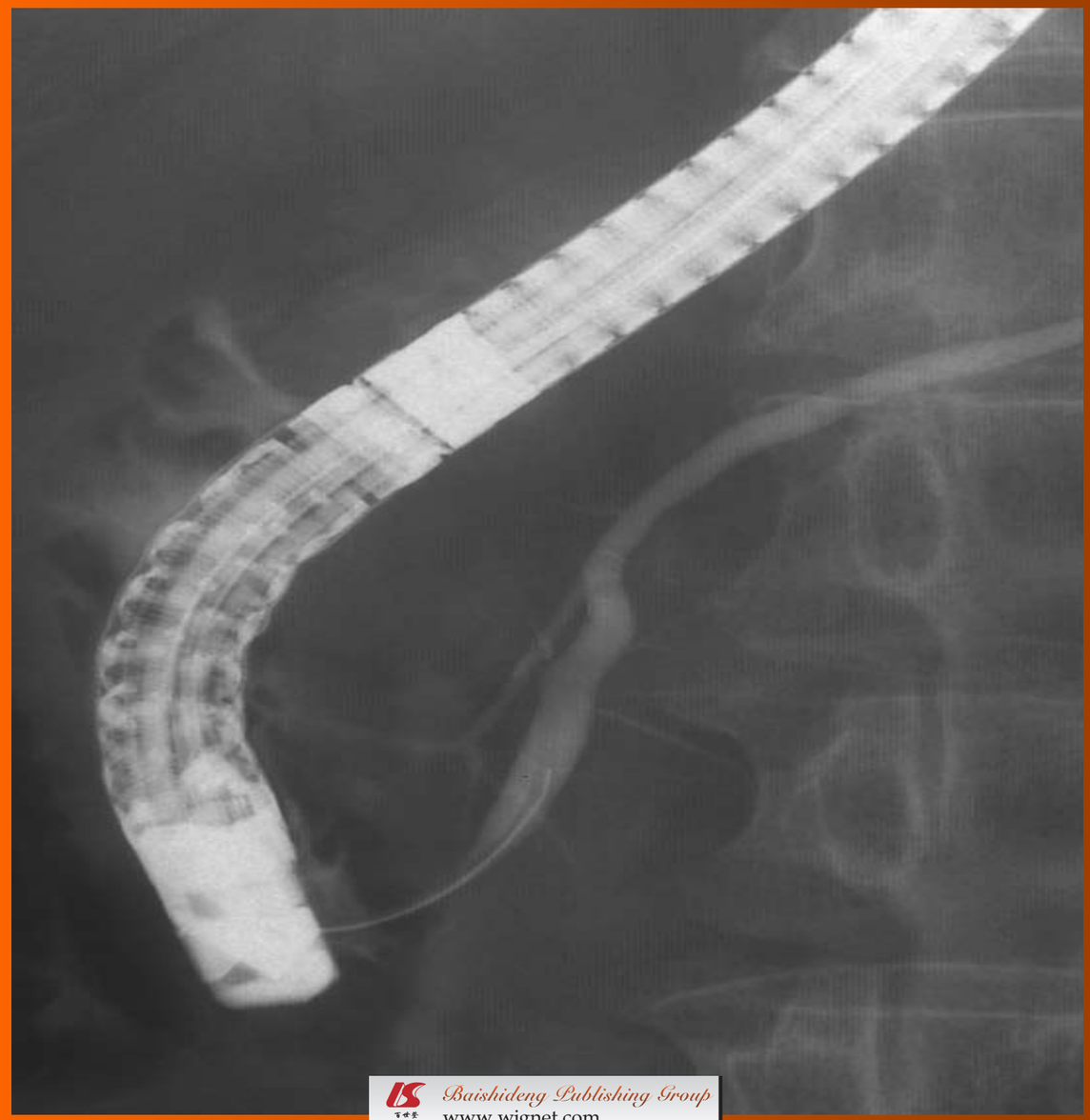

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Room 903, Building D, Ocean International Center,
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EDITORIAL

miRNAs in precancerous lesions of the gastrointestinal tract

Matteo Fassan, Carlo M Croce, Massimo Rugge

Matteo Fassan, Massimo Rugge, Department of Medical Diagnostic Sciences and Special Therapies, Surgical Pathology and Cytopathology Unit, University of Padova, 35100 Padova, Italy
Matteo Fassan, Department of Oncological and Surgical Sciences; General Oncology Unit, University of Padova, 35100 Padova, Italy
Carlo M Croce, Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210, United States
Massimo Rugge, Istituto Oncologico del Veneto - IOV-IRCCS, 35100 Padova, Italy
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Correspondence to: Massimo Rugge, MD, FACG, Full Professor of Surgical Pathology, Department of Medical Diagnostic Sciences and Special Therapies, the Surgical Pathology and Cytopathology Unit, University of Padova, Via Aristide Gabelli, 61, 35121 Padova, Italy. massimo.rugge@unipd.it
Telephone: +39-49-8218990 Fax: +39-49-8272277
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Abstract

In spite of the well-established understanding of the phenotypic lesions occurring in the shift from native epithelia to invasive (adeno) carcinoma, the molecular typing of the precancerous changes in the gastrointestinal tract remains unreliable. In recent years, no biomarkers have aroused as much interest as the miRNAs, a class of non-coding RNA molecules that function as endogenous silencers of numerous target genes. Aberrant miRNA expression is a hallmark of human disease, including cancer. Unlike most mRNAs, miRNAs are both long-living *in vivo* and very stable *in vitro*. Such characteristics allow their testing in paraffin-embedded tissue samples, which is essential in the biological profiling of small (phenotypically characterized) preneoplastic lesions of the gastrointestinal tract (as well as in other fields of human pathology). The upcoming challenge lies in the reliable identification of disease-specific targets of dysregulated miRNAs, to enable miRNA testing in the clinical management of the secondary prevention of gastrointestinal cancer.

Key words: miRNA; Dysplasia; Barrett's esophagus; Atrophic gastritis; Inflammatory bowel disease

Peer reviewer: Wendy Wilhelmina Johanna de Leng, PhD, Department of Pathology, Internal address H04.312, Heidelberglaan 100, Postbox 85500, Utrecht, 3508 GA, The Netherlands

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INTRODUCTION

The molecular mechanisms involved in the multistep processes of gastrointestinal (GI) cancers are one of the most intriguing research fields in pathology.

In spite of our well-established understanding of the phenotypic lesions occurring in the shift from native epithelia to invasive (adeno)carcinoma, the molecular typing of the precancerous changes in the GI tract remains unreliable due to: (1) discrepancies in their histological classification; (2) their inherent biological heterogeneity; and (3) their variability on molecular biology testing. As a result, no reliable molecular data are currently available that can be implemented with confidence in GI cancer secondary prevention strategies[1,2].

Molecular profiling, as achievable using well-characterized histology samples, would ideally be done using formalin-fixed, paraffin-embedded (FF-PE) tissue

Table 1 miRNAs associated with different histological lesions of the gastrointestinal tract

Organ	Pathology	Overexpressed miRNAs	Downregulated miRNAs	Ref.
Esophagus	Barrett's mucosa	*miR-15b; miR-21; miR-25; miR-143; miR-145; miR-192; miR-194; miR-196a; miR-200c; miR-215*	*let-7a; let-7c; miR-125b; miR-203; miR-205; miR-486-5p*	[30,31-33,35,37]
	LG-IEN	*miR-192; miR-196a; miR-215*	*let-7c; miR-203; miR-205*	[32,35]
	HG-IEN	*miR-15b; miR-21; miR-125b; miR-192;miR-196a; miR-200a*; miR-215*	*let-7a; let-7c; miR-181b; miR-193b; miR-203; miR-205; miR-486-5p*	[30,31-33,35,36]
	Neosquamous epithelium	*miR-143*	-	[38]
Stomach	*H. pylori*-related gastritis	*miR-21; miR-146a; miR-155; miR-223*	*let-7f; miR-34b; miR-34c; miR-124a-1; miR-124a-2; miR-124a-3; miR-141; miR-200a; miR-203; miR-204; miR-455*	[43,45,47,49,50,53,56,58]
	Atrophic gastritis	-	*let-7a*	[55]
Colon	Traditional adenoma	*miR-21; miR-135*	*miR-143; miR-145*	[65]
	Serrated adenoma	*miR-21; miR-181b*	-	[70,71]
	Hyperplastic polyp	*miR-181b*	-	[71]
	Crohn's disease	*miR-9; miR-9*; miR-21; miR-22; miR-23b; miR-26a; miR-29b; miR-29c; miR-30a; miR-30b; miR-30c; miR-31; miR-34c-5p; miR-106a; miR-126; miR-126*; miR-127-3p; miR-130a; miR-133b; miR-146a; miR-146b-5p; miR-150; miR-155; miR-181c miR-191; miR-196a; miR-223; miR-324-3p; miR-375*	*miR-19b; miR-629*	[75,79]
	Ulcerative colitis	*let-7f; mIR-7; miR-16; miR-21; miR-23a; miR-24; miR-29a; miR-29b; miR-31; miR-126; miR-126*; miR-127-3p; miR-135b; miR-223; miR-324-3p; miR-195; miR-196a*	*miR-188-5p; miR-192; miR-215; miR-320a; miR-346; miR-375; miR-422b*	[75,79]
	IBD dysplasia	*miR-31*	-	[80]

LG-IEN: Low-grade-intraepithelial neoplastic lesions; HG-IEN: High-grade-intraepithelial neoplastic lesions; *H. pylori*: *Helicobacter pylori;* IBD: Inflammatory bowel disease.

samples. Unfortunately, molecular profiling from FF-PE samples is patchy, which makes it difficult to integrate molecular data (gene expression arrays, among others) with histological information consistently[3-5].

Hence the priority to overcome this methodological impasse. Despite efforts made by the scientific community to identify biomarkers for human cancer, no such biomarkers have aroused as much interest as the miRNAs, a class of endogenous, small, non-coding RNAs that modulate gene expression by causing target mRNA degradation or inhibiting their translation[6-10]. Since their initial discovery in *Caenorhabditis elegans* (*C. elegans*) in 1993[11], an enormous amount of research has been published, indicating that the biological function of miRNAs is crucial to most cellular processes. In humans, aberrant miRNA expression is a hallmark of various diseases, including cancer[9,10]. Unlike most mRNAs (due to their molecular structure), miRNAs are long-living *in vivo* and very stable *in vitro*[3-5]. These particular features are fundamental to their analysis in FF-PE samples, supporting a potentially central role for miRNAs in the molecular study of preneoplastic GI lesions.

Focusing on the similar, multistep carcinogenic cascade occurring in both esophageal and gastric adenocarcinomas, and on the colorectal carcinogenic processes, this Editorial briefly discusses the role of miRNAs in preneoplastic GI pathology.

miRNAs AS DIAGNOSTIC TOOLS

miRNA expression profiling has the potential for differentiating between normal and pathological lesions, and among preneoplastic lesions, it may be able to classify different subtypes[9,10].

Several reports have already demonstrated the excellent reproducibility and accuracy of miRNA expression profiling in archived FF-PE specimens. In gastroenterology, as in other fields of human pathology, integrating genome-wide profiling with the functional characterization of miRNAs (their overexpression or downregulation) and the identification of miRNA-specific gene targets currently represents the approach most likely to yield advances in the new field of non-coding RNA research[6-8,12].

In FF-PE specimens, miRNA expression could also be visualized at cellular/sub-cellular level (*in situ* hybridization) and this particular characteristic makes miRNAs potentially suitable for supporting routine diagnostic surgical pathology practice[13,14].

Aberrant miRNA expression signatures have been extensively investigated in preneoplastic GI diseases and several key oncogenic miRNAs have consistently been found dysregulated (Table 1)[6-8,12,15-21]. In some cases, specific miRNA expressions have been linked to cancer-associated pathways, indicating a role for them in GI carcinogenesis. The systematic molecular evaluation of the GI mucosa (always supported by the "advanced" histological and clinical characterization of the specimens) not only provides new basic biological information, but can also pave the way to risk-stratified patient management programs and innovative therapeutic measures (Figure 1).

Therefore, miRNAs represent both potential cancer prognostic markers and therapeutic targets for the treatment of human malignancies. Further understanding of the biological roles of miRNAs in cancer is required, particularly the experimental validation of miRNA targets.

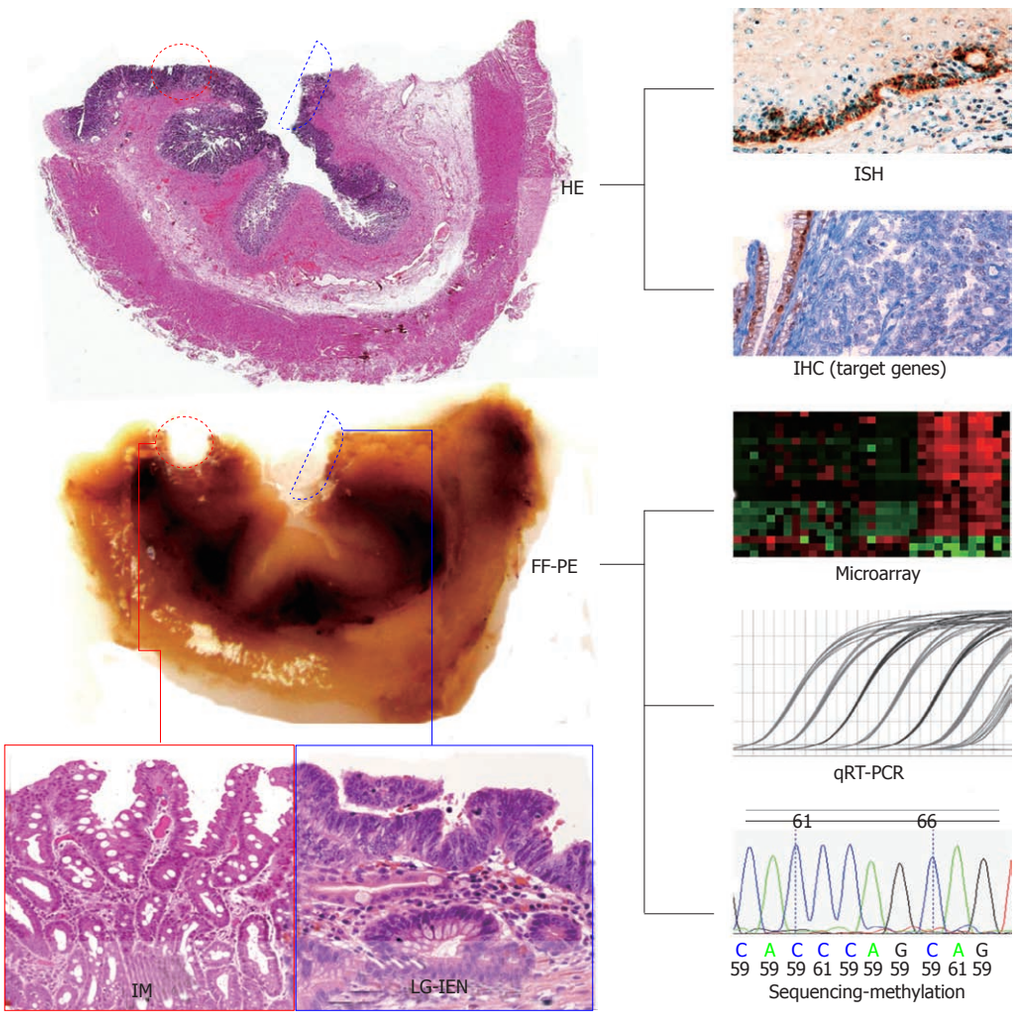

Figure 1 miRNA analysis in formalin-fixed, paraffin-embedded samples. Routine histological analysis (hematoxylin and eosin) enables adequate selection of preneoplastic lesions (in this case, intestinal metaplasia and low-grade-intraepithelial neoplastic lesions obtained from a resected Barrett's adenocarcinoma specimen). Either miRNA-specific *in situ* hybridization or immunohistochemical analysis of the miRNA gene targets could be performed starting from the original paraffin block. To obtain total RNA or DNA, lesions are macro-dissected from the paraffin block (or micro-dissected from unstained formalin-fixed, paraffin-embedded sections). The genome material can be further analyzed using modern genomic techniques. HE: Hematoxylin and eosin; FF-PE: Formalin-fixed, paraffin-embedded; IM: Intestinal metaplasia; LG-IEN: Low-grade-intraepithelial neoplastic lesions; ISH: *in situ* hybridization; IHC: Immunohistochemistry; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

ONCOGENIC CASCADES IN GASTRO-ESOPHAGEAL CARCINOMA

In the last 25 years, it has been demonstrated that full-blown gastroesophageal cancers are the final outcome of long-standing biological processes that include a progressive accumulation of genotypic and phenotypic changes triggered by persistent inflammatory conditions, i.e., chronic acid/bile reflux in the esophagus and longstanding gastritis, due primarily to *Helicobacter pylori* (*H. pylori*) infection, in the stomach[1,2].

Such natural histories have been described as Barrett's model of carcinogenesis for esophageal adenocarcinoma and Pelayo Correa's multistage cascade for gastric adenocarcinoma[1,2].

At first, both conditions lead to the replacement of native by metaplastic epithelia [columnar epithelia in Barrett's esophagus (BE) and intestinal metaplasia of the native gastric glands][1,2]. The phenotypic shift involved in these processes is due to the combined effect of changes in the expression of genetic factors, epigenetic silencing, transcription factors, signaling pathways and growth factors. None of the biological mechanisms underlying the metaplastic transformation have been clarified in detail as yet, however, and genome-wide microarray expression analysis seems to be the most promising line of investigation.

There are significant differences in the pathogenetic, morphological (histochemical and immunohistochemical) and clinical impact of metaplasia in the two above-mentioned anatomical settings. Intestinal metaplasia (IM), however, is known as the "carcinogenic field" in which both esophageal and gastric intraepithelial neoplastic lesions (IENs), be it low-grade-IENs or high-grade (HG)-IENs and subsequent adenocarcinomas may develop[22-27].

The Barrett's cascade

BE is characterized by the native stratified squamous epi-

thelium lining the esophagus being replaced by a columnar epithelium with intestinal differentiation [Barrett's mucosa (BM)][2]. From the clinical point of view, only a histological diagnosis of IEN is currently considered in the definition of high-risk BE populations, confirming the need for prognostically useful, novel molecular biomarkers[28]. In this scenario, several reports have investigated miRNA expression profiling in Barrett's carcinogenesis[15,29-33].

In their seminal work, Feber *et al*[29] investigated miRNA expression profiling in a small series of frozen specimens (nine native squamous esophageal mucosa, five BM, one HG-IEN, and 10 Barrett's adenocarcinoma). Among the miRNA expression signatures observed, miR-203 and miR-205 were downregulated and miR-21, miR-192 and miR-194 were overexpressed in esophageal adenocarcinoma samples[29]. It is important to bear in mind that miR-194 is involved in the lineage commitment decisions of the intestinal epithelium system, with a major role in the maturation of the intestinal epithelium[34]. The authors also considered 10 squamous cell carcinoma specimens in their analysis, showing that specific miRNA expression profiles correspond to specific esophageal tumor histotypes[29].

Two further papers with consistent results focused on the miRNA expression signatures associated with disease progression[30,31]. Our group provided comprehensive information on the miRNA profile coupled with each single step in the natural history of Barrett's carcinogenesis[32]. To confirm our microarray and quantitative RT-PCR data, we also performed miRNA-specific *in situ* hybridization analyses on FF-PE tissues and identified HMGA2 (a let-7c mRNA target, which is a small, non-histone chromosomal protein that can modulate transcription by altering chromatin architecture) as a promising biomarker of BM transformation on immunohistochemical analysis. Based on these microarray findings, Bansal *et al*[33] recently have investigated the prognostic impact of miRNA expression in determining BE progression, demonstrating that miRNAs could pinpoint high-risk BE patients with a reasonable clinical accuracy.

In exploring the molecular mechanisms implicated in BM transformation and progression, Maru *et al*[35] have particularly focused on the role of miR-196a. As previously demonstrated by the same research group, miR-196a is overexpressed in neoplastic samples, and its expression levels increase proportionally with the dedifferentiation of the IEN[35]. Moreover, they have shown that miR-196a plays a part in the downregulation of the *SPRR2C*, *S100A9* and *KRT5* genes, the expression of which is characteristically decreased or lost during the neoplastic transformation of esophageal tissue[35]. These findings strongly support a role for miR-196a as a novel therapeutic target in the treatment of esophageal cancers and as a valuable early marker of cancer-prone BE.

Another important oncomiR is miR-21, which is upregulated in various human tumors, and has been found to be involved in Barrett's adenocarcinoma[29,31]. Recent studies have shown that miR-21 promotes cell transformation by repressing tumor suppressor genes such as *PTEN*, *PDCD4*, *RECK* and *TPM1*[3]. Our group has demonstrated significant miR-21 upregulation in samples of HG-IENs and adenocarcinoma, consistent with *PDCD4* downregulation[36].

The dysregulation of not just one, but multiple miRNAs could be implicated in cancer progression. miRNAs could be organized as a cluster of genes expressed by a single transcription unit (i.e., polycistron), which goes to show how complex miRNA research may be. The miR-106b-25 polycistron on chromosome 7q22.1 (i.e., miR-25, miR-93 and miR-106b) has been found to be increasingly activated in successive stages of Barrett's carcinogenesis, with potentially proliferative, antiapoptotic, and cell cycle promoting effects *in vitro* and tumorigenic effects *in vivo* by targeting *p21* and *Bim*[37].

From the therapeutic standpoint, argon plasma coagulation (thermal ablation) is one of the options for the surgical treatment of BE patients. BM ablation usually eventually results in the formation of neosquamous epithelium. IM can recur, however (so cancer could subsequently develop too) even after apparently complete BM ablation. Biomarkers might therefore be helpful in clinical decision-making for BE patients, by providing information on the likely clinical behavior of the mucosa after ablative therapy. That is why Dijckmeester *et al*[38] investigated miR-143 and miR-205 expression in neosquamous epithelium and BM. Only miR-143 expression was significantly higher in neosquamous and native squamous esophageal mucosa than in samples of BM[38]. It is worth adding that miR-143 is highly expressed in colonic tissues and has a significant role in suppressing colorectal cancer cell growth by inhibiting *KRAS* translation[39].

Different genetic polymorphisms could be implicated in miRNA-related carcinogenesis. The biogenesis of miRNAs is complex and involves multiple proteins and RNAs. Although miRNA expression profiles have frequently been reported to correlate with the etiology, classification, progression and prognosis of numerous human cancers, the effect of common genetic variants [i.e., single nucleotide polymorphisms (SNP)] of miRNA-related genes on susceptibility to cancer remains unclear[9,10]. Ye *et al*[40] have demonstrated that seven SNPs were significantly associated with the risk of esophageal adenocarcinoma, pointing to intriguing new fields of translational research.

Correa's cascade

Despite a steady decline in the related mortality in the past few decades, gastric cancer is still the second cause of death due to cancer worldwide[41]. *H. pylori*-associated gastritis is the most common gastric adenocarcinoma precursor (leading to intestinal-type, or the so-called "epidemic"gastric cancer)[42].

Matsushima *et al*[43] have investigated the miRNA expression profiling of *H. pylori*-positive mucosa samples, finding 31 significantly dysregulated miRNAs. The severity of both active and chronic inflammatory infiltration

significantly correlated with the expression of several miRNAs, supporting a biological role for miRNAs in host immune response to *H. pylori* infection[44]. Among others, miR-155 has been suggested as a central effector of *H. pylori*-induced immune response: *H. pylori* infection raises miR-155 expression levels in gastric epithelial cell lines and gastric mucosal tissues by activating nuclear factor-κB and the activator protein-1 pathway, and *via* the Foxp3 transcription factor in T cells[45,46]. The overexpressed miR-155 could also negatively modulate the release of the proinflammatory cytokines interleukin-8 and GRO-a, leading to chronic *H. pylori*-related infection[47].

Another important *H. pylori*-induced miRNA is miR-146a, which seems to have a role in a negative feedback loop that modulates the inflammatory damage by targeting *IRAK1* and *TRAF6*[47]. Two common SNPs in its pre-miRNA sequence (rs2910164 and rs11614913) have also been associated with a greater susceptibility to gastric cancer in the Chinese and Japanese populations[48].

Among healthy volunteers, individuals with *H. pylori* infection show higher methylation levels of miR-34b, miR-34c, miR-124a-1, miR-124a-2 and miR-124a-3[49,50]. These data strongly suggest that *H. pylori* infection (in addition to protein-coding genes) induces DNA methylation of miRNA genes, also indicating that miRNA silencing is an early event in the oncogenic process and the result of a global epigenetic field defect.

Another important miRNA that is dysregulated in chronic gastritis is miR-27a. Arisawa *et al*[51] have shown a close correlation between miR-27a genome polymorphism and the onset of advanced gastric mucosa atrophy in Japanese men, suggesting a definitive role for miR-27a in the development of the "cancerization field. These findings are further supported by the fact that miR-27a has been found upregulated in gastric cancer samples; it correlates with gastric cancer lymph node metastasis; and functions as an oncogene by targeting the tumor suppressor gene prohibitin[52].

miR-21 seems to play a fundamental part in gastric carcinogenesis, being constantly upregulated in gastric adenocarcinoma (as already demonstrated in others miscellaneous solid tumors, as well as Barrett's adenocarcinoma)[53]. *In vitro*, miR-21 upregulation has been shown to enhance significantly migration and capacity for invasion of gastric cancer cell lines[53]. On the other hand, miR-21 knockdown causes a significant reduction in cell proliferation and a significant increase in apoptosis[53]. miR-21 is also significantly overexpressed in *H. pylori*-infected gastric mucosa, suggesting an early, important involvement of miR-21 in the development of gastric cancer[53].

The tumor suppressor let-7a is one of the founder members of the miRNA family, which was first identified in *C. elegans*. In gastric cancer, let-7a is downregulated and negatively regulates HMGA2 expression (like let-7c)[54,55]. let-7a downregulation has already been identified in chronic atrophic gastritis samples[56].

The oncogenic miR-106a, a member of the miR-106a-92 cluster, has been found upregulated in gastric cancer tissues and it has been significantly associated with negative clinicopathological parameters[56,57]. The miR-106a-92 cluster has a marked gene structure homology with other miRNA clusters recognized as being oncogenic, i.e., miR-17-92 and miR-106b-25. On this point, Petrocca *et al*[58] have observed dysregulation of the miR-106b-25 cluster (i.e., miR-106b, miR-92 and miR-25) determined by transcription factor E2F1 expression in *H. pylori*-related gastric carcinogenesis and gastric cancer cell lines. miR-206b and miR-93 directly regulate E2F1 expression, establishing an miRNA-related negative feedback loop[58]. The upregulation of these miRNAs interferes with the transforming growth factor (TGF)-β tumor suppressor pathway, hindering the expression of CDKN1A and BCL2L11[58]. TGF-β controls the turnover of intestinal, as well as gastric, cells leading them to cell cycle arrest followed by apoptosis. By downregulating two of the most important downstream effectors in the TGF-β pathway, the miR-106b-25 cluster ensures the development of a resistance to TGF-β-mediated cell cycle arrest and apoptosis, and may represent an interesting novel therapeutic target for the treatment of gastric cancer. In the same study, the authors identified a seven-miRNA signature associated with chronic *H. pylori*-related gastritis, which included the above-mentioned miR-155[58].

ONCOGENIC CASCADES IN COLORECTAL ADENOCARCINOMA

The historically well-established pathway of sporadic colorectal cancer is the so-called adenoma-carcinoma sequence, formally described by Morson in 1962[59]. Approximately 60% of sporadic colorectal cancers (CRCs) are consistent with this phenotypic sequence. The lesions included in this "cascade" are tubular, tubulovillous and villous adenomas (with different IEN grades)[60].

More recently, however, molecular genetic findings have highlighted two further pathways: (1) serrated carcinogenesis, in which the sessile serrated adenoma is considered the precursor lesion; and (2) the mixed-type sequence, combining the molecular features of both the traditional and the serrated pathways[60,61].

A fourth route is the one resulting from longstanding/relapsing inflammation associated with inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC)[62,63]. The first report of CRC in IBD came from Crohn and Rosenberg in 1925[64]. From a molecular point of view, the IBD-related carcinogenic process seems to follow a temporal sequence of genetic alterations different from the situation seen in sporadic cancers. The IBD-related CRC risk increases in long-standing colitis and parallels the anatomical extent and severity of the related inflammation. From the pathological standpoint, IBD-associated CRC can arise from: (1) raised dysplastic lesions or dysplasia-associated lesions or masses; and (2) endoscopically flat dysplastic lesions. In both cases, the lesions can blend easily with the gross inflammatory abnormalities commonly encountered in IBDs, making their endoscopic detection difficult even for the experienced

endoscopist, resulting in the need for innovative dysplasia-specific biomarkers[61].

Sporadic colorectal cancer

A whole constellation of experimental studies on CRC has provided insight into the miRNA-mediated, regulatory links to well-known oncogenic and tumor suppressor signaling pathways[20,65-67].

Global miRNA downregulation is a common feature of colorectal carcinogenesis. As described previously for gastroesophageal diseases, CpG island hypermethylation has been described as a mechanism for miRNA silencing in CRC samples too. Balaguer *et al*[68] also have demonstrated high rates of miR-137 CpG island methylation in colorectal adenomas, suggesting that the epigenetic silencing of specific tumor-suppressor miRNAs is an early event in the multistep colorectal carcinoma cascade.

Several investigations on paired samples of colorectal neoplasia and normal mucosa have demonstrated reduced levels of miR-143 and miR-145 in both colonic adenoma and carcinoma[39]. These two miRNAs reveal a tumor suppressor-like activity *in vitro* by targeting *KRAS* (miR-143) and the insulin receptor substrate 1 (*IRS-1*; miR-145), among others[39].

Among the overexpressed miRNAs, miR-21 (an important regulator of PDCD4 expression) has frequently been found dysregulated in CRC samples[3,69]. Consistent with these data, we found that miR-21 was significantly upregulated in IEN and CRC biopsy samples, suggestive of a diagnostic role for this miRNA in discriminating between neoplastic and non-neoplastic lesions[70]. We observed a similar PDCD4 dysregulation in serrated adenomas too, suggesting a major oncogenic function for miR-21 that is acquired early in different pathways of colorectal carcinogenesis. Similar results were have been reported by Schmitz *et al*[71], who have found significant miR-21 overexpression in sessile serrated adenomas by comparison with both normal mucosa and hyperplastic polyps (though miR-21 expression alone failed to distinguish between the histological lesions considered).

Sequence variations in the miRNA-binding sites of *CD86* and *INSR* have been associated with a higher risk of CRC (with ORs of 2.74 and 1.94, respectively)[72,73]. These data, integrated with genome-wide association studies supported by high-resolution SNP arrays and next-generation sequencing technology, could help clinicians to assess CRC genetic susceptibility more accurately, with the prospect of stratifying patients according to their "molecular" cancer risk.

Inflammatory bowel diseases

IBDs result from an abnormal immune response to environmental factors in genetically susceptible hosts. miRNAs have been increasingly recognized as important in the development of both the innate and the adaptive immune system, and dysregulated miRNA expression has already been described in several immune-related diseases. miRNA expression profiling in active CD and UC has been the object of several studies, which have consistently shown unique disease-specific miRNA signatures[74-77]. Supporting the role of miRNAs in immune system dysregulation, Wu *et al*[78] have demonstrated that miR-192 (which is significantly upregulated in UC) blocks tumor necrosis factor α-induced stimulation by targeting the chemotactic cytokine *MIP-2a*. Fasseu *et al*[79] have studied both active and inactive IBD samples, and have demonstrated that miRNAs are crucial players in the onset/relapse of active inflammation. In particular, they have found two subsets of 14 (UC) and 23 (CD) miRNAs with a significant dysregulation in comparison to healthy controls (Table 1), and among these, eight that were commonly dysregulated in non-inflammed UC and CD (miR-26a, miR-29a, miR-29b, miR-30c, miR-126*, miR-127-3p, miR-196a, and miR-324-3p)[79]. What is more, underlying the importance of miRNA dysregulation in IBD onset, several miRNA genes with altered expression co-localized with acknowledged IBD-susceptibility loci[79].

miR-31a has been indicated as a specific marker of IBD-related dysplasia[80]. It is overexpressed in both IBD-related dysplasia and IBD-associated CRC (and also significantly increased in sporadic CRC, although to a lesser degree than in IBD-related neoplasia). Preliminary studies have demonstrated the angiogenic potential of miR-31 *via* the targeting of *FIH-1* in CRC-derived cell lines[80].

Beyond their histopathological applications, miRNAs offer a number of practical advantages due to their relatively high stability *in vivo*, and in the circulation in particular. In addition, they do not require the use of specific antibody-linked detection reagents of protein biomarkers, and they offer the specificity of nucleic acid detection methods such as RT-PCR. In IBD patients, differences in circulating immune cells in CD and UC are reflected by altered miRNA expression, supporting the usefulness of peripheral blood miRNA analysis as an innovative non-invasive class of biomarkers[81].

CONCLUSION

Preneoplastic lesions within the GI tract include a broad spectrum of phenotypic alterations, which may be difficult to assess only on the basis of their phenotypical features and are hard to stratify in different prognostic classes.

As this review shows, miRNAs and miRNA-related gene expression and polymorphism have a central role in assessing the individual (patient-specific) cancer susceptibility and cancer progression. The upcoming challenge lies in the reliable identification of disease-specific targets of dysregulated miRNAs, to enable miRNA testing in clinical practice. The miRNA revolution is only just beginning!

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EDITORIAL

Impact of liver diseases on the development of type 2 diabetes mellitus

Po-Shiuan Hsieh, Yen-Ju Hsieh

Po-Shiuan Hsieh, Yen-Ju Hsieh, Department of Physiology and Biophysics, National Defense Medical Center, 114 Taipei, Taiwan, China
Po-Shiuan Hsieh, Department of Medical Research, Tri-Service General Hospital, 114 Taipei, Taiwan, China
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Correspondence to: Po-Shiuan Hsieh, MD, PhD, Professor and Head of Department of Physiology and Biophysics, National Defense Medical Center, 114 Taipei, Taiwan, China. pshsieh@hotmail.com
Telephone: +886-2-87923100-18622 Fax: +886-2-87924827
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Abstract

The prevalence of type 2 diabetes mellitus (T2DM) is higher in patients who have liver diseases such as nonalcoholic fatty liver disease, chronic viral hepatitis, hemochromatosis, alcoholic liver disease and cirrhosis. It is suggested that there is a pathogenic link between the presence of T2DM and the severity of liver injury. However, evidence related to the impact of hepatic inflammation on the development of T2DM has not yet emerged. This article provides an overview of the evidence for an increased prevalence of diabetes in a range of liver diseases, the impact of liver diseases on insulin resistance and β cell dysfunction, and the potential mechanisms whereby coexistent liver diseases exacerbate the development of T2DM.

Key words: Hepatic inflammation; Insulin resistance; β cell dysfunction; Type 2 diabetes mellitus

Peer reviewer: Rudi Beyaert, Professor, Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology and Ghent University Technologiepark 927, B-9052 Gent, Belgium

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INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) is higher in patients who have suffered from certain liver diseases. Therefore, it is speculated that there is a pathogenic link between liver disease progression and T2DM. For instance, nonalcoholic fatty liver disease (NAFLD) has been proposed as a term that encompasses a spectrum of fatty liver disease from steatosis to non-alcoholic steatohepatitis (NASH) through cirrhosis to end-stage liver disease[1,2]. Clinical investigations and epidemiologic studies have associated NAFLD and NASH in particular with the metabolic syndrome[3,4], with T2DM[1] as the pivotal pathogenic factor. However, the cause-effect relationship between NAFLD/NASH and diabetes remains elusive. On the other hand, cross-sectional and longitudinal studies have shown that chronic hepatitis C virus (HCV) infection is associated with an increased risk of developing insulin resistance and T2DM[5]. Moreover, high alanine aminotransferase level is associated with decreased hepatic insulin sensitivity and predicts the development of T2DM[6]. Pradhan *et al*[7] have reported that elevated levels of the inflammatory markers interleukin 6 (IL-6) and C-reactive protein (CRP, hepatic acute phase protein) were associated with the development of T2DM in healthy middle-aged women in a prospective, nested-control study. However, only limited evidence is emerging to identify the possible impact of liver diseases on the development of T2DM.

IMPACT OF LIVER DISEASES ON INSULIN RESISTANCE

Fatty liver diseases and insulin resistance

Fatty liver disease (FLD), whether it is alcoholic fatty liver disease (AFLD) or nonalcoholic fatty liver disease (NAFLD), encompasses a morphological spectrum consisting of hepatic steatosis (fatty liver) and steatohepatitis. The indistinguishable spectrum of histological features of both AFLD and NAFLD suggests a possible convergence of pathogenetic mechanisms at some critical juncture that enables the progression of steatohepatitis toward cirrhosis and liver cancer. Moreover, fat accumulation in the liver is, independent of body mass index and intraabdominal and overall obesity, characterized by several features of insulin resistance in normal weight and moderately overweight subjects[8].

Although NAFLD/NASH is generally considered as the result of insulin resistance syndrome including obesity, T2DM and hyperlipidemia, some recent studies implicate that NAFLD could also be a prediabetic condition. For example, Fan *et al*[9] conducted a retrospective study on a cohort of 358 individuals with hepatic ultrasound-defined fatty liver and 788 age-, sex- and occupation-matched controls for 4-7 years, which showed that metabolic syndrome components were present at a greater frequency among those with fatty liver than among controls. Population-based studies showed an association between elevated liver enzymes (as inflammatory markers of NAFLD) and a future potential for development of the metabolic syndrome[10,11].

On the other hand, it has also been reported that subjects with alcoholic liver disease have been shown to be at increased risk for T2DM[12]. Wei *et al*[13] showed that subjects with high alcohol intake (> 270 g/wk) have a 2-fold increased risk of developing T2DM compared with those with moderate alcohol intake (60-120 g/wk) in a prospective follow-up study of 8663 men. This association was independent of other risk factors, such as age, obesity, blood pressure, smoking and family history of diabetes. However, a similar relationship was not shown in a study with female subjects[14].

The pathogenesis of alcoholic fatty liver disease and NASH is multifactorial and includes several overlapping events. The accumulation of fat in hepatocytes (steatosis) and the onset of steatohepatitis may reflect successive stages in FLD. The "two-hit" hypothesis proposed by Day and James in 1998[15] postulates that the steatotic liver is susceptible to secondary insults including a vulnerability to reactive oxygen species, gut-derived endotoxins, and adipocytokines such as tumor necrosis factor-α (TNF-α) and other cytokines. The first "hit" is thought to be an accumulation of fatty acids and triglycerides within the liver, possibly due to insulin resistance. Chronic stress such as portal endotoxemia (the second "hit") leads to mitochondrial dysfunction and Kupffer cell adaptive changes[16,17], which in turn result in hepatocyte survival adaptation[18] and subsequent necrosis and/or apoptosis. Concomitant release of liver-derived inflammatory cytokines, i.e., TNF-α[19] and acute-phase proteins such as CRP, LPS binding protein and serum amyloid-P component[20] may induce some extrahepatic effects and then affect the development of T2DM. However, this hypothesis has not been directly examined in human and animal studies.

Viral hepatitis and insulin resistance

Recent studies have suggested that HCV infection is associated with an increased risk of development of T2DM, and that T2DM is more common among patients with chronic HCV infection than in patients with other liver diseases or in the general population, irrespective of whether or not hepatic cirrhosis is present[21]. Moreover, the conclusion of a prospective, case-cohort study conducted within a community-based cohort of 1084 persons aged 44-65 years suggests that pre-existing HCV infection may increase the incidence of T2DM in persons with known risk factors[22]. A community-based cohort survey performed in southern Taiwan enrolled 4958 persons aged ⩾ 40 years without T2DM. After a follow-up of 7 years, 474 cases of incident T2DM were recorded: overall, 14.3% of anti-HCV positive, 7.5% of HBsAg positive, and 8.6% of seronegative individuals developed T2DM during the study. Compared to anti-HCV negative individuals, anti-HCV positive persons had a higher cumulative incidence of T2DM ($P < 0.0001$)[23].

Hepatogenous diabetes

Up to 96% of cirrhotic subjects have impaired glucose tolerance or diabetes[24]. The term "hepatogenous diabetes" is used to describe the close association between cirrhosis and impaired glycemic control. There is less association with other T2DM-related risk factors such as age, obesity, smoking history, family history of diabetes and hypertension[24]. Cirrhosis may contribute to the development of T2DM through numerous factors such as reduced insulin clearance with peripheral hyperinsulinemia[25], which could contribute to the development of insulin resistance through the down-regulation of insulin receptors. Nevertheless, interaction of the cause of liver cirrhosis with environmental factors may also play a significant role in the link between cirrhosis and diabetes rather than development of cirrhosis alone.

IMPACT OF LIVER DISEASES ON β CELL DYSFUNCTION

The pancreas and the liver are in close proximity and many of the blood vessels and ducts in these organs are anatomically associated with each other. It was suggested that liver disease, regardless of the etiology, may predispose the patient to develop acute or chronic pancreatitis in an analysis of 107 754 adult autopsies in Japan[26]. In addition, exocrine pancreatic function has been reported to be damaged in chronic liver disease[27] and chronic viral hepatitis[28]. An animal study showed exacerbation of

acute pancreatitis in the presence of chronic liver injury in rats[29].

On the other hand, pancreatogenic diabetes, regarded as a form of "secondary diabetes"[30], accounts for 1% to 2% of all diabetic patients in North America but as many as 15% to 20% of diabetic patients in the Southeast Asian continents[31]. Many issues remain unknown regarding the etiology of pancreatic inflammation in pancreatogenic diabetes. In addition, clinical studies showed that the impairment of exocrine pancreatic function was more frequently seen in subjects with alcoholic and non-alcoholic liver diseases[32]. A recent investigation has been undertaken to clarify the effect of the inflammatory change of fatty liver on the development of β cell dysfunction in T2DM. It showed that the inflamed liver induced by mild portal endotoxemia was concomitantly combined with an impairment of pancreatic insulin secretion[33], suggesting that this may be a detrimental factor in the pathogenesis of β cell failure during development of T2DM and also pancreatogenic diabetes.

MECHANISMS UNDERLYING THE EFFECTS OF LIVER DISEASES ON T2DM

Gut microbiota

Wigg *et al*[34] reported a higher prevalence of small intestinal bacterial overgrowth (SIBO) and increased circulating TNF-α levels in patients with NASH. In addition, inflammatory liver damage in various rat models of SIBO is improved by antibiotic treatments[34,35]. A recent study from Brun *et al*[36] has demonstrated that genetically obese mice (*ob*/*ob* and *db*/*db* mice) display enhanced intestinal permeability leading to increased intraportal endotoxemia that can contribute to the liver inflammatory damage. These studies provide evidence to suggest that portal endotoxemia is a major risk factor in the pathogenesis of NASH. Furthermore, a recent investigation demonstrated that mild portal endotoxemia induced by low-dose intraportal LPS infusion intensified the inflammatory changes of fructose-induced fatty liver and also caused low-grade systemic inflammation in the absence of an increase in blood endotoxin levels, providing strong evidence to support the causal role of portal endotoxemia in development of NASH[37] .

On the other hand, Backhed *et al*[38] showed that germ-free mice gained significantly less weight and fat mass than conventionalized mice, and were protected against high-fat diet-induced glucose intolerance and insulin resistance, suggesting that a bacterially related factor/mechanism other than energy harvesting may be responsible for the development of diet-induced obesity and diabetes. In addition, gut microbiota, especially bacterial LPS, has recently been speculated to contribute to the low-grade inflammation in obesity, diabetes, NAFLD and cardiovascular disease[39]. Cani *et al*[40,41] showed that there was a significant increase in circulating LPS levels in mice on high-fat feeding for 2-4 wk. They reproduced metabolic endotoxemia in these high-fat fed mice by chronically infusing low-dose LPS for 4 wk to develop the same phenotype as those on a high-fat diet such as obesity, insulin resistance, diabetes, hepatic steatosis and adipose tissue macrophage infiltration. However, this high-fat associated phenotype was not exhibited in LPS receptor knockout mice (CD-14KO) with a high-fat diet. Moreover, CD14KO mice were hypersensitive to insulin, even when they were fed a normal diet, suggesting that CD14 could be a modulator of insulin sensitivity under physiological conditions.

Furthermore, recent study using chronic low-dose portal LPS infusion in rats to simulate low-grade portal endotoxemia and hepatic injury showed that increasing oxidative (malondialdehyde) and inflammatory TNF-α and IL-6 markers with pathogenic changes were not only exhibited in liver but also in the pancreas of experimental rats[33]. This data also showed that the sequential effects of inflamed fatty liver could further impair pancreatic β cell function in the absence of change in homeostasis model assessment-insulin resistance index, suggesting that the low-grade hepatic inflammation induced by intraportal low-dose LPS infusion is the significant detrimental factor for the early development of T2DM.

HCV infection

It is suggested that HCV may alter glucose homeostasis by its direct action[42], or *via* indirect mechanisms such as through cytokine stimulation. For instance, in the transgenic mouse model[43], the core-encoding region of HCV is sufficient to induce insulin resistance. The effect was reversed by treatment with anti-TNF-antibodies, which suggested an increased level of serine phosphorylation of IRS-1 as induced by TNF-α. Nevertheless, direct but genotype-specific mechanisms have been reported[44], in which down-regulation of peroxisome proliferator-activated receptor-γ (PPARγ) and up-regulation of SOCS-7 were observed in cells transfected with the core protein of genotype 3, whereas the core protein of genotype 1b activated the mammalian target of rapamycin. These findings were confirmed by using agonists for PPARγ or short interfering RNAs for SOCS-7[45]. Accordingly, the *in vitro* study of Kawaguchi *et al*[46] demonstrated that HCV proteins inhibit insulin signaling.

In addition, studies on chronically infected patients have suggested that increased oxidative stress and intrahepatic inflammation may also play a role[47]. In fact, an increased intrahepatic TNF-α response, which results in insulin resistance and a higher risk of developing T2DM in chronic HCV, has been described[48]. It is necessary to further investigate at a more in-depth level the causal relationship between HCV-induced hepatic inflammation and the development of T2DM.

Hepatic inflammation and obesity-associated insulin resistance

Subclinical inflammation is predictive of both cardiovascular diseases and T2DM. Inflammatory changes in visceral adiposity in obesity and chronic hepatic inflammation are etiologically and functionally intertwined, and

both may be associated with chronic systemic inflammation in the pre-diabetic state. To isolate the potential systemic effects of chronic subacute hepatic inflammation, Cai *et al*[49] conducted a study with non-obese transgenic mice expressing constitutively active I-Kappa-B kinase-β (IKK-β) in hepatocytes. Their data suggest that chronic low-grade hepatic inflammation could cause systemic insulin resistance mediated by elevated circulating IL-6 levels. Arkan *et al*[50] recently presented similar findings in mice lacking IKK-β in hepatocytes. Liver-specific deletion of IKK-β resulted in relative insulin sensitivity in the liver when animals were placed on a high-fat diet or were intercrossed with the *ob/ob* model of genetic obesity, but development of insulin resistance in muscle and fat occurred.

Hepatic fat accumulation

However, a recent study in rats with hepatic overexpression of glycerol-sn-3-phosphate acyltransferase 1 demonstrated that increased flux through the pathway of hepatic *de novo* triacylglycerol synthesis can cause hepatic and systemic insulin resistance in the absence of increased hepatic inflammation, suggesting that an excess flux of lipid intermediates in the pathway of triacyglycerol synthesis are sufficient to cause insulin resistance[51]. Accordingly, studies showing that liver fat content, much more strongly than visceral fat mass, determines insulin sensitivity in humans, support a direct and major role of fatty liver in the pathogenesis of insulin resistance[52,53].

Reactive oxygen species

Oxidative stress due to generation of reactive oxygen species and/or decreased antioxidant defenses[54] has been proposed as the root cause underlying the development of insulin resistance, β cell dysfunction, impaired glucose tolerance and T2DM. In both nonalcoholic steatohepatitis and experimental steatohepatitis, hepatic expression of CYP2E1 is increased, leading to oxidative stress, which has been demonstrated to impair insulin signaling[55].

Hepatokines

Recent studies have suggested that the mechanisms of fatty liver-induced metabolic diseases may differ from those of expanded adipose tissue mass. The new concept proposed is that the fatty liver releases factors in the circulation, similarly to expanded and inflamed adipose tissue (adipokines), which can be called hepatokines, and they have direct effects on target tissues. There is strong evidence to support the concept that hepatokines such as protein fetuin-A, sex hormone-binding globulin and selenoprotein P play an important role in the pathogenesis of insulin resistance and also subclinical inflammation[56,57].

CONCLUSION

The prevalence of T2DM is higher in patients who have liver diseases such as NAFLD, chronic viral hepatitis, alcoholic liver disease and cirrhosis. However, the cause-effect relationship between liver diseases and T2DM remains ambiguous. This article provides an overview of the evidence for the impact of chronic liver diseases on T2DM and examines up-to-date studies about the possible underlying mechanism. A better understanding of the deleterious factors which affect progression of chronic liver diseases are of clinical importance in order to monitor and treat T2DM patients with liver diseases.

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TOPIC HIGHLIGHT

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Role of genetics in the diagnosis and prognosis of Crohn's disease

Epameinondas V Tsianos, Konstantinos H Katsanos, Vasileios E Tsianos

Epameinondas V Tsianos, Konstantinos H Katsanos, Vasileios E Tsianos, 1st Division of Internal Medicine and Hepato-Gastroenterology Unit, Department of Medicine, Medical School, University of Ioannina, Leoforos Stavrou Niarxou, PO Box 1186, 45110 Ioannina, Greece

Author contributions: Tsianos EV designed and critically revised the paper; Katsanos KH designed and wrote the paper; Tsianos VE performed research for acquisition of data.

Correspondence to: Epameinondas V Tsianos, MD, PhD, FEBG, AGAF, Professor of Internal Medicine, 1st Division of Internal Medicine and Hepato-Gastroenterology Unit, Department of Medicine, Medical School, University of Ioannina, Leoforos Stavrou Niarxou, PO Box 1186, 45110 Ioannina, Greece. etsianos@uoi.gr
Telephone: +30-26510-07501 Fax: +30-26510-07016
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Abstract

Considering the epidemiological, genetic and immunological data, we can conclude that the inflammatory bowel diseases are heterogeneous disorders of multifactorial etiology in which hereditability and environment interact to produce the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. Several genes have so far been related to the diagnosis of Crohn's disease. These genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the strongest and most replicated associations with Crohn's disease have been demonstrated with *NOD2*, *IL23R* and *ATG16L1* genes. Many genes have so far been implicated in the prognosis of Crohn's disease and many attempts have been made for classification of genetic profiles in Crohn's disease. *CARD15* seems to be not only a susceptibility gene, but also a disease-modifier gene for Crohn's disease. Enriching our understanding of Crohn's disease genetics is of value, but when combining genetic data with functional data the outcome could be of major importance to clinicians.

Key words: Crohn's disease; Genetics; Polymorphism; Diagnosis; Prognosis; Genome-wide scan; Genetic consortium

Peer reviewers: Charles P Heise, MD, Associate Professor, Department of Surgery, School of Medicine, University of Wisconsin, 600 Highland Avenue, Madison, WI 53792, United States; Alberto Tommasini, MD, Professor, Laboratory of Immunopathology, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Via dell'Istria 65/1, Trieste 34137, Italy; Emanuel K Manesis, MD, Professor of Medicine, Athens University School of Medicine, Liver Unit, Euroclinic, 19 Mavromateon Street, Athens 1034, Greece

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EVOLVING ROLE OF GENETICS IN CROHN'S DISEASE

Despite decades of research the etiology of inflammatory bowel diseases (IBD) remains largely unexplained, but considering together the epidemiological, genetic and immunological data, we can conclude that IBD are heterogeneous disorders of multifactorial etiology in which hereditability (genetic) and environment (microbial, behavior) interact to produce the immunological background of the disease. It is probable that patients have a genetic predisposition for the development of the disease

coupled with disturbances in immunoregulation. The disease can then be triggered by any of a number of different unknown environmental factors and sustained by an abnormal immune response to these factors. Accordingly, the intensive interaction between intestinal epithelial cells and immune competent cells is critical to maintain and perpetuate the chronic inflammatory process characteristic for IBD[1].

Early epidemiologic evidence for the role of genetic factors in the pathogenesis of Crohn's disease (CD) came from studies demonstrating higher rates of CD among individuals of Caucasian and Jewish ethnicity, familial aggregation of CD and higher concordance rates for both twins developing CD in monozygotic compared with dizygotic twins. The search for specific CD susceptibility genes, however, has been difficult due to complex genetics, including factors such as the lack of simple Mendelian inheritance patterns, involvement of several genes, and the influence of environmental factors and intestinal microflora on disease development. More than 30 distinct genomic loci encode genes involved in a number of homeostatic mechanisms and have been suggested to be involved in CD etiopathogenesis and prognosis[2].

Until very recently, two main approaches could be undertaken to identify genes in complex diseases: the positional cloning approach, based on linkage analysis, and the candidate gene approach, based on association studies. Linkage analysis studies the co-segregation of the disease with a marker within families. The candidate gene approach uses case-control cohorts or trios of affected offspring with both parents. Here, a specific gene with known or potential interest for the disease is studied. The allelic frequencies (in the case of case-control study) or the transmission of a single nucleotide polymorphism (SNP) towards affected offspring (in the case of trios) are analyzed, and differences between patients and controls, or distortion of transmission towards affected children, will point towards implication of the gene in the pathogenesis of the disease under investigation.

Despite the large numbers of genome-wide association studies (GWAS) established to date, most diseases have only managed to explain some additional percentage of the hereditability estimates. In an attempt to explain some of this missing hereditability, researchers have adopted several complementary strategies. Larger cohorts of cases are being collected, through either further patient recruitment or collaborations. The meta-analysis data generated to date have demonstrated how increasing the cohort sample size generates additional statistical power to detect smaller and smaller odds ratios[3]. Advances in technology, and particularly bioinformatics, have now made it possible to perform GWAS using common copy number variation probes. Many groups are looking to high-throughput sequencing technology, with the aim of sequencing candidate gene regions identified by GWAS, to hopefully identify either the causal or rare variants[4,5]. Several GWAS have been published in the last decade and have identified many genes associated with Crohn's disease (Table 1). Among these, there are recognition-related genes such as *NOD1* and *TLRs*, other susceptibility genes including *DLG5, OCTN* and *HLA,* and the newest susceptibility genes in CD resulting from GWAS: *IL23R* gene, *ATG16L1* gene and *IRGM* gene[6].

THE ROLE OF GENES IN THE DIAGNOSIS OF CROHN'S DISEASE

Several genes have so far been related to the diagnosis of Crohn's disease. These genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the strongest and most replicated associations with CD have been demonstrated with *NOD2*, *IL23R* and *ATG16L1* genes.

Genes related to innate pattern recognition receptors

***NOD2/CARD15* gene:** *NOD2/CARD15* (Caspase Recruitment Domain Family member 15) acts as a pattern recognition receptor (PRR); this locus has been characterized as the *IBD1* locus on 16q12-13[7].

Fine mapping of the *IBD1* locus identified the underlying gene on chromosome 16 as the *CARD15* (previous *NOD2*) gene. *CARD15* represents homology with the *R* genes in plants; genes that confer resistance to infection[8]. Thirty nonconservative polymorphisms have been identified within the gene, which are associated with CD, but only three are common (Arg702Trp, Gly908Arg and Leuc1007insC). These three common variants account for approximately 82% of the mutated alleles. *CARD15* is associated with CD only and not with ulcerative colitis. *CARD15* codes for a protein expressed in monocytes, macrophages, dendritic cells, epithelial cells and Paneth cells. *CARD15* is involved in the recognition of bacterial peptidoglycan-derived muramyl dipeptide through the leucine-rich repeat region. Of importance, the frameshift mutation 1007fsinsC that leads to a truncated protein lacking the 33 distal amino acids was associated with impaired activation of the transcription factor NF-kappa B after stimulation.

It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins. Although nucleotide-binding oligomerization domains (NODs) are expressed at low levels in absorptive and secretory intestinal epithelial cells, Paneth cells in the small intestine have been recognized as the predominant site of expression of *NOD2* in the epithelium. Furthermore, *NOD2* mutations have been associated with decreased expression of antimicrobial peptides, the α-defensins, by Paneth cells. In addition, a distinct gene polymorphism resulting in low β-defensin 2 gene copy number has been associated with a predisposition to colonic Crohn's disease. In addition, *NOD2* plays important roles in the promotion of antibacterial T-helper-17 (Th-17) cells in the IL-23-IL-1-IL-17 axis.

CARD15 variants are found in 35% to 45% of white

Table 1 Genetic polymorphisms related to Crohn's disease

Diagnosis of CD	
Innate pattern recognition receptors	*NOD2/CARD15, OCTN, TLR*
Epithelial barrier homeostasis	*IBD5, DLG5*
Molecular mimicry and autophagy	*ATG16L1, IRGM, LRRK2*
Lymphocyte differentiation	*IL23R, STAT3*
Secondary immune response and apoptosis	*MHC, HLA*
Prognosis of CD	
Age of CD onset	*TNFRSF6B, CXCL9, IL23R, NOD2, ATG16L1, CNR1, IL-10, MDR1, DLG5, IRGM*
CD behavior	
Stenotic/stricturing behavior	*NOD2, TLR4, IL-12B, CX3CR1, IL-10, IL-6*
Penetrating/fistulizing behavior	*NOD2, IRGM, TNF, HLADRB1, CDKAL1*
Inflammatory behavior	*HLA*
Granulomatous disease	*TLR4/CARD15*
CD location	
Upper gastrointestinal	*NOD2, MIF*
Ileal	*IL-10, CRP, NOD2, ZNF365, STAT3*
Ileocolonic	*ATG16L1, TCF-4 (TCF7L2)*
Colonic	*HLA, TLR4, TLR1, -2, -6*
CD activity	*HSP70-2, NOD2, PAI-1, CNR1*
Surgery	*NOD2, HLA-G*
Dysplasia and cancer	*FHIT*
Extraintestinal manifestations	*CARD15, FcRL3, HLADRB*103, HLAB*27 HLA-B*44, HLA-B*35, TNFα-308A, TNF-1031C, STAT3*
Pharmacogenetics in CD	*CARD15, NAT, TPMT, MDR1, MIF, DLG5, TNF, LTA*

NOD: Nucleotide binding and oligomerization domain; CD: Crohn's disease; LRR: Leucine-rich repeat; OCTN: Organic cation transporter; TLR: Toll-like receptor; DLG5: Discs large homolog 5; LTA: Lymphotoxin α; CARD: Caspase recruitment domain; STAT3: Signal transducer and activator of transcription 3; MHC: Major histocompatability complex; PAI-1: Type 1 plasminogen activator inhibitor; FHIT: Fragile histidine triad; NAT: N-acetyltransferase; MIF: Macrophage migration inhibition; HLA: Human leucocyte antigen; TPMT: Thiopurine methyltransferase; MDR1: Multidrug resistance gene 1; TNF-α: Tumour necrosis factor-α; Tcf: T-cell factor; Atg: Angiotensinogen; FCRL: Fc receptor-like; ZNF: Zinc finger; TNFRSF: Tumor necrosis factor receptor superfamily; IRGM: Immunity-related GTPase family, M; HLA: Human leukocyte antigen; LRRK2: Leucine-rich repeat kinase 2; CNR: Cadherin-related neuronal receptor.

CD patients, with the exception of Scandinavian, Irish and Scottish patients[9,10], in whom the prevalence is much lower. Genotype relative risks of 3 (simple mutation) and 10-44 (double mutations) have been reported in European Caucasians[9,10]. However, *CARD15* mutation is not frequent or even absent in African-American populations, and in Indian, Chinese and Japanese populations[11-13]. Other *CARD* related genetic loci that have been associated with CD diagnosis are the *CARD4* (*NOD1*), *CARD8* and *CARD9* loci[14,15].

Organic cation transporter genes: Organic cation transporters (OCTNs, 5q31-33) are membrane transporters for drugs and positively charged endogenous metabolites. The novel OCTN subfamily may also transport carnitine, which is essential for metabolism of lipids and is involved in transport of light chain fatty acids into mitochondria for beta-oxidation. Early studies in this field reported on two functional mutations in the carnitine/*OCTN* cluster on *5q31* (the *IBD5* locus) that were associated with Crohn's disease. As membrane transporters of organic cations, OCTNs are therefore important in the maintenance of intracellular homeostasis. In humans *OCTN1* and *OCTN2* map to *IBD5* on *5q31*. An *OCTN3* has recently been described in humans[16].

Toll-like receptor genes: Host response to microbial pathogens includes self-defense mechanisms such as defensins, PRRs, pathogen-associated molecular patterns and toll-like receptors (TLRs). TLRs recognize conserved motifs on pathogens that are not found in higher eukaryotes and initiate "innate" (rapid and non-specific) immune responses[17]. Subsequently, specific receptors recognizing chemo-attractant molecules mobilize phagocytic leukocytes and induce their migration to inflammatory sites. There, leukocytes encounter the invading microorganisms and ingest them through the activation of phagocytic receptors that mediate the uptake process. Innate immune responses are linked to the generation of corresponding adaptive immune responses and studies of genetically engineered or cellularly manipulated animal models have generated a great deal of new information[18].

Leucocyte-epithelial interactions are of special interest, as exposure of epithelial TLRs to microbial ligands has been shown to result in transcriptional upregulation of inflammatory mediators, whereas ligation of leucocyte TLRs modulates specific antimicrobial responses[19]. It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins. In addition, it has been shown that *NOD2* mutations lead to loss of negative regulatory effects on TLR signaling while activation of the *CARD* domain results in activation of NF-κB[20].

TLRs are the most important receptors of the innate immune system. They are expressed by immune cells and by intestinal epithelial cells in IBD patients. In humans, at least 10 different TLRs are described and each recognizes a specific pathogen-associated molecular pattern. A trans-

mission disequilibrium test on Belgian IBD trios with CD demonstrated preferential transmission of the *TLR4* Asp299Gly polymorphism from heterozygous parents to affected children[21]. *TLR9* modulates CD susceptibility and there is interaction between other polymorphisms such as *NOD2*, *IL23R* and *DLG5*[22,23].

Genes related to epithelial barrier homeostasis

The gastrointestinal tract uses a system of tolerance and controlled inflammation to limit the response to dietary or bacteria-derived antigens in the gut[24]. When this complex system breaks down, by means of either a chemical or pathogenic insult in a genetically predisposed individual, the resulting immune response may lead to IBD[25]. Genes or loci involved in the maintenance of epithelial barrier integrity and associated with Crohn's disease are the *IBD5* and the Discs large homolog 5 (*DLG5*)[26].

The *DLG5* gene is a 180-kb protein containing 1900 amino acids. DLG5 protein harbors a caspase activation and recruitment domains (*CARD*), is a further CD susceptibility gene of the *CARD* family and contributes to *CARD*-mediated mechanisms of host defense. In fact, the *DLG5* gene associated protein is a member of the MAGUK (Membrane Associated Guanylate Kinase) family of scaffolding proteins. Scaffolding proteins organize protein complexes at cellular junctions to integrate the tethering of adhesion molecules, receptors and intracellular signaling enzymes. Of interest is a population variation regarding *DLG5* variants. For example, the *DLG5* R30Q variant has not been confirmed in other European studies[27,28]. Other genes of potential importance in the same panel are the *PTGER4*, *ITLN1*, *DMBT1*, *BPI* and *XBP1* genes[29].

Genes related to molecular mimicry and autophagy

The innate immune system is the first line of defense against infection. Of interest, virulence factors from bacteria and viruses have been identified that manipulate host innate immune signaling pathways through molecular mimicry. These microbial proteins contain signaling domains that bear sequence and structural similarity to their host targets, thereby potentially sabotaging host immunity by hijacking crucial signaling pathways and uncoupling receptor activation from effector induction. Several protein families have evolved to function as receptors or sensors of pathogen invasion. There are two types of signaling domains for the above receptors: the TIR domain for the TLRs, and the Pyrin domain or *CARD* for the *NOD*-like receptors (*NLRs*) and retinoic acid-inducible gene 1-like receptors or helicases.

Molecular mimicry has been invoked as one of the mechanisms responsible for the activation of autoreactive cells by microbial peptides that have structural similarities to self peptides, but there is also evidence that antigenically unrelated infections or specific inflammatory signals can result in autoaggressiveness and induction of organ-specific autoimmunity, including the gut. The extent and severity of this loss of tolerance is still being defined, as it has been demonstrated that loss of tolerance in IBD patients is not exclusive for bacterial antigens and occurs also for orally administered soluble proteins[30]. This subversion of innate immune signaling through molecular mimicry is closely related to the phenomenon of autophagy. Autophagy is the tightly orchestrated cellular 'housekeeping' process responsible for the degradation of damaged and dysfunctional cellular organelles and protein aggregates, and is well recognized to play an important role in maintaining cellular homeostasis under physiological and pathophysiological conditions. Regulated degradation and turnover of subcellular components is essential for normal cellular function, growth and development. The major catabolic pathway responsible for the disposal of obsolete or damaged organelles and protein aggregates is autophagy (i.e., "self-digestion"). During this process organelles and proteins are encircled in a double-membrane vesicle (the autophagosome), delivered to lysosomes and the substrates for adenosine-triphosphate generation, and products can be recycled to synthesize new proteins, high-energy phosphates and other cellular components. Autophagy has evolved as a conserved mechanism for cell survival under conditions of starvation and stress. In addition to (macro)autophagy, characterized by the sequestration of organelles and proteins within an autophagosome, there are two additional subtypes of self-digestion: microautophagy which refers to protrusion of the lysosomal membrane *per se* around a region of cytoplasm; and chaperone-mediated autophagy in which degradation is restricted only to those proteins with a consensus peptide sequence recognized by specific chaperone complexes[31]. Autophagy is now considered to be important for host defense against intracellular microorganisms. The associations of the autophagy-associated genes with Crohn's disease strongly support the hypothesis that abnormal innate immune responses to intracellular pathogens contribute to the pathogenesis of Crohn's disease. In fact, the pathological characteristics of human Crohn's disease represent "granuloma" formation. The mechanisms of granuloma formation remain unclear. Recent studies have demonstrated functional roles for IL-23 in the differentiation and promotion of Th-17 cells. Autophagy genes that have been related to CD diagnosis are the *ATG16L1*[32,33], *IRGM* and the *LRRK2* genes[34]. Unraveling the mechanisms of such molecular mimicry is crucial to our understanding and clinical intervention of infectious diseases and inflammatory disorders of unknown etiopathogenesis, including Crohn's disease.

Genes related to lymphocyte differentiation

***IL23R* gene:** Dysregulated cytokine production by mucosal lymphocytes and macrophages has been implicated in the pathogenesis of CD. In fact, an exclusive increase of $CD4^+$ T cells in inflammatory bowel disease and their recruitment as intraepithelial lymphocytes has been demonstrated[35]. $CD4^+$ T cells secreting interleukin-17 [T helper type 17 (Th-17) cells] have emerged as a key effector population driving colitis in animal models previously

associated with exaggerated T helper type 1 responses.

Of the genes involved in the differentiation of Th-17 lymphocytes the *IL23R* gene has been proved of great importance and has been related to Crohn's disease[36,37].

IL23R, consisting of an *IL-12β1* and an *IL23R* chain, is highly expressed on memory T cells. *IL23* is a novel cytokine formed *via* the binding of *IL12p40* to a p19 protein. After binding to the *IL23* receptor, *IL23* preferentially activates memory T cells. IL23 does exhibit some similar biological activities to *IL-12*; however, *IL-12* is more involved in the differentiation of naïve T-cells into Th1 lymphocytes and subsequent *IFNγ* production. *IL23*, on the other hand, mediates proinflammatory activities in part by the production of *IL17* through activation of Th17 lymphocytes[38].

Signal transducer and activator of transcription 3 gene: Signal transducers and activators of transcription 3 (*STAT3*) play an important role in various autoimmune disorders including IBD[39,40]. *STAT3* was initially identified as an acute phase response factor, an inducible DNA binding protein that binds to the *IL-6* responsive element within the promoters of hepatic acute phase protein genes, and is involved in IL-6 dependent T-cell proliferation through prevention of apoptosis. Subsequent studies indicate that *STAT3* becomes activated in response to a wide variety of cytokines and growth factors. Recent studies have revealed that *STAT3* activation plays distinctly different roles between innate immune responses and acquired immune responses in colitis. *STAT3*-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing the survival of pathogenic T-cells. In contrast, *STAT3*-mediated activation of innate responses contributes to the suppression of colitis. Emerging data indicate that *STAT3* is one of the crucial targets for the treatment of IBD. However, as the receptors of these cytokines and growth factors are present in both innate and acquired cells, activation of *STAT3* is likely to occur in both cell types. Therefore, as the function of *STAT3* is a double-edged sword, careful attention should be directed toward the cell population that is being targeted when one contemplates *STAT3* inhibition or activation in human IBD[41]. Within the same panel, other than *STAT3* genes, and with probable importance, are the *TNFSF15*, *JAK2*, *CCR6* and *ICOSLG* genes[42-44].

Genes related to secondary immune response, apoptosis and other pathways

Chemokines play a central role in the pathogenesis of IBD as they are able to trigger multiple inflammatory actions including leukocyte activation and chemoattraction, granule exocytosis, production of metalloproteinases for matrix degradation and upregulation of the oxidative burst[45]. Therefore, further support is given for genes that relate to secondary immune response, apoptosis and other pathways. For example, in the *IBD4* locus 4 several interesting candidate genes, which may be relevant in the pathogenesis of CD, lie within this region (e.g., genes regulating apoptosis, signal transduction proteins, chemokine receptors, T cell receptors, metalloproteinases).

Gene expression profiles from colon lamina propria fibroblasts have demonstrated several functional changes in some proteins coded from the corresponding genes: collagen types I , IV, XIV; matrix metalloproteinase 1; cathepsin K; stroma cell-derived factor-1; chitinase3-like-1; and many others[46]. The major histocompatibility complex (MHC) has been extensively investigated. Human leucocyte antigen (HLA) class II molecules present partially digested antigen to the T-cell receptor and play a central role in the immune response. In CD, the MHC and HLA studies have yielded conflicting and heterogenous results. *HLADR1* has been implicated in CD[47].

Many other genes, loci and chromosomes involved in CD have also been advocated in several studies that, however, still require wide replication and association with clinical practice. These include *CNR1, MCP-1*[48], *PTPN2* (protein tyrosine phosphatase)[49], *PTPN22*, *NKX-3, IL-18 RAP/IL-18R1, IL12/IL23* pathway[50], *PTGER4, MST1/BSN/MST1R*[50-52], *IL-2/IL-21*[53], *TYK2, JUN, NAT2*[54], *IL-10, NELL1, NKX2-3*[55], *Cyclin Y*, Hect domain, 1q24, 10q21, 5p13, RCC1-like domain, *ICOSLG, CDKAL1*[56], 13q13.3, 1p35.2, 3p29, 5p13.1[57,58], X chromosome[59], *NLRP3*[60], Vitamin D receptor (*VDR*) polymorphisms[61] and many others as well.

Genes in family and ethnic group studies

Linkage studies performed in complex genetic disorders such as CD frequently use model-free analytic methods, which are non-parametric analyses that do not assume Mendelian recessive or dominant models of inheritance.

The strongest risk factor for IBD is having a relative with the same disease. First-degree relatives of patients with CD have a 12-to-15 times greater risk of developing CD than do people of comparable age in the general population[61]. Familial clustering can also result from exposure to common environmental risk factors. Twin studies are very useful to determine the degree of genetic *vs* non-genetic etiologies for a trait. Today, there is no evidence of a separate entity of familial IBD[62,63]. Based on the current literature, phenotypic differences between familial and sporadic cases of IBD are weak. Available data are to be accepted with caution, however, as they are mostly retrospective and may be biased. *CARD15* explains around 20% of the genetic predisposition to Crohn's disease[64]. The relative risk of developing CD in the presence of one mutation is 2-4, but increases dramatically in the case of two mutations (compound heterozygous or homozygous).

Although *NOD2* provides no clear familial predisposition, unaffected relatives do carry an increased rate of *CARD15* variants (37.1%) compared to controls, and it would be interesting to see if they will eventually develop symptoms[65-67]. In addition, maternal transmission of *CARD15* variants seems protective with a lower ratio of affected/unaffected children when compared to fathers[68,69]. In the light of the foregoing data, it seems that genetic counseling should be carried out with caution. In addition, families should not receive genetic counsel-

ing/information about age at onset and disease severity. Ethnic group studies and ethnic variation were firstly demonstrated in Jewish populations, and those studies are of major importance in this context[70].

THE ROLE OF GENES IN PROGNOSIS OF CROHN'S DISEASE

This is a major issue that greatly concerns patients. Many genes have so far been implicated in the prognosis of Crohn's disease and numerous attempts have been made to classify the genetic profiles in Crohn's disease. Of interest, *CARD15* seems not only a susceptibility gene, but also a disease-modifier gene for CD. Of the many studies published on the clinical relevance of *CARD15* mutations, there are several providing data on disease location, and the majority of them support a significant association of *CARD15* mutations with ileal disease site, while some demonstrate a connection with the absence of colonic location. Some studies also provide data supporting the relevance of *CARD15* variants with stricturing disease behavior, and also penetrating behavior. Other pertinent studies have revealed an association with early onset of the disease. These investigations also support the theory that pediatric Crohn's is a "more genetic disease" consistent with other polygenic disease models. Other reports provide data on an increased risk or need of surgery related to CD[71].

Differences among studies are difficult to explain, and we could argue about the low number of patients in some of the studies, the disease variability among Caucasians and, finally, differences regarding disease assessment and interobserver agreement. Whether the described relationship between the *CARD15* variants and both stenosing phenotype and increased need for surgery in CD patients is a true association, or only reflects the high proportion of ileal CD developing bowel stenosis and, therefore, requiring surgery, is still a matter of controversy.

Genes related to age of Crohn's disease onset

With respect to age of CD onset and more specifically to childhood or early-onset Crohn's disease, many genes/loci have been implicated: *TNFRSF6B*, *CXCL9*[72], *IL23R*[73,74], *NOD2*[75], *ATG16L1 rs2241880*[76], *CNR1*[77], *IL-10*[78], *MDR1*[79]. Of interest, *DLG5* seems protective for female children[80], while there are also studies not supporting the relationship of genes and early onset of CD[81] or supporting the relation of *IL-10* and *IRGM* with adult onset[82].

Genes related to Crohn's disease behavior

Genes related to stenotic/stricturing behavior in CD are: *NOD2/CARD15*[83], *TLR4*[84], *IL-12B*[85] and *CX3CR1*[86,87]. Of importance, *NOD2/CARD15* has been also related to acute intestinal obstruction[88]. *IL-10* and *IL-6* are also potentially related to stenotic/structuring behavior in CD, while genetic variants of several metalloproteinases and their inhibitors would be excellent candidate genes, since these molecules are considered to play a key role in the abnormal fibrogenesis that underlies the development of bowel stenosis in CD patients. Genes related to penetrating/fistulizing behavior in CD are as follows: *NOD2*, *IRGM*, *TNF*[89], *HLADRB1*[90], the C-allele in *CDKAL1* rs6908425 SNP is associated with *NOD2*(-) perianal fistula, whereas *OCTN* and the near *IL-12B* gene rs12704036 T-allele have a relationship with non-perianal fistula[91]. Inflammatory CD behavior has been related to *HLA* variation[92] while granulomatous disease has been related with *TLR4/CARD15* variants[93].

Genes related to Crohn's disease location

Upper gastrointestinal Crohn's disease has been related to *NOD2*[94] and *MIF* variants[95]. Ileal CD has been related to the following genes: *IL-10*[96], *CRP* gene[97], *NOD2*, *ZNF365* and *STAT3*[98]. Genes/loci associated with ileocolonic CD are 3p21, *ATG16L1*[98] and *TCF-4* (*TCF7L2*)[99]. No role for phenotype in *IL23R* gene has been demonstrated[100] while a detailed genotype-phenotype analysis revealed weak associations of the *IL23R* rs10024819 variant with ileal involvement and stenoses in carriers of the TT genotype. Finally, the *HLADRB1*0701* has been associated with ileal CD, but only in patients who have no *CARD15* variants[101]. Colonic CD has been related to the following genes: *HLA* region (associated with inflammatory colonic phenotype); and *TLR4*[102], *TLR1*, *-2*, *-6*[103]. The *TNF* gene showed a negative association with stricturing behavior or colonic location[104]. For *IBD5* and *OCTN1* and *2*, results have not been consistent but associations with perianal and ileal disease have been reported.

Genes related to Crohn's disease activity

Genes implicated in disease activity are the following: *HSP70-2* heat shock protein gene[105], *NOD2*[106], *PAI-1* (Type 1 plasminogen activator inhibitor[107]), while the combination of *NOD2* and *PAI-1* predicted complicated disease behavior[108]. Of importance, *NOD2* predicted lower weight in children[109], and *CNR1* low body mass index[110].

Genes related to surgery

The *NOD2* gene has been related to early pediatric surgery[111], stenosis and need for surgery[112], previous surgeries[113], increased number of surgeries[107] and surgical costs[114]. *NOD2* has no relation to the risk of re-operation[115]. Finally, *HLA-G* has been associated with higher risk for ileocolonic resectio[116].

Genes related to dysplasia and cancer

The *FHIT* gene (fragile histidine triad gene) located at 3p14.2 has been identified as a candidate tumor-suppressor gene. The gene spans the t(3;8) translocation breakpoint of familial renal cell carcinoma and contains the FRA3B fragile site. It encodes the human diadenosine triphosphate hydrolase, which *in vitro* cleaves the diadenosine substrate into ADP and AMP. It has been suggested that *FHIT* gene plays a role in the pathogenesis of IBD and the development and progression of a subgroup of IBD-related carcinomas at an early phase[117-119].

Genes related to extraintestinal manifestations and concomitant diseases

Extraintestinal manifestations are common in CD. Genes related to CD extraintestinal manifestations have been reported, as follows. Peripheral arthritis was related to *FcRL3*[120], *HLADRB*103, HLAB*27 HLA-B*44*, *HLA-B*35*, *TNFa-308A*[121]. *CARD15* has been related to spondylarthropathy[122] and uveitis[123] but not to sacroileitis[124]. *TNF-1031C* was associated with erythema nodosum while certain *HLA* alleles (*HLA-B27, HLA-B35* and *HLA-B44*) were connected with different disease behavior and extraintestinal manifestations such as arthropathy, eye and skin manifestations. Genes/loci related to other chronic diseases concomitant to CD are 10p12.2 (sarcoidosis and CD)[125], *STAT3* (multiple sclerosis and CD)[126] and a parallel genetic fingerprint between leprosy and CD[127].

Pharmacogenetics in Crohn's disease

Pharmacogenetics is of major importance in CD therapeutics and prognosis. Genes have been implicated in influencing the efficacy and side effects of drugs and reflect a complex interplay regarding absorption, elimination and transport. Future studies need to be large and prospective with uniformly phenotyped patients, and correlating genetic associations with functional data. In addition, hypotheses such as whether observations about drug response in IBD lead us to IBD etiology or whether the genes that control the drug response are related to genes that control the disease still remain unanswered. Pharmacogenetic studies to date have found no association between *CARD15* variants and prediction of response to various IBD therapies. In addition, responses to azathioprine (AZA), steroids and infliximab are not related to *NOD2*[128]. Of note, *NOD2* was only related to antibiotic failure[129]. For mesalazine, variability in drug acetylation was demonstrated many years ago with patients divided into slow and rapid acetylators, because of polymorphisms in the N-acetyltransferase (*NAT*) genes. Two isoenzymes NAT1 and NAT2 have been identified in humans and more than 50% of Caucasians are NAT2 slow acetylators. Mesalazine is acetylated in the liver by NAT1 into N-acetyl-5 aminosalicylates and excreted in the urine[47].

The clinical usefulness of pharmacogenetics in CD is limited to AZA and thiopurine methyltransferase (TPMT) at this moment. The human *TPMT* gene, consisting of 10 exons, is located on chromosome 6p22.3. The hereditary nature of *TPMT* deficiency in humans was initially identified in a study of *TPMT* activity in red blood cells (RBC). This and subsequent studies determined the distribution of *TPMT* activity in RBC to be trimodal; 90% of persons have high activity, 10% have intermediate activity and 0.3% have low or no detectable enzyme activity. To date, numerous mutant *TPMT* alleles have been identified, including the three most frequent alleles (*TPMT*2, TPMT*3A* and *TPMT*3C*), which account for 80%-95% of intermediate or low TPMT enzyme activity cases. The prevalence of the most frequent SNPs in the *TPMT* gene has been reported to vary worldwide. However, it is of interest that studies on the prevalence of *TPMT* SNPs in large IBD cohorts are lacking.

Although AZA is an effective drug for maintenance of remission in IBD, it is associated with side effects. Clinically sound pharmacogenetic studies over the last two decades have shown that polymorphisms in the *TPMT* gene locus play a significant role in the occurrence of various side effects of thiopurine drugs including life-threatening bone marrow toxicity, a serious dose-related toxicity[130-134].

The G2677T variant in the *MDR1* gene predicted gastrointestinal and unspecified intolerance to azathioprine and methotrexate in inflammatory bowel disease patients. These findings suggest a role for MDR1/P-gp in the mechanism of action of azathioprine and methotrexate[135,136].

Twin studies have linked polymorphisms of the *VDR* gene with bone mineral density in healthy women, and in addition *VDR* is an important regulator of calcium metabolism and bone cell function and influences calcium absorption from the intestine. *VDR* polymorphisms have also been implicated in susceptibility to Crohn's disease[137].

The *HLADR* region has been associated with failure to budesonide[138] while *DLG5R30Q* predicted response to steroids[139]. Other genes such as *MIF* (macrophage migration inhibition)[140] and *MDR* have also been related to steroid therapy[136]. In addition, 1082 AA *IL-10* genotype was associated with steroid dependency, whereas the allele 113A of the *DLG5* gene conferred resistance to steroids.

Regarding response to infliximab, the data for the *TNF* gene are conflicting. Specifically, there are conflicting data regarding the role of *FcGR3A*, which has been supported by some authors[141,142], but was not confirmed in patients in the ACCENT I study. Response to infliximab is not related to *TNFa-308*[143] or *TNFR1* and *TNFR2*[144] or *NOD*[145] or *CRP* gene[136]. The association between the Fas ligand-843 TT genotype and lack of response to infliximab seemed to be the most relevant observation[136]. The relationship between infliximab response and the lymphotoxin alpha gene is also conflicting[144].

WHAT LIES AHEAD

Gene-to-gene crosstalk and epistasis

With new methodologies such as genome-wide association studies, microarrays and fine SNP analysis becoming available during the last decade, our investigative armamentarium has been considerably enriched. As many studies with complex statistics arise, we understand increasingly the real crosstalk present among genes and the need for a genetic panel for disease diagnosis and prognosis. It is now evident that gene-to-gene interaction and epistasis modulate disease activity and susceptibility[146]. Some data have come to light. A genome-wide scan in a Flemish population of IBD affected families supports the

Table 2 Predicted future developments in the genetics of Crohn's disease

What lies ahead in the genetics of crohn's disease
Gene-to-gene crosstalk and epistasis
Genome-wide association studies
Microarrays
Fine single nucleotide polymorphism analysis
Genetic consortium studies and genome-wide scans
Genome-wide association studies
Genetic consortium studies
Future perspectives
Functional studies to understand the mechanisms
Combining genetic data with functional data
Combination of a panel of clinical, biochemical, serological and genetic factors
Functional consequences of polymorphisms
Molecular and cellular mechanisms leading to Crohn's disease
Predict disease outcomes
Redesigning the methods of treatment

existence of *IBD4* on 14q11, and has shown additional evidence for the existence of other susceptibility loci (1p, 4q and 10p). This study has further demonstrated that epistasis and gene-to-gene interactions (*CARD15-TLR4*) are also present in IBD and that population heterogeneity is not to be underestimated[147]. Crosstalk has been demonstrated for *TLR9* with *NOD2, IL23*R and *DLG5*, and epistasis has been shown between *IL23*R and *DLG5*. Also, potential epistasis between *IL23*R variants and the three other previously described CD susceptibility genes *CARD15*, *SLC22A4* and *SLC22A5* (*OCTN 1* and *2*) has been shown[116].

Genetic consortium studies and genome-wide scans

Over the past few years, a combination of progress in high-throughput genotyping technology and growing knowledge about the human genome through the International HapMap project and the Human Genome Project have enabled GWAS for several complex diseases. To understand the approach to conducting GWAS in this setting, it is important to expound on the concept of linkage disequilibrium, which refers to the nonrandom association of alleles at nearby loci. Specifically, linkage disequilibrium refers to adjacent alleles assorting together nonindependently from generation to generation because they are tightly linked and thus less likely to become separated by recombination. Genetic consortium studies are of major importance, and homogeneity in methodology issues is of paramount value[148-151]. Appropriate study design[152], power analysis[153] and overall data analysis and meta-analysis[154] are mandatory. Accurate estimation of sample sizes required in a genetic association study is essential before commencing genotyping, to ensure that the study is sufficiently powered to detect the subtle genetic effects that contribute to most complex diseases. The extensive genetic variation and complex linkage disequilibrium across even a small genomic region will give rise to several alternative scenarios. Genetic variation across a region studied should be carefully evaluated and consideration should be given to possible linkage disequilibrium and allelic heterogeneity when evaluating power of an association study. As larger datasets are studied and combined, as genotyping platforms provide even greater depth of coverage of the genome, and as modest hits are followed up in large independent panels, so the vast majority of true signals should be identified. These robust genetic data will truly provide a solid platform for functional studies to understand the mechanisms by which these genetic variants predispose to Crohn's disease. Finally, studies at post-transcriptional level become more and more urgent[155]. Enriching our understanding of CD genetics is important, but when combining genetic data with functional data the outcome could be of major importance. In fact, improved understanding of immune mechanisms, on which manifold genetic and environmental traits might converge, and which ultimately mediate all phenomena in inflammatory bowel disease, holds promise (Table 2).

CONCLUSION

The recent advances in the understanding of CD genetics have been tremendous[156]. Starting with the susceptibility area, whole genome linkage and association scans have already led to the identification of a number of susceptibility genes (*NOD2/CARD15, DLG5, OCTN1* and *2, NOD1, IL23R, PTGER4*, *ATG16L1* and *IRGM*) of which the *NOD2/CARD15* gene is the most replicated and understood at present. Although it is clear that genetic research in IBD has advanced our understanding of the clinical heterogeneity of the disease, new efforts are required and point towards the complex combination of a panel of clinical, biochemical, serological and genetic factors, in order to achieve the optimal prediction of both clinical behavior and response to therapy.

Genome-wide association studies have allowed an unprecedented rapid unraveling of the genetic basis of IBD; however there will be much more follow-up work needed in this field. First, ongoing work including meta-analysis of the CD genome-wide association studies will probably reveal additional CD susceptibility genes. It will then be essential to investigate the functional consequences of polymorphisms in these genes so that the molecular and cellular mechanisms leading to CD can be better characterized. Finally, genotype-phenotype correlation studies should help clinicians predict disease outcomes with more accuracy, including the risk for complications, need for surgery, and response to therapy, and finally lead to redesigning the methods of treatment of CD patients.

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ORIGINAL ARTICLE

Hepatocellular carcinoma-specific immunotherapy with synthesized α1,3- galactosyl epitope-pulsed dendritic cells and cytokine-induced killer cells

Ying Qiu, Ming-Bao Xu, Mark M Yun, Yi-Zhong Wang, Rui-Ming Zhang, Xing-Kai Meng, Xiao-Hui Ou-Yang, Sheng Yun

Ying Qiu, Rui-Ming Zhang, Xing-Kai Meng, Xiao-Hui Ou-Yang, Sheng Yun, The First Teaching Hospital, Inner Mongolia Medical College, Huhhot 010050, Inner Mongolia Autonomous Region, China
Ming-Bao Xu, The Armed Police General Hospital, Beijing 100036, China
Mark M Yun, Medical School of UCL, London WC1E 6BT, United Kingdom
Yi-Zhong Wang, Sheng Yun, Aerospace Medical College, Peking University, Beijing 100049, China
Ying Qiu, Department of Clinical Sciences, King's College Hospital, London SE5 9NU, United Kingdom
Sheng Yun, Clinical Sciences Centre, MRC, Hammersmith Hospital Campus, London W12 0NN, United Kingdom
Author contributions: Qiu Y and Xu MB contributed equally to this paper, they performed the majority of the work; Yun MM analyzed the data; Wang YZ contributed to the flow cytometric analysis of blood samples; Zhang RM, Meng XK and Ou-Yang XH coordinated and collected the tumor samples; Yun S proposed the study and wrote the first draft.
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Correspondence to: Sheng Yun, Professor, Department of Oncology, The First Teaching Hospital, Inner Mongolia Medical College, No. 1, Tongdao BeiJie, Huhhot 010050, Inner Mongolia Autonomous Region, China. sheng.yun@hotmail.co.uk
Telephone: +86-471-6637582 Fax: +86-471-6965931
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Abstract

AIM: To evaluate the safety and clinical efficacy of a new immunotherapy using both α-Gal epitope-pulsed dendritic cells (DCs) and cytokine-induced killer cells.

METHODS: Freshly collected hepatocellular carcinoma (HCC) tumor tissues were incubated with a mixture of neuraminidase and recombinant α1,3-galactosyltransferase (α1,3GT) to synthesize α-Gal epitopes on carbohydrate chains of the glycoproteins of tumor membranes. The subsequent incubation of the processed membranes in the presence of human natural anti-Gal IgG resulted in the effective phagocytosis to the tumor membrane by DCs. Eighteen patients aged 38-78 years with stage Ⅲ primary HCC were randomLy chosen for the study; 9 patients served as controls, and 9 patients were enrolled in the study group.

RESULTS: The evaluation demonstrated that the procedure was safe; no serious side effects or autoimmune diseases were observed. The therapy significantly prolonged the survival of treated patients as compared with the controls (17.1 ± 2.01 mo *vs* 10.1 ± 4.5 mo, $P = 0.00121$). After treatment, all patients in the study group had positive delayed hypersensitivity and robust systemic cytotoxicity in response to tumor lysate as measured by interferon-γ-expression in peripheral blood mononuclear cells using enzyme-linked immunosorbent spot assay. They also displayed increased numbers of CD8-, CD45RO- and CD56-positive cells in the peripheral blood and decreased α-fetoprotein level in the serum.

CONCLUSION: This new tumor-specific immunotherapy is safe, effective and has a great potential for the treatment of tumors.

Key words: Hepatocellular carcinoma; α-Gal epitope; Dendritic cell; Tumor-associated antigen; Dendritic cell-activated cytokine-induced killer cell

Peer reviewers: C Bart Rountree, MD, Assistant Professor of Pediatrics and Pharmacology, Penn State College of Medicine, 500 University Drive, H085, Hershey, PA 17033, United States;

Mahir M Ozmen, Professor, Department of Surgery, Hacettepe University, Ankara 06830, Turkey

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INTRODUCTION

Primary hepatocellular carcinoma (HCC), the most common neoplasm of the liver, is generally resistant to conventional treatment. The prognosis of the patients is poor, especially at the late stage, with a mean survival of less than ten months[1-3]. This poor prognosis has led to the interest in various alternative therapies. Immunotherapy is particularly appealing because of its specificity against tumor cells, absence of serious side effects and its potential to eradicate residual tumors after conventional treatment.

One area of active research is immunotherapy with cytokine-induced killer cell (CIK)[1-3]; unfortunately, its efficiency is limited because of low specificity to tumor cells. Another approach is tumor-associated antigen (TAA)-pulsed dendritic cell (DC) therapy, but the outcome is still not satisfactory because of the low immunogenicity of TAA[4-6].

DCs are the most effective antigen-presenting cells (APCs) responsible for initiating an immune response[7]. Upon exposure to tumor cells, isolated tumor antigen, and even to tumor mRNA, DCs are capable of presenting endogenous and exogenous antigens to naïve T cells in a human leukocyte antigen (HLA)-restricted manner and expressing several adhesive and costimulatory surface molecules. However, the poor immunogenicity of TAA may be the reason why tumor cells fail to adequately stimulate DCs for effective presentation to immune cells[8]. A possible method for increasing the uptake of TAAs by DCs is to complex them with an IgG antibody. These immune complexes would bind to Fcγ receptors (Fcγ-Rs) on DCs and induce phagocytosis of TAAs. The augmentation of antigen processing, presentation by immune complex and subsequent activation of immune responders has been demonstrated in several studies. For example, targeting tetanus toxoid (TT) to APC Fcγ-Rs by complexing it with anti-TT IgG results in a 10- to 1000-fold increase in Th-cell activation[9,10]. Similarly, the targeting of autologous tumor cell vaccines by DC Fcγ-Rs uptake would also lead to an effective immune response against the tumor cells[11]. Such targeting was achieved by complexing the tumor cell membranes *in situ* with naturally existing antibodies against the α-Galactosyl epitope (Gal-α1, 3Gal-β1, 4-GlcNAc-R, α-Gal)[12]. These anti-Gal antibodies have been shown to be detrimental in xenotransplantation[13] and to destroy retroviral vectors used for gene therapy[14]. In humans, the α-Gal epitope on the cell membrane is absent, but the natural anti-Gal antibody is abundant in serum[15]. Expression of the α-Gal epitope on tumor cells could result in *in situ* binding of the patient's natural anti-Gal IgG. The binding complex can then opsonize DC phagocytosis and enhance TAA presentation to naïve T or CIK cells, which are then activated and attack the remaining tumor cells *in vivo*. However, patients with malignancies usually have very low immunity, especially in cellular immune responses. The cause of the low immunity is not quite clear. One possible explanation is that the patient's T cells have been anergized by tumor antigens in the absence of APCs and cannot be activated again with the same antigen *in vivo*.

To maximize APC phagocytosis and the activation of tumor-specific T/CIK cells, we used newly differentiated T/CIKs from bone marrow stem cells instead of circulating T cells. Here, we present a new therapy for HCC using α-Gal epitope-expressing tumor cell-pulsed DCs to activate tumor-specific T/CIKs *in vitro*. The activated immune responders were then expanded in the presence of a high concentration of cytokines *ex vivo*.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from all the patients who participated in the study before treatment according to the guidelines of the Ethics Committee of the Armed Police General Hospital, which approved this study. Inclusion criteria were a Karnofsky score of ≥ 60[16], the lowest possible maintenance dose of glucocorticoid therapy (prednisone < 5mg/d) and normal baseline hematological parameters before treatment. These parameters included: hemoglobin ≥ 10.0 g/dL; total granulocyte count ≥ 4000/μL; platelet count ≥ 100 000/μL; blood urea nitrogen ≤ 30 mg/dL; creatinine ≤ 2 mg/dL; alkaline phosphatase and aspartate aminotransferase levels less than 2 times the normal upper limit; prothrombin and activated partial thromboplastin not more than 1.4 times higher than the control; and total bilirubin < 35 μmol/L. Exclusion criteria included cachexia, severe jaundice, a large amount of ascites, human immunodeficiency virus positive status, drug addiction, pregnancies, severe pulmonary and cardiac diseases, treatment with a large dose of steroids and a history of an autoimmune disorder or prior history of other malignancies.

Nine patients with clinical stage Ⅲ, histologically-proven primary HCC were enrolled in the study group. There were 2 women and 7 men ranging in age from 38 to 78 years (56.8 ± 13.9 years, Table 1). After surgical removal of the tumor masses, the patients received radio-, chemo- or interventional therapies. They were monitored after the treatment by computed tomography (CT). None of the patients was receiving steroids at the time of treatment.

There were 9 patients with clinical stage Ⅲ, histologically-proven HCC in the control group. This group con-

Table 1 Outcomes of immunotherapy in the study group

Patient no.	Sex	Age (yr)	Pre-treatment KPS	No. of injection	DTH positive ⩾ 5 mm	Adverse events	Tumor markers AFP	Imaging response	Survival months till now
1	F	38	70	4	60 mm	Fever, rash	Decrease	PD	14.4[D]
2	M	46	100	5	70 mm	Fever, rash, erythema	Decrease	PD	19.6[A]
3	M	55	75	6	45 mm	Fever, multiple erythema	Decrease	PR	18.8[A]
4	M	65	90	2	60 mm	Fever	Decrease	NC	16.8[D]
5	M	72	70	5	25 mm	Fever, rash	Decrease	NC	15.2[D]
6	M	78	90	6	65 mm	Fatigue, fever	Decrease	PR	16.0[D]
7	M	40	80	4	60 mm	Fever, rash	Decrease	NC	15.6[D]
8	M	54	70	6	5 mm	Fever	Increase	PD	17.6[D]
9	F	63	95	7	50 mm	Rash	Decrease	PR	20.0[A]

AFP: α-fetoprotein; PR: Partial response; PD: Progress disease, NC: No change; A: Still alive; D: Dead; KPS: Karnofsky performance status; DTH: Delayed-type hypersensitivity.

sisted of one woman and 8 men, aged 45-66 years. They all underwent similar conventional treatment as those of the study group.

Preparation of primary hepatocellular carcinoma cell suspension

The surgically removed tumor samples were immediately placed in ice-cold phosphate buffered saline (PBS) containing 3 mmol ethylenediaminetetraacetic acid (EDTA). A fine scalpel was used to remove as much adjacent non-tumor tissues as possible. The tumor tissue was then dispersed using a curved needle to create a cell suspension, which was filtered using a 70 μm cell strainer (BD Biosciences, United Kingdom). The cells were resuspended (2×10^8 cells/mL) in ice-cold PBS containing 3 mmol EDTA. The cells were irradiated five times at a total dose of 100 Gy, with a 5-min break during which the cells were placed on ice. They were then washed twice with ice-cold PBS.

Synthesis of α-Gal epitopes on intact tumor cells

Tumor cells prepared as described above were first resuspended at a concentration of 2×10^8 cells/mL in saline containing 0.1 mol Tris-HCl pH 7.0, 15 mmol $MnCl_2$, and 1 mmol UDP-Gal (Sigma-Aldrich, United Kingdom) and then incubated with 1 mU/mL neuraminidase (Sigma-Aldrich, United Kingdom) and 50 μg/mL recombinant bovine α1,3-galactosyltransferase (α1,3GT, produced in our lab) at 37 ℃ for 1 h with constant rotation. The cells were washed with RPMI-1640 medium, resuspended in RPMI-1640 medium (Gibco, Beijing) supplemented with 10% AB serum and incubated on ice for 1 h to promote maximum binding of the natural anti-Gal antibody to newly synthesized α-Gal epitope on the tumor cell surface. The cells (2×10^8 cells/mL) were washed and sonicated in ice water using a Sonicator-3000 (Misonix, Inc., United States) to destroy all tumor cells and centrifuged briefly to remove the viscous DNA. Finally, the tumor cell lysate was aliquoted and stored at -80 ℃ for future use.

Synthesis of the α-Gal epitope on tumor cells was analyzed by flow cytometry with 10 μg/mL purified human natural anti-Gal antibody[17], followed by staining with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human immunoglobulin antibody (Dako, Denmark). Intact tumor cells incubated with neuraminidase only were used as a negative control because they lack the α-Gal epitope.

Generation of dendritic cells from peripheral blood mononuclear cells

A concentrated 50-mL leukocyte fraction was generated through restricted peripheral blood leukapheresis. The peripheral blood mononuclear cells (PBMCs) were then purified using Ficoll-Hypaque (Sigma-Aldrich, United Kingdom) density gradient centrifugation. The cells were resuspended in RPMI-1640 medium supplemented with 10% human AB group serum, plated at a concentration of 5×10^6 cells/mL and allowed to adhere to tissue culture plates for DC preparation. The adherent cells were further cultured at 37 ℃ for 5 d in PRMI-1640 in the presence of 1000 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Amoytop Biotech, Xiamen, China), 500 U/mL recombinant human IL-4 (Amoytop Biotech, China), and 1% penicillin/streptomycin (Sino-American, Beijing). They were next grown for an additional 2 d in the presence of 1000 U/mL tumor necrosis factor-α (TNF-α) (R&D Systems, Inc., Shanghai). The semi-adherent and non-adherent cells were harvested by pipetting and used as DCs for pulsing with the tumor cell lysate prepared as described above[18].

The DCs were incubated with mouse anti-human CD3, CD4, CD8, CD14, CD16, CD19, CD40, CD86, CD80, CD83, CD56, CD45RO, MHC I and HLA-DR monoclonal antibodies (BD Biosciences, Shanghai) for 30 min at 4 ℃. Species- and isotype-matched monoclonal antibodies were used as negative controls. Subsequently, the cells were washed and incubated with FITC-conjugated rabbit anti-mouse antibody (Dako, Denmark) and analyzed using FACSCalibur (Becton Dickinson, United States).

Pulsation of autologous dendritic cells by α-Gal expressing tumor cell lysate

The abovementioned DCs (1×10^7 - 4×10^7/mL) were co-cultured with the α-Gal-expressing HCC cell lysate (equal to 2×10^8 cells) in PRMI-1640 medium supplemented with 10% human AB group serum and incubated in 5% CO_2 at 37 ℃ overnight. The co-incubated DCs

were washed twice and resuspended in PRMI-1640 medium at a concentration of 1×10^7 cells/mL in preparation for the activation of tumor-specific T/CIKs.

Generation of tumor-specific T/cytokine-induced killer cells

The non-adherent mononuclear cells obtained from patient bone marrow were resuspended in PRMI-1640 supplemented with 10% human AB group serum at a concentration of 1×10^7 cells/mL. IFN-γ (Amoytop Biotech, Xiamen, China; 1000 U/mL) was added on day 1; Muromonab CD-3 (OKT3) monoclonal antibody (Sunbio, China; 50 ng/mL), IL2 (Canerotc, China; 500 U/mL) and rIL-1α (Amoytop Biotech, Xiamen, China; 100 U/mL) were added on day 2. Fresh complete medium containing IL2 was added every two or three days to expand the CIKs.

The CIKs were collected on day 12 and co-cultured with the tumor cell lysate-pulsed DCs in PRMI-1640 (a ratio of DC:CIK, 1:30) supplemented with 10% AB serum for an additional 72 h in the presence of IFN-γ, OKT3, and IL2. The co-cultured cells were harvested in 2% human albumin saline for injection.

Clinical study design

The patients of the study group received the first injection on the third day after completing the radio- or chemotherapy, and the subsequent doses were administered every week. The first single dose was initiated with 2×10^9 T/CIK cells and increased to 20×10^9 cells per injection.

Patients were monitored for immediate and delayed toxicities. The survival in months was calculated from the time of hospitalization to the time of death or to the present time. Treatment responses were evaluated by clinical observations, laboratory tests and radiological findings. CT scan was performed every 3 mo to evaluate the lesions after the injections. Tumor size was estimated by direct measurement of the volume of the abnormal enhancement region on the CT. The responses were classified into the following categories: (1) disappearance of the entire tumor, classified as complete response; (2) a reduction in tumor size by 25% or more, as partial response (PR); (3) either a decrease in tumor size by less than 25% or an increase in tumor size by less than 25%, classified as no change (NC); and (4) an increase in tumor size by 25% or more, classified as progressive disease (PD).

Delayed-type hypersensitivity reaction

To test the delayed-type cytotoxicity response, 1 μg of autologous tumor cell lysate with no α-Gal epitopes was administered intradermally into the forearm before and after the injections. A positive DTH reaction was recorded as ≥ 5 mm in diameter of rash or any size of blister at the injection site after 48 h.

Interferon-γ enzyme-linked immunospot assay

Human interferon-γ secretion by effectors was assessed by enzyme-linked immunosorbent spot assay (ELISPOT). Multiplescreen 96-well assay plates (Dakewe, Shenzhen, China) were precoated overnight at 4 ℃ with anti-IFN-γ antibody according to the manufacturer's instructions. After washed with PBS and 0.05% Tween-20, the plates were blocked for 1 h at 37 ℃ with 100 μL of RPMI-1640 supplemented with 1% bovine serum albumin (Sigma-Aldrich, Beijing). Mononuclear cells from the patients were plated in triplicate wells at a density of 1×10^4 cells/100 μL of PRMI-1640 medium supplemented with 10% AB serum, cultured overnight, washed and incubated with anti-IFN-γ mAb-biotin (Dakewe, Shenzhen, China). After washing, goat anti-biotin antibodies (Dakewe, Shenzhen, China) were added and incubated for 1 h at 37 ℃, and then 30 μL of activator solution (Dakewe, Shenzhen, China) was used to develop the spots. The number of spots in each well was counted using the Bioreader 4000-PRO-X (Bio-Sys, Germany). The cutoff criterion for positive spots was defined as a spot size more than 3 times the SD greater than the mean value of the spot diameter obtained in the absence of the DCs.

RESULTS

Dendritic cells phenotype

Peripheral mononuclear cells (1.5×10^8 - 6.6×10^8) were induced to differentiate into DCs in the presence of GM-CSF, IL-4 and TNF-α. The final yield of DCs was 1×10^7 - 4×10^7 cells after 7 days of incubation. The phenotype of the purified DCs was 100% HLA class I positive, > 90% CD1a, CD80, and HLA-DR positive, > 70% CD83 and CD86 positive, and CD3, CD4, CD8, CD14, CD19 and CD56 negative.

Synthesis of α-Gal epitopes on intact HCC cells

Intact tumor cells from the 9 patients were isolated for the synthesis of α-Gal epitopes (Figure 1). The tumor cells expressed abundant α-Gal epitopes on their surface after incubation with both neuraminidase and recombinant bovine α1, 3GT as indicated by the extensive binding of human purified natural anti-Gal antibody. However, the cells incubated with recombinant α1,3GT in the absence of neuraminidase weakly expressed the α-Gal epitope at a level that was 10-50 folds lower than the cells incubated with both neuraminidase and recombinant α1,3GT. The reason for this result is that most of the N-acetyllactosamine units on HCC cells were capped by sialic acid and could be exposed by neuraminidase.

Safety of α-Gal-pulsed dendritic cells and tumor-specific cytokine-induced killer cells

The DCs pulsed with tumor cell membrane expressing synthesized epitopes were co-cultured with autologous CIKs to induce tumor-specific CIKs. The cell mixture was administered to the patients for 2-7 times with a mean of 5.

The patients did not develop any grade Ⅲ or Ⅳ adverse effects associated with the treatment. These effects are defined by the National Cancer Institute Common Toxicity Criteria. No other serious side effects were ob-

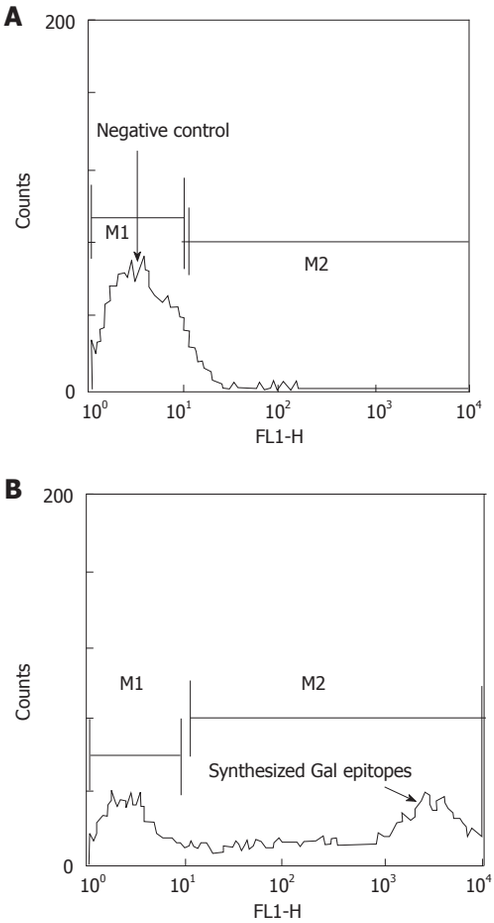

Figure 1 Synthesis of α-Gal epitopes on tumor cell membranes. A: Negative control, tumor cells treated with neuraminidase only, cells with no synthesized epitopes; B: Tumor cells treated with both neuraminidase and α1,3GT, > 60% tumor cells with synthesized α-Gal epitopes.

served other than death from tumor progression. There were no substantial changes in blood tests, such as anti-dsDNA, anti-ANA antibodies, *etc.* All patients developed a mild fever, which lasted 1-3 d after the second and third injections. Five patients showed mild to median rash or multiple erythema, suggesting the presence of delayed-type DTH. All symptoms vanished within 1-2 wk (Table 1).

Delayed-type hypersensitivity reaction

To test the cell-mediated immune responses, tumor lysate with no α-Gal epitopes was administered intradermally into the forearm before and after treatment. A positive DTH skin test reaction was defined as ⩾ 5 mm in diameter after 48 h arbitrarily. As a result, the reaction was less than 5 mm in diameter in all studied patients before immunotherapy. However, after treatment, only one patient demonstrated a negative response and all other patients showed a positive reaction; patients 1, 2, 4, 6, 7 and 9 displayed a reaction of more than 50 mm in diameter (Table 1).

Clinical responses

There were no statistically significant differences between the study and control groups in age (56.8 ± 13.9 years *vs* 49.7 ± 19.7 years; $P = 0.39$). The overall survival in

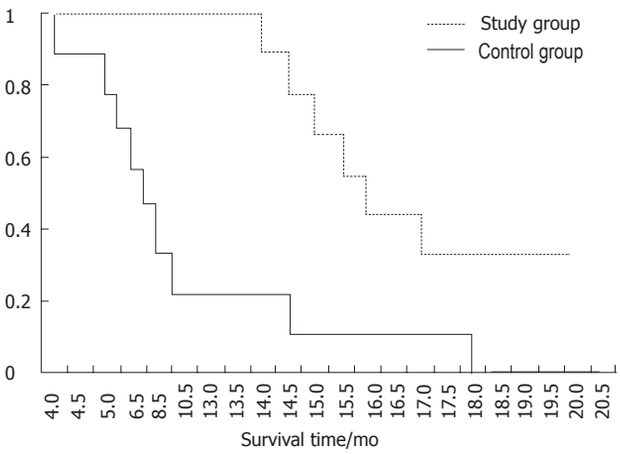

Figure 2 Kaplan Meier survival curves of the patients with stage Ⅲ hepatocellular carcinoma. Study group, $n = 9$; control group, $n = 9$; $P = 0.00121$.

months was determined from the date of hospitalization to the date of death or present time. The response to the treatment was evaluated by clinical observations and laboratory findings.

The quality of life in the study group improved significantly, e.g., the Karnofsky performance status score increased to 90 in 8 patients after treatment. The median survival time for the study and control groups was 17.1 mo and 10.1 mo, respectively. The Kaplan Meier test showed that the survival curves were significantly different between the two groups ($P = 0.00121$) (Figure 2). Three patients in the study group were still alive and in stable condition at the time the paper was written, and they have survived for more than 2 years (Table 1); and three patients showed a partial response in the size of the tumor as shown on CT. The lab test indicated that the AFP in the serum decreased in 8 patients after three injections (Table 1).

Increase of tumor-specific T cells in patients' peripheral blood

The phenotype (CD3, CD4, CD8, CD16, CD19, CD45RO and CD56) of the patients' peripheral blood lymphocytes was analyzed using flow cytometry before and after treatment. A moderate increase in the number of CD3- and CD4-positive cells was observed after immunotherapy; the increase in number of CD45RO+, CD8+ and CD56 cells was significant (Table 2).

The tumor-specific cytotoxicity of PBMCs was assessed by IFN-γ ELISPOT assay. PBMCs were isolated and stimulated with α-Gal-expressing tumor cell lysate. Untreated PBMCs were used as a negative control. The tumor antigens were found to generate strong tumor-specific T cell responses by virtue of their ability to induce increased frequencies of IFN-γ-producing T cells after immunotherapy (Table 2).

DISCUSSION

Previous immunotherapy treatment for primary HCC used passive[19], adoptive[20], and non-specific strategies

Table 2 Ratios of post- and pre-immunotherapy in cell populations and interferon-γ expression levels

Patient no.	CD8+ post *vs* pre	CD45RO+ post *vs* pre	CD56+ post *vs* pre	IFN-γ fold increase post *vs* pre
1	5.09	6.78	4.81	15.32
2	5.22	4.97	5.66	12.25
3	6.33	3.78	4.77	12.45
4	4.81	1.42	1.39	11.11
5	2.15	2.43	2.17	3.88
6	3.67	3.66	5.86	7.94
7	3.78	3.94	2.81	8.31
8	0.55	2.02	0.65	1.03
9	5.23	6.67	3.55	12.73

IFN: Interferon; CD: Cluster of differentiation..

that yielded limited benefits[21]. There are several possible reasons for the weak and inefficient immune responses against tumor cells. The first and perhaps the most important reason is that the antigenicity of TAA is not strong enough to stimulate the host immune system and cannot be recognized as "foreign"[22]; thus, TAA cannot generate strong tumor-specific immune responses. Another explanation for the inability of TAAs to induce an efficient tumor-specific immune response is the lack of costimulatory molecules on tumor cells[23,24]. The activation of naïve T cells requires recognition by the T-cell receptors (TCRs) of TAA peptides in association with the major histocompatibility complex (MHC) (Signal 1), as well as a costimulatory signal (Signal 2) that is not antigen-specific. The interaction of TCRs on naive T cells with TAAs on tumors without the delivery of signal 2 is thought to result in T cell anergy; this anergy leads to the tolerance of TAAs[23,24], which is similar to the tolerance of normal self-antigens during development. In contrast, if the antigenicity of TAA has been artificially amplified, the uptake of tumor cell membranes by APCs would result in proteolytic degradation of the TAAs, followed by presentation of the TAA peptides in association with MHC class I and class Ⅱ molecules. These molecules together with costimulatory molecules on APCs would activate naive T helper (Th) cells[25].

The second reason is that T cell function is impaired in most patients with malignant tumors[26]. The immunity in tumor patients is generally quite low, and it may be difficult for the circulating T cells to be activated due to anergy to tumor antigens. To overcome these problems, an ideal method is to enhance the TAA, pulse patient DCs with TAA-enriched tumor cells and use the newly differentiated naïve T/CIK cells from bone marrow stem cells. The DCs then activate the patients' naïve T/CIK cells to produce tumor-specific immune responders *ex vivo*.

This new approach consists of four steps to fulfill the ideal requirements for immunotherapy described above. The first step was to increase the antigenicity of TAA based on the bio-synthesis of α-Gal epitopes on the tumor membrane. In the second step, the adhesive cells from PBMCs were induced to differentiate into DCs. At the same time, the isolated non-adhesive mononuclear cells from bone marrow were induced to naïve T/CIKs. In the third step, α-Gal epitope TAAs pulsed DCs. In the final step, the TAA-pulsed DCs and the T/CIKs were co-cultured for a short time to promote TAA delivery to naïve T/CIKs.

In this study, the naïve T/CIKs activated by TAA-pulsed DCs elicited significant cytotoxic responses against tumor cells in 8 of the 9 patients as determined by ELISPOT in the restimulated PBMCs. IFN-γ increased over 10 folds after three injections in patients 1, 2, 3, 4 and 9. While determining whether the injection of DCs and CIKs could increase the T cell reaction against a tumor antigen, we found that the numbers of CD3-, CD4-, CD8-, CD4RO- and CD56-positive cells increased after treatment. Another important finding is that patients treated with the tumor-specific immune cells had a prolonged survival than the control patients. These findings indicate the therapeutic relevance of the observed enhancement of tumor-specific cytotoxicity to HCC.

However, the immunotherapy increased the tumor marker AFP in the serum after the first treatment. We hypothesize that the injection of the immune responders destroyed tumor cells and gradually released the AFP. The specific binding of α-Gal epitope and its natural anti-Gal antibody promotes DC delivery of TAA to T lymphocytes and CIKs *in vitro*. The *ex vivo*-activated, tumor-specific immunoresponse cells may account for the robust increase in the survival of late-stage HCC patients in this study as compared with the control group. This study used α-Gal epitopes added to the tumor cell surface to increase tumor antigenicity, TAA-pulsed DCs and *ex vivo*-activated CIKs rather than TAA-pulsed DCs alone or CIKs alone, which represented a divergence from previously reported immunotherapeutic trials.

In summary, this new therapeutic technique can significantly increase the tumor-specific immune responders in circulation and the survival of advanced HCC patients with no serious side effects. At the same time, clinical studies in other tumors, such as pancreatic carcinoma, lung cancer and lymphoma, are being conducted to reassess the role that the therapy may play in prolonging the survival of the patients.

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COMMENTS

Background

Hepatocellular carcinoma (HCC), the most common primary neoplasm of the liver, is generally resistant to conventional treatment. This led to the interest in studies of alternative approaches. Immunotherapy is particularly appealing because of the potential specificity of the immune response in eradicating residual tumor cells after conventional treatment. However, the biggest obstacles of tumor immunotherapy are the low antigenicity of tumor cells and the weak immunity of the patients.

Research frontiers

Previous reports have suggested that synthesis of the immunostimulatory xenoantigen α-Gal on malignant cells could be used as a means to enhance tumor-associated antigen (TAA) immunogenicity and that treatment with cytokine-

induced killer cells (CIKs) may benefit patients with various types of tumors. Although the α-Gal epitope can increase the antigenicity of tumor cells and CIKs can kill tumor cells, the combination of α-Gal epitope-capped TAAs pulsed dendritic cells (DCs) with the newly differentiated naive T/CIKs to treat tumors has never been reported. In this study, the authors demonstrate that the novel technique has a great potential for tumor treatment.

Innovations and breakthroughs

The authors described a novel immunotherapy for advanced HCC. DCs were pulsed by co-culturing with patient's α-Gal epitope expressing tumor cells to promote phagocytosis *in vitro*. The pulsed DCs were employed to activate newly differentiated T cells from bone marrow stem cells which had not been anergized by the tumor antigen. The highly active, tumor-specific immunoresponders were expanded *ex vivo*.

Applications

The combination of the α-Gal epitope-expressing tumor cell-pulsed DCs and the newly differentiated naive T/CIKs could increase specific anti-tumor immune responses and prolong the survival of HCC patients with no serious side effects. This study represents a new strategy for therapeutic intervention in the treatment of patients with HCC.

Terminology

α-Gal epitope-pulsed dendritic cells (DCs): DCs are potent antigen presenting cells (APCs) that possess the ability to stimulate naïve T cells. The α-Gal epitope expressing tumor cells are co-cultured with DCs in the presence of natural anti-Gal antibody to promote phagocytosis and presentation of tumor antigens to T lymphocytes. Cytokine-induced killer cells (CIKs): CIKs are a heterogeneous subset of *ex-vivo* expanded T lymphocytes which present a mixed T phenotype. They have been described as highly efficient cytotoxic effector cells capable of recognizing and lysing tumor cell targets in a non-major histocompatibility complex restricted fashion. CIKs could also be activated and attack tumor cells specifically after contact with tumor specific DCs.

Peer review

This is a study looking for the role of *ex vivo* activated tumor specific CIK therapy on HCC. This is definitely a well planned and performed and well written study. The authors describe a novel immunotherapy for advanced HCC, and the data is compelling.

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ORIGINAL ARTICLE

Decreased hepatic peroxisome proliferator-activated receptor-γ contributes to increased sensitivity to endotoxin in obstructive jaundice

Xin Lv, Jian-Gang Song, Hong-Hai Li, Jun-Ping Ao, Ping Zhang, Ye-Sheng Li, Shao-Li Song, Xiang-Rui Wang

Xin Lv, Jian-Gang Song, Xiang-Rui Wang, Department of Anesthesiology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China
Xin Lv, Department of Anesthesiology, Shanghai Pneumology Hospital, Tongji University, School of Medicine, Shanghai 200433, China
Hong-Hai Li, Jun-Ping Ao, Ping Zhang, State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai Cancer Institute, Shanghai 200127, China
Ye-Sheng Li, Department of Special Treatment, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, Shanghai 200438, China
Shao-Li Song, Department of Nuclear Medicine, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China
Author contributions: Lv X, Song JG contributed equally to this work, performed the majority of experiments and wrote the manuscript; Li HH, Ao JP, Zhang P, Li YS, Song SL performed part of experiment and statistical analysis; Wang XR designed the study.
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Correspondence to: Xiang-Rui Wang, MD, Department of Anesthesiology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, No.1630,Dongfang Road, Shanghai 200127, China. xiangruiwang@163.com
Telephone: +86-21-68383198 Fax: +86-21-68383198
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Abstract

AIM: To investigate the role of hepatic peroxisome proliferator-activated receptor-γ (PPAR-γ) in increased susceptibility to endotoxin-induced toxicity in rats with bile duct ligation during endotoxemia.

METHODS: Male Sprague-Dawley rats were subjected to bile duct ligation (BDL). Sham-operated animals served as controls. DNA binding were determined by polymerase chain reaction, Western blotting analysis, and electrophoretic mobility shift assay, respectively. BDL and sham-operated rats received a non-lethal dose of intraperitoneal lipopolysaccharide (LPS) injection (3 mg/kg, i.p.). Additionally, the potential beneficial effects of the PPAR-γ agonist rosiglitazone were determined in BDL and sham-operated rats treated with a non-lethal dose of LPS. Survival was assessed in BDL rats treated with a non-lethal dose of LPS and in sham-operated rats treated at a lethal dose of LPS (6 mg/kg, i.p.).

RESULTS: PPAR-γ activity in rats undergoing BDL was significantly lower than in the sham-controls. Hepatic *PPAR-γ* gene expression was downregulated at both the mRNA and protein levels. In a parallel group, serum levels of pro-inflammatory cytokines were nearly undetectable in the sham-operated rats. When challenged with a non-lethal dose of LPS (3 mg/kg), the BDL rats had approximately a 2.4-fold increase in serum IL-6, a 2.7 fold increase in serum TNF-α, 2.2-fold increase in serum IL-1 and 4.2-fold increase in serum ALT. The survival rate was significantly lower as compared with that in sham-operated group. Additionally, rosiglitazone significantly reduced the concentration of TNF-α, IL-1β, IL-6 and ALT in sham-operated rats, but not in BDL rats, in response to LPS (3 mg/kg). Also, the survival was improved by rosiglitazone in sham-operated rats challenged with a lethal dose of LPS, but not in BDL rats, even with a non-lethal dose of LPS (3 mg/kg).

CONCLUSION: Obstructive jaundice downregulates hepatic PPAR-γ expression, which in turn may contribute to hypersensitivity towards endotoxin.

Key words: Obstructive jaundice; Endotoxemia; Liver; Peroxisome proliferator-activated receptor-γ; Rosiglitazone

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INTRODUCTION

The high incidence of perioperative septic complications in patients with obstructive jaundice is well documented. This may be related to the high frequency of endotoxemia during cholestasis. It has been reported that up to 50%-70% of patients with obstructive jaundice, even without identifiable source of infection in many cases, have endotoxemia [1-3]. Increased bacterial translocation and absorption of endotoxins, caused by changes in plasma lipoproteins that bind endotoxin, loss of intestinal mucosal integrity, and lack of bile salts in the intestinal lumen, may result in endotoxemia[4-7]. On the other hand, enhanced susceptibility toward endotoxin-induced toxicity, characterized by exaggerated release of several proinflammatory cytokines, more severe organ damage, and reduced survival after non-lethal endotoxemia, is also believed to be responsible for the high morbidity and mortality of patients with obstructive jaundice[8-11]. Previous studies have suggested that prolonged stimulation or over-activation of the Kupffer cells (KCs), the largest numbers of resident macrophages in the liver, is a critical factor in the exaggerated inflammatory response to endotoxin[12-14].

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand activated transcription factor that belongs to the nuclear receptor family. It is a key regulator of adipocyte differentiation, lipid metabolism, glucose homeostasis, and cell proliferation. Activation of PPARγ could produce anti-inflammatory effects by repressing the expression of inflammatory cytokines in activated macrophages and monocytes[15,16]. In endotoxemia and sepsis, it has been reported that hepatic PPAR-γ expression in KCs is downregulated, possibly by increased plasma tumor necrosis factor-α (TNF-α). Decreased PPAR-γ expression, in turn, increased the production of proinflammatory cytokines and tissue injury[17]. During obstructive jaundice, portal or systemic endotoxemia is frequently observed. Also, circulating proinflammatory cytokines (e.g., TNF-α and interleukin-6) are increased even in the absence of exogenous stimuli under cholestatic conditions[18-21]. We therefore speculated that the PPAR-γ expression and function in the liver are decreased by endotoxemia and/or increased proinflammatory cytokines during obstructive jaundice.

Here we examined whether hepatic PPAR-γ expression and function are decreased in a rat model of obstructive jaundice, to seek for a possible correlation between alteration of PPAR-γ expression and increased susceptibility to endotoxin.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250-300 g) were purchased from the Animal Center of Shanghai Jiao Tong University School of Medicine (Shanghai, China) and were housed in an air-filtered room at 22-25 ℃ on a 12-h light/dark cycle, with unlimited access to water and standard rat chow. All experimental procedures were in accordance with the institutional animal care guidelines and approved by the local ethic committees.

Bile duct ligation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The common bile duct was ligated and divided after laparotomy. Sham-operated rats underwent the same procedure without bile duct ligation.

Experimental protocol

Three sets of experiments were carried out. In the first experiment, rats were sacrificed at day 7 after the surgery to analyze PPAR-γ expression and activation in the liver. In the second experiment, endotoxemia was established by a non-lethal dose of lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4; Sigma, 3 mg/kg, i.p.) in the BDL or sham-operated rats. Blood was collected for analysis of pro-inflammatory cytokines (e.g., TNF-α, IL-6 and IL-1β) and alanine aminotransferase (ALT) 2 h after LPS challenge. Survival was also monitored in another group. In the third experiment, the potential beneficial effects of the PPAR-γ agonist rosiglitazone were examined in BDL and sham-operated rats treated with a non-lethal dose of LPS (3 mg/kg, i.p.). Survival was examined in BDL rats treated with a non-lethal dose of LPS and in sham-operated rats treated at a lethal dose of LPS (6 mg/kg, i.p.). Rosiglitazone (3 mg/kg, i.p.) or vehicle (10% dimethyl sulfoxide) was administered intraperitoneally as a bolus 15 min prior to LPS injection.

RNA extraction and peroxisome proliferator-activated receptor-γ gene expression

Total RNA was extracted from hepatic tissues using TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol. Primers were obtained from Shanghai Sangon Biologic Engineering and Technology and Service (Shanghai, China). The following primer sequences were used: for PPAR-γ mRNA (forward: 5'-ACACCATGCTGGCCTCCCTGA-3'; reverse: 5'-AAGCCTGGGCGGTCTCCACT-3'; size 220 bp), and for β-actin (forward: 5'-CCACACCCGCCACCAGTTCG-3'; reverse: 5'-CTTGCTCTGGGCCTCGTCGC-3'; size 205 bp). Amplifica-

tion and detection were performed with an ABI PRISM 7300 real-time polymerase chain reaction (PCR) System (Applied Biosystems, Foster City, California, United States) as follows: 30 s at 95 ℃, and 40 cycles at 95 ℃ for 5 s and at 60 ℃ for 31 s. The DNA-binding dye SYBER Green I for the detection of PCR products was used. The reaction mixture (RT-PCR kit, Code DRR063A, Takara) contained 25 μL Premix Ex Taq, 1 μL forward and reverse primers, 1 μL ROX reference dye, 4 μL cDNA (equivalent to 20 ng total RNA) in a final volume of 50 μL. The amount of gene transcript was measured using a comparative (2-[DELTA][DELTA]CT) method by Applied Biosystems. The reference gene β-actin was used for normalization of the expression data.

Western blotting analysis of peroxisome proliferator-activated receptor-γ proteins

The nuclear extracts were prepared from liver tissues using a nuclear extract kit (Active Motif, Carlsbad, CA), following the manufacturer's protocol. Protein concentration was measured by the bicinchoninic acid assay method (Pierce). Liver nuclear extracts containing equal amounts of protein were separated in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE). Separated protein bands were transferred onto a nitrocellulose filter. The filter was blocked with 5% non-fat milk, primary rabbit-anti-mouse PPAR-γ antibody (1:200 dilution, Santa Cruz Technologies, Santa Cruz, CA) and β-actin monoclonal antibody (1:4000 dilution, Sigma-Aldrich) were used. After incubation with a horseradish peroxidase-conjugated secondary antibody, chemoluminescence agent A was mixed with B at equal volumes and evenly smeared onto the filter. The chemiluminescence signals were quantified using a Chemi-Smart 3000 (Vilber Lourmat; Marne-la-Vallée, France).

Electrophoretic mobility shift assay of peroxisome proliferator-activated receptor-γ activation

Electrophoretic mobility shift assay (EMSA) was used to detect specific binding of the transcription factor PPAR-γ to its specific DNA consensus sequence. Nuclear protein/DNA binding reactions were conducted for 20 min at room temperature in a 20-μL reaction volume containing 2 μL 10 × binding buffer, 1 μL polydI-dC, 1 μL 50% glycerol, 1 μL 1% NP-40, 1 μL of 1 mol/L KCl, 1 μL 100 mmol/L $MgCl_2$, 1 μL 200 mmol/L EDTA (all the reagents are included in the LightShift Chemiluminescent EMSA kit; Pierce), 20 fmol biotin-labeled probe (PPAR-γ consensus sequence 5'-GGGGTCAGTAAGTCAGAGGCCAGGGA-3'), and 2 μL nuclear extract. Binding reactions were analyzed using 8% PAGE. After blotting to a nylon membrane, the labeled oligonucleotides were detected with the LightShift Chemiluminescent EMSA kit (Pierce). The relative intensity of the bands was analyzed using an LAS-1000 luminoimage analyzer (Fuji Film, Tokyo, Japan).

Measurement of serum cytokines and alanine aminotransferase

Serum concentrations of TNF-α, IL-1β, or IL-6 were quantified using enzyme-linked immunosorbent assay (R&D Systems). Serum ALT levels were determined with an autoanalyzer (Model 7600, Hitachi Co., Tokyo, Japan).

Statistical analysis

The data were expressed as means ± SE. Data were analyzed by analysis of variance, followed by the Student-Newman-Keuls test. The survival curve was estimated by the Kaplan-Meier method and statistical significance was assessed by log-rank test. *P* values less than 0.05 were considered as significant.

RESULTS

Peroxisome proliferator-activated receptor-γ activation and expression are decreased upon bile duct ligation-induced cholestasis

We started the investigation by measuring modification of *PPAR-γ* activation and expression upon BDL and consequent cholestasis. We measured levels of PPAR-γ activation through electrophoretic mobility shift assay of PPAR-γ DNA binding in nuclear extracts from liver of treated and control rats (Figure 1A). PPAR-γ activity in rats undergoing BDL was significantly lower than in the sham-controls (0.62 ± 0.07 *vs* 1.00 ± 0.08, $P = 0.0257$). Hepatic *PPAR-γ* gene expression was downregulated at both the mRNA (1 ± 0.09 *vs* 0.09 ± 0.03, $P = 0.0000002$, Figure 1B) and protein levels (0.70 ± 0.02 *vs* 0.59 ± 0.01, $P = 0.0089$, Figure 1C).

Enhanced susceptibility toward endotoxin-induced toxicity in rats with pre-existing cholestasis

In a parallel group, serum levels of pro-inflammatory cytokines were nearly undetectable in the sham-operated rats. Biliary obstruction *per se* resulted in the induction of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 (66.0 ± 6.5 *vs* 27.9 ± 3.2, $P = 0.00036$; 56.0 ± 7.6 *vs* 19.7 ± 2.6, $P = 0.0011$; 60.2 ± 5.8 *vs* 30.7 ± 3.5, $P = 0.0014$, Table 1), and liver injury, as indicated by increased circulating level of ALT (119.7 ± 11.1 *vs* 41.2 ± 3.2, $P = 0.00005$, Table 1). When challenged with a non-lethal dose of LPS (3 mg/kg), the BDL rats had approximately 2.75-fold increase in serum TNF-α, 2.21-fold increase in serum IL-1β, 2.41-fold increase in serum IL-6 and 4.17-fold increase in serum ALT (2364.7 ± 196.3 *vs* 861.1 ± 44.2, $P = 0.00006$; 2373.0 ± 265.1 *vs* 1073 ± 97.7, $P = 0.00098$; 10802.0 ± 853.2 *vs* 4476.7 ± 430.2, $P = 0.00005$; 305.0 ± 37.5 *vs* 73.2 ± 7.7, $P = 0.0001$, Table 1). The survival rate was significantly lower than in the sham-operated group (20% *vs* 90%, $P = 0.000009$, Figure 2). These results indicated that enhanced susceptibility toward endotoxin-induced toxicity is present in BDL rats, which is consistent with previous studies.

Peroxisome proliferator-activated receptor-γ agonist rosiglitazone does not protect jaundiced rats from endotoxemia

To further verify a possible correlation between alterations of PPAR-γ function and increased susceptibility

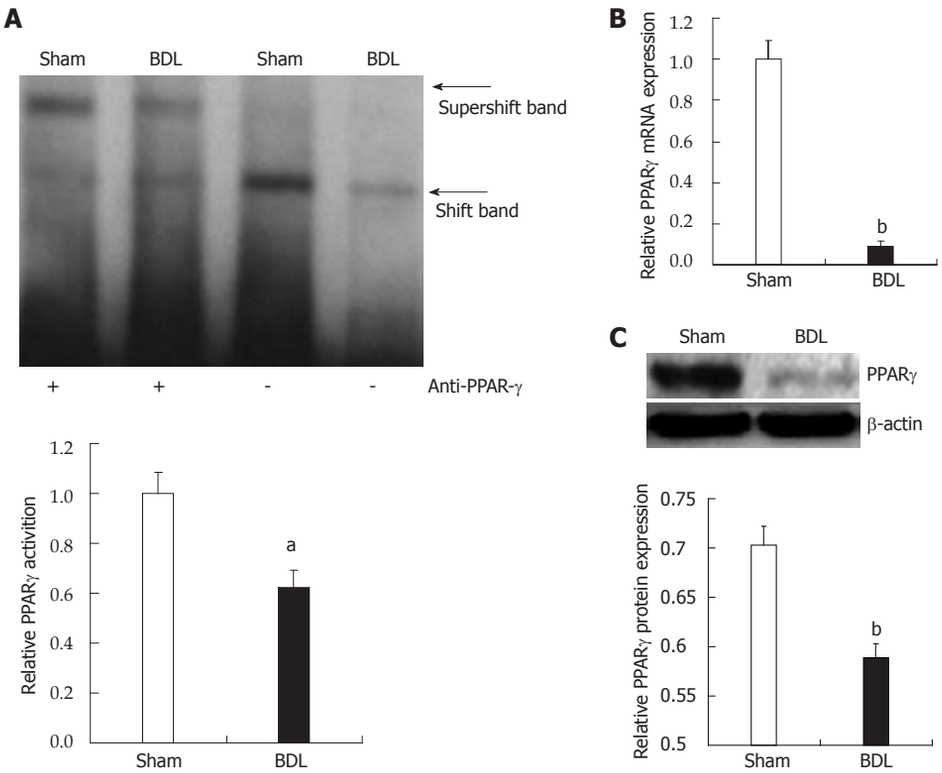

Figure 1 Modified measurement of peroxisome proliferator-activated receptor-γ activation and expression upon bile duct ligation and consequent cholestasis. A: Electrophoretic mobility shift assay (top panel) of peroxisome proliferator-activated receptor (PPAR)-γ. DNA binding in nuclear extracts from liver, and relative densitometric analysis (lower panel). Data are expressed as mean ± SE (n = 3). [a]P < 0.05 for bile duct ligation (BDL) group *vs* sham group; B: PPAR-γ mRNA expression in the liver. PPAR-γ expression in the liver of BDL rats decreased significantly compared with the sham-group determined by quantitative real-time reverse transcription-polymerase chain reaction. Data are expressed as mean ± SE (n = 6). [b]P < 0.01 for BDL group *vs* sham group; C: Western blotting analysis (top panel) of PPAR-γ in nuclear extracts from liver, and relative densitometric analysis (lower panel, n = 3). Data are expressed as mean ± SE (n = 3). [b]P < 0.01 for BDL group *vs* sham group.

Table 1 Serum alanine aminotransferase, tumor necrosis factor-α, IL-1β and IL-6 levels in rats after administration of lipopolysaccharide (n = 6, mean ± SE)

	Sham	BDL	Sham + LPS	BDL + LPS
ALT (U/L)	41.2 ± 3.2	119.7±11.1[a]	73.2 ± 7.7	305.0 ± 37.5[a]
TNF-α (pg/mL)	27.9 ± 3.2	66.0 ± 6.5[a]	861.1 ± 44.2	2364.7 ± 196.3[a]
IL-1β (pg/mL)	19.7 ± 2.6	56.0 ± 7.6[a]	1073 ± 97.7	2373.0 ± 265.1[a]
IL-6 (pg/mL)	30.7 ± 3.5	60.2 ± 5.8[a]	4476.7 ± 430.2	10802.0 ± 853.2[a]

[a]P < 0.01 for bile duct ligation group *vs* Sham group. BDL: Bile duct ligation; LPS: Lipopolysaccharide; ALT: Alanine aminotransferase; TNF-α: Tumor necrosis factor-α.

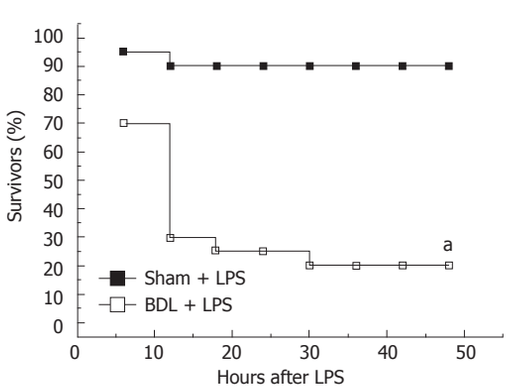

Figure 2 Survival rates of rats after administration of lipopolysaccharide (3 mg/kg, i.p.). The survival curve was estimated by the Kaplan-Meier method and the log-rank method was used to compare differences in survival rates between groups (n = 20). [a]P < 0.01 for bile duct ligation group *vs* sham group. BDL: Bile duct ligation; LPS: Lipopolysaccharide.

to endotoxin, we investigated whether PPAR-γ agonist rosiglitazone had anti-inflammatory effects in BDL rats. Treatment with rosiglitazone significantly reduced the concentration of TNF-α, IL-1β, IL-6, and ALT in sham-operated rats, but not in BDL rats, in response to LPS (3 mg/kg) (Table 2). The survival was improved by rosiglitazone in sham-operated rats challenged with a lethal dose of LPS (6 mg/kg) (50% *vs* 20%, P = 0.040) but not in BDL rats, even with a non-lethal dose of LPS (35% *vs* 25%, P = 0.49) (Figure 3). These data indicated that endogenous anti-inflammatory pathway of nuclear receptor PPAR-γ is impaired by obstructive jaundice, which in turn may be at least in part, responsible for the increased susceptibility to endotoxin.

DISCUSSION

In this study, both the expression and function of PPAR-γ in the liver were significantly depressed in rats with BDL as compared with the sham-operated rats. Concomitant to the decrease in PPAR-γ DNA binding, we observed a markedly increased susceptibility to endotoxin, evidenced by higher degree of liver injury, enhanced proinflammatory cytokine release, and a higher mortality. The PPAR-γ

Table 2 Effect of pretreatment with rosiglitazone on serum alanine aminotransferase, tumor necrosis factor-α, IL-1β and IL-6 levels in rats after administration of lipopolysaccharide ($n = 6$, mean ± SE)

	Sham + vehicle	BDL + vehicle	Sham + ROSI	BDL + ROSI
ALT (U/L)	78.6 ± 8.1	329.4 ± 40.5	42.3 ± 5.7[a]	303.0 ± 46.6
TNF-α (pg/mL)	770.5 ± 52.5	2017.9 ± 167.5	517.0 ± 69.7[a]	1984.0 ± 181.1
IL-1β (pg/mL)	1096.1 ± 101.7	2345.2 ± 194.7	648.4 ± 111.4[a]	2226.7 ± 209.8
IL-6 (pg/mL)	4479.4 ± 377.4	10774.4 ± 675.4	2758.5 ± 497.7[a]	9847.1 ± 865.1

[a]$P < 0.05$ for vehicle (dimethyl sulfoxide) pretreatment *vs* rosiglitazone pretreatment group. BDL: Bile duct ligation; ROSI: Rosiglitazone; ALT: Alanine aminotransferase; TNF-α: Tumor necrosis factor-α.

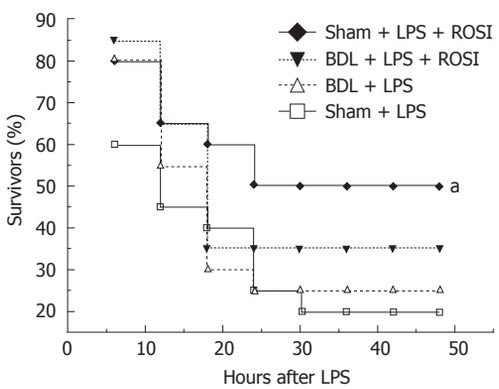

Figure 3 Effects of pretreatment with rosiglitazone (3 mg/kg, i.p.) on survival rates in rats after administration of lipopolysaccharide (6 mg/kg for sham-group and 3 mg/kg for bile duct ligation group, i.p.). The survival curve was estimated by the Kaplan-Meier method and the log-rank method was applied to compare differences in survival rates between groups (n = 20). Rosiglitazone pretreatment significantly improve the survival in sham + lipopolysaccharide (LPS) (6 mg/kg) group but not in bile duct ligation + LPS (3 mg/kg) group, [a]$P < 0.05$ *vs* sham + LPS group. BDL : Bile duct ligation; ROSI: Rosiglitazone.

agonist rosiglitazone failed to protect BDL rats against endotoxemia.

Despite the use of broad-spectrum antibiotics and improvement in surgical technique, patients with cholestatic liver disease continue to experience a high incidence of postoperative morbidity and mortality[22,23]. Belghiti *et al*[24] showed that the mortality of patients with obstructive jaundice is much higher (21%) than that of the patients with normal liver function. Among the multiple complications from obstructive jaundice, gram-negative bacterial sepsis is the most common cause of secondary morbidity and mortality[25]. Animal studies have demonstrated that obstructive jaundice exaggerates the release of several cytokines after endotoxin administration, including TNF-α, IL-1β, and IL-6[8-11].

KCs are the largest macrophage population and form the first line of defense against microorganisms entering the portal circulation. It has been demonstrated that the KCs are the primary source of circulating TNF-α and IL-6 in response to LPS[26]. In the context of appropriate immune response, KC activation plays a protective role during the acute phase of hepatopathies. However, in response to prolonged bacterial or endotoxin challenge, KC over-activation may increase the severity of organ damage and lethality. With regards to obstructive jaundice, previous studies indicate that liver KCs are involved in the exaggerated cytokine secretion after endotoxin challenge[12,13]. In jaundiced animals undergoing endotoxin challenge, blockade of KC function with gadolinium chloride or TNF-α antibody has been shown to improve survival and to suppress the systemic proinflammatory response[14]. Consequently, altered function of KCs during cholestasis is thought to play a decisive role in the increased susceptibility to endotoxin-induced toxicity.

PPARs are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Recently, it has been found that PPAR-γ activation inhibits the expression of several inflammatory response genes in activated macrophages, including the genes encoding inducible nitric oxide synthase, TNF-α, gelatinase B, and COX-2[27,28]. Moreover, activation of PPAR-γ reduces the organ injury/dysfunction caused by endotoxin[29] and by hemorrhage and resuscitation[30], as well as systemic inflammation caused by zymosan[31] and by cecal ligation and puncture[32] in rodents. In this study, the expression and function of hepatic PPAR-γ were decreased during obstructive jaundice. PPAR-γ agonist rosiglitazone failed to protect BDL rats from lethal endotoxemia, suggesting that the downregulation of PPAR-γ in jaundiced rats is of functional significance.

It should be pointed out that hepatic PPAR-γ is expressed in both KCs and hepatocytes (HCs). We did not examine the source of PPAR-γ activity in this study. Nevertheless, previous studies have indicated that PPAR-γ gene expression decreased significantly in KCs at 20 h after sepsis by cecal ligation and puncture, whereas PPAR-γ expression in HCs was not altered. Moreover, when isolated KCs or HCs from normal rats were stimulated with LPS or TNF-α for 20 h, KC PPAR-γ protein levels were all significantly decreased. In contrast, neither LPS nor TNF-α affects PPAR-γ protein levels in HCs either in monoculture or in co-culture with KCs. These findings suggest that the KCs and HCs respond to LPS and TNF-α differentially[17]. Therefore, we believe that the downregulated PPAR-γ expression in the liver during obstructive jaundice is most likely due to decreased PPAR-γ in KCs but not in HCs.

Recent studies have indicated the potential efficacy of PPAR-γ ligands as novel therapeutic approaches in sepsis, inflammation and reperfusion injury. However, our observation that treatment with a PPAR-γ ligand fails to provide protection in cholestatic animals suggests that activation of the endogenous PPAR-γ pathway may need to be tailored to the specific conditions, e.g., obstructive jaundice.

Evidence suggests that the PPAR-γ expression is a function of the inflammatory response. For instance, in rats subjected to sepsis by cecal ligation and puncture or

double-hit hemorrhage and sepsis, PPAR-γ expression is downregulated in the liver, bronchial epithelium, and vascular endothelium[33-35]. In mice subjected to endotoxin administration, PPAR-γ expression is also markedly reduced in adipose tissues, heart and lungs[36-39]. Consistent with these reports, the current study demonstrated that biliary obstructive jaundice significantly increases serum amounts of TNF-α, IL-6 and IL-1β. Thus, increased proinflammatory cytokines (e.g., TNF-α) during cholestasis may result in downregulation of the PPAR-γ in the liver.

In conclusion, biliary obstructive jaundice downregulates hepatic PPAR-γ expression and function, which in turn may contribute to enhanced susceptibility to endotoxin-induced toxicity.

COMMENTS

Background

Biliary obstructive jaundice increases susceptibility to endotoxin-induced toxicity. However, the underlying molecular mechanisms are not fully understood.

Research frontiers

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor family of ligand-activated transcription factors. Activation of PPARγ could produce anti-inflammatory effects. In this study, the authors demonstrated that hepatic PPAR-γ is down-regulated upon obstructive jaundice, and provided some evidence suggesting that the down-regulation of PPAR-γ contributes to the hypersensitivity to endotoxin.

Innovations and breakthroughs

The expression and function of hepatic PPAR-γ were significantly decreased in the bile duct ligation rats compared with the control rats. Down-regulation of PPAR-γ was accompanied by exaggerated inflammatory response. Treatment with the PPAR-γ agonist rosiglitazone protected sham-operated, but not BDL rats, from endotoxemia.

Applications

Recent studies have suggested the potential efficacy of PPAR-γ ligands as novel therapeutic approaches in sepsis, inflammation, and reperfusion injury. The treatment with a PPAR-γ ligand fails to provide protection of cholestatic animals suggests that activation of the endogenous PPAR-γ pathway needs to be tailored to the specific conditions, e.g., obstructive jaundice.

Peer review

This is an elegant study from a biological observation to a potential clinical aspect.

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BRIEF ARTICLE

Impact of early or delayed elective resection in complicated diverticulitis

Kai Bachmann, Geeske Krause, Tamina Rawnaq, Lena Tomkotter, Yogesh Vashist, Shanly Shahmiri, Jakob R Izbicki, Maximilian Bockhorn

Kai Bachmann, Geeske Krause, Tamina Rawnaq, Lena Tomkotter, Yogesh Vashist, Shanly Shahmiri, Jakob R Izbicki, Maximilian Bockhorn, Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany

Author contributions: Bachmann K analyzed and interpreted the data, and drafted the manuscript; Krause G and Rawnaq T acquired, analyzed and interpreted the data; Tomkotter L drafted the manuscript; Vashist Y revised the manuscript; Shahmiri S drafted the manuscript and statistical analysis; Izbicki JR studied concept and designed and studied supervision; Bockhorn M studied concept, and designed and drafted the manuscript.

Correspondence to: Kai Bachmann, MD, Department of Surgery, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. k.bachmann@uke.de
Telephone: +49-40-741057346 Fax: +49-40-741044995
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Abstract

AIM: To investigate the outcomes of early and delayed elective resection after initial antibiotic treatment in patients with complicated diverticulitis.

METHODS: The study, a non-randomized comparison of the two approaches, included 421 consecutive patients who underwent surgical resection for complicated sigmoid diverticulitis (Hinchey classification Ⅰ-Ⅱ) at the Department of Surgery, University Medical Center Hamburg-Eppendorf between 2004 and 2009. The operating procedure, duration of hospital and intensive care unit stay, outcome, complications and socioeconomic costs were analyzed, with comparison made between the early and delayed elective resection strategies.

RESULTS: The severity of the diverticulitis and American Society of Anesthesiologists score were comparable for the two groups. Patients who underwent delayed elective resection had a shorter hospital stay and operating time, and the rate of successfully completed laparoscopic resections was higher (80% *vs* 75%). Eight patients who were scheduled for delayed elective resection required urgent surgery because of complications of the diverticulitis, which resulted in a high rate of morbidity. Analysis of the socioeconomic effects showed that hospitalization costs were significantly higher for delayed elective resection compared with early elective resection (9296 € ± 694 € *vs* 8423 € ± 968 €; $P = 0.001$). Delayed elective resection showed a trend toward lower complications, and the operation appeared simpler to perform than early elective resection. Nevertheless, delayed elective resection carries a risk of complications occurring during the period of 6-8 wk that could necessitate an urgent resection with its consequent high morbidity, which counterbalanced many of the advantages.

CONCLUSION: Overall, early elective resection for complicated, non-perforated diverticulitis is shown to be a suitable alternative to delayed elective resection after 6-8 wk, with additional beneficial socioeconomic effects.

Key words: Complicated diverticulitis; Resection of sigmoid; Delayed elective resection; Early elective resection; Socioeconomic effects

Peer reviewers: Dr. Bernardino Rampone, Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, 53100 Siena, Italy; Naoki Ishii, MD, Department of Gastroenterology, St. Luke's International Hospital, 9-1 Akashi-cho, Chuo-ku, 104-8560 Tokyo, Japan

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INTRODUCTION

Diverticular disease of the sigmoid colon is common in Western countries with a prevalence of 27%[1,2]. It increases with age from approximately 10% in those younger than 40 years to 33% in those older than 45 years, and then to 50%-70% in those older than 80 years. The average age of presentation with diverticulitis is 62 years. In the United States, approximately 130 000 patients per year are admitted to hospital due to diverticulitis. The increase in the number of cases diagnosed in recent years is, in part, due to improvements in non-invasive diagnostic techniques, especially the use of computed tomography (CT) scanning[3].

The risk of recurrence after a first attack of diverticulitis ranges from 5%-43%[4]. Complications (which include fistula formation, abscess, bleeding or perforation) occur in 15%-20% of cases and require surgical intervention[5,6]. The treatment of patients with complicated sigmoid diverticulitis is associated with a significant morbidity and mortality that is mainly influenced by the severity of the disease and the global medical status of the patient. For elective sigmoid resections, the postoperative morbidity rate is 15%-20%[5] and the mortality rate ranges from between 0%-17%, compared to 30% in patients with perforated diverticulitis[6-8].

The appropriate timing of elective resection for uncomplicated diverticulitis is a subject of controversy, with discussion focused mainly on the number of previous attacks. Surgery is generally suggested after one to four episodes of diverticulitis[9]. The American Society of Colon and Rectal Surgeons recommends that the decision regarding resection be made on a case-by-case basis[10]. Even in patients with clear indication for elective resection, the optimal timing of the operation remains unclear as little data exists, especially for cases of complicated diverticulitis. Therefore, this trial analyzed the impact of both delayed and early elective resections on outcome, mortality and morbidity, and socioeconomic cost.

MATERIALS AND METHODS

Study design and patients

The study analyzed 421 consecutive patients in whom elective surgical resection for complicated sigmoid diverticulitis had been planned at the Department of Surgery at the University Medical Center Hamburg-Eppendorf between 2004 and 2009. The study was approved by the institutional review board of the hospital. The stage of the diverticulitis was classified according to the modified Hinchey score[11], which is based on the CT scan at the time of diagnosis. Only patients with clear indication for surgery with complicated diverticulitis, but without perforation (Hinchey Ⅰ-Ⅱ), were included in this trial. Data, which included the sex and age of the patient, medication and the complications experienced, were obtained from clinical records. Data concerning previous medical history, co-morbidities and the American Society of Anesthesiologists (ASA) score at the time of the presentation and classification of the disease were recorded, along with the operating procedure and duration of the operation, durations of the hospital and intensive care unit stays, outcome and complications. Clinical follow-up data was obtained by review of the hospital records and by direct communication with the patients or their attending physicians. The socioeconomic costs of the treatment were evaluated in all cases by review of the total amount invoiced to the health insurance companies.

Operation and antibiotic treatment

On the basis of clinical features and CT scan, elective resection of the sigmoid was planned, with laparoscopic resection the operation of choice, where possible, for all patients except those who had undergone previous major abdominal surgery. The operating surgeon decided whether to perform a primary anastomosis with or without diverting stoma, or a Hartman's procedure depending on the intraoperative findings. The patients were assigned by their surgeon to either early or delayed elective surgery without randomization. The group assigned to early elective resection was treated with antibiotics (ceftriaxone and metronidazole) for 2-4 d before the early elective resection was performed. The delayed elective group was treated with intravenous antibiotics (ceftriaxone and metronidazole) for 5-7 d depending on the clinical course of the patient. Once their symptoms had settled, the patients were discharged and an elective resection was performed 6-8 wk later.

Statistical analysis

SPSS® for Windows® Version 13.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Data are presented as mean ± SD. The continuous data for the different groups were compared using the Student's *t* test. For all non-continuous variables, cross-tables were generated, followed by calculation of the *P* value by using the χ^2 test/Fisher's exact test. Statements of significance refer to *P* values for two-tailed tests of less than 0.05.

RESULTS

Patient characteristics

Overall, 421 patients who underwent surgical resection for sigmoid diverticulitis were included. To analyze the impact of the timing of the operation, early elective surgery was compared to delayed elective surgery after 6-8 wk. Early elective surgery was performed in 272 patients at a median of 2 d after admission to hospital. Elective resection after 6-8 wk was planned for 149 patients; however, eight of these required urgent surgery during this period because of the occurrence of severe complications following their initial conservative management.

At the time of surgery, the mean age of the patients was 63 ± 13 years; 184 patients (44%) were men, 237 patients (56%) women. Preoperatively, 14 patients (3%) were classified as ASA 1239 (57%) as ASA 2159 (38%) as ASA 3 and 9 patients (2%) as ASA 4. In 362 patients (86%), complicated diverticulitis or a small confined pericolic or

Table 1 Patient characteristics *n* (%)

	Early elective resection (*n* = 272)	Delayed elective resection (*n* = 149)	*P* value
Age (mean ± SD)	63.5 ± 13.1	64.2 ± 12.6	0.57
Sex			
Male	121 (44.5)	63 (42.3)	0.66
Female	151 (55.5)	86 (57.7)	
Classification of diverticulitis			
Hinchey 1	230 (84.6)	132 (88.6)	0.28
Hinchey 2	42 (15.4)	17 (11.4)	
ASA classification			
ASA 1	10 (3.7)	4 (2.7)	0.69
ASA 2	150 (55.1)	89 (59.7)	
ASA 3	107 (39.3)	52 (34.9)	
ASA 4	5 (1.8)	4 (2.7)	

ASA: American Society of Anesthesiologists.

mesenteric abscess was diagnosed (Hinchey I); while 59 patients (14%) presented with a distant or complex abscess without perforation (Hinchey II).

The distribution of patients who underwent early and delayed elective resection was largely comparable with regards to sex, age, ASA classification and severity of the diverticulitis (Hinchey classification, Table 1).

The operation performed

Overall, 323 patients (77%) underwent laparoscopic resection, while open surgery, including conversions, was performed in 98 patients (23%). Primary anastomosis without a diverting stoma was possible in 387 patients (92%), while 30 patients (7%) received a primary anastomosis and diverting stoma. In four patients (1%) a Hartman's procedure with descendostomy was necessary due to peritonitis. No significant difference in mortality rates between the various procedures (primary anastomosis, with or without diverting stoma, or Hartman´s procedure) was identified.

In patients who underwent delayed elective resection after 6-8 wk, the rate of successfully completed laparoscopic resection was significantly higher, as the inflammation had settled down compared with the early elective resection group (80% *vs* 75%, $P = 0.032$).

Outcome

Three patients (0.7 %) died in hospital. For delayed elective resection, the rate of wound infections was 7.1% *vs* 11.0% for early surgery; the rate of re-operations 5.7% *vs* 8.1%; these differences were not statistically significant. However, the operating time (149 min *vs* 166 min; $P < 0.001$) and the hospital stay (13 d *vs* 16 d, $P = 0.002$) were significantly shorter (Table 2).

Complications whilst waiting for delayed elective surgery

It is important to note that in eight patients initially planned for delayed elective surgery, urgent surgery was necessary before the 6-8 wk period had elapsed. Reasons for proceeding to urgent surgery were recurrent episodes with covered perforation or perforation with fecal peritonitis in four patients (on days 24, 29, 34 and 41), clinical deterioration in three patients (on days 3, 5 and 6) and re-presentation with acute diverticular bleeding in one patient. This subgroup of patients had a higher rate of complications. Three patients underwent a Hartman´s procedure and four underwent re-operations either for a planned second look and lavage, or because of anastomotic leakage; one of these patients died. On analysis of outcome based on the intention to treat, the advantages of delayed elective resection are lost with the exception of the shorter operating time and hospital stay (Table 3).

Table 2 Outcome following early and delayed elective resections performed for complicated diverticulitis *n* (%)

	Early elective resection (*n* = 272)	Delayed elective resection performed (*n* = 141)	*P* value
Operation			
Minimally invasive surgery	204 (75.0)	119 (84.4)	0.032
Open surgery	68 (25.0)	22 (15.6)	
Procedure			
Hartman	1 (0.4)	0 (0.0)	0.75
Protective stoma	17 (6.3)	8 (5.7)	
Primary anastomosis without stoma	254 (93.4)	133 (94.3)	
Surgical complications			
Wound infection	30 (11.0)	10 (7.1)	0.22
Anastomotic leakage	14 (5.1)	6 (4.3)	0.81
Reoperation	22 (8.1)	8 (5.7)	0.43
Medical complications			
Uriary tract infection	16 (5.9)	7 (5.0)	0.82
Pneumonia	9 (3.3)	4 (2.8)	1
Overall morbidity	41 (15.1)	16 (11.3)	0.16
Mortality	1 (0.4)	1 (0.7)	1
Socioeconomic data (mean ± SD)			
Duration of hospital stay (d)	16.1 ± 9.0	13.3 ± 7.9	0.002
Duration of operation (min)	166.2 ± 44.2	149.1 ± 39.1	< 0.001
Duration of ICU stay (d)	0.64 ± 0.90	0.72 ± 0.91	0.47
Overall costs (€)	8423 ± 968	9296 ± 694	< 0.001

ICU: Intensive care unit.

Socioeconomic effects

The socioeconomic costs of the hospital stay for the two treatment approaches were compared. Early elective resection was found to reduce the overall costs from 9296 € ± 694 € to 8423 € ± 968 € ($P < 0.001$). This is mainly due to costs incurred during the initial intravenous antibiotic treatment. With the inclusion of the eight patients who needed urgent surgery, the advantage for early surgery is even greater.

DISCUSSION

New insights from this study

Taken together, our results suggest that delayed elective resection has a better outcome in complicated diverticulitis than early elective resection as long as no acute complications occur during the 6-8 wk. The occurrence of an

Table 3 Outcome of planned early and delayed elective resections for complicated diverticulitis (intention to treat analysis) *n* (%)

	Early elective resection (*n* = 272)	Planned for delayed elective resection[1] (*n* = 149)	*P* value
Operation			
Minimally invasive surgery	204 (75.0)	119 (79.9)	0.28
Open surgery	68 (25.0)	30 (20.1)	
Procedure			
Hartman	1 (0.4)	3 (2.0)	0.153
Protective stoma	17 (6.3)	13 (8.7)	
Primary anastomosis without stoma	254 (93.4)	133 (89.3)	
Surgical complications			
Wound infection	30 (11.0)	16 (10.7)	0.92
Anastomotic leakage	14 (5.1)	8 (5.4)	1.0
Reoperation	22 (8.1)	12 (8.1)	1.0
Medical complications			
Uriary tract infection	16 (5.9)	7 (4.7)	0.66
Pneumonia	9 (3.3)	5 (3.4)	1.0
Overall morbidity	41 (15.1)	23 (15.4)	0.92
Mortality	1 (0.4)	2 (1.3)	0.26
Socioeconomic data (mean ± SD)			
Duration of hospital stay (d)	16.1 ± 9.0	14.2 ± 10.0	0.043
Duration of operation (min)	166.2 ± 44.2	148.9 ± 38.4	< 0.001
Duration of ICU stay (d)	0.64 ± 0.90	1.4 ± 4.0	0.004
Overall costs (€)	8423 ± 968	9941 ± 3563	< 0.001

[1]It includes the eight patients who required emergency surgery during the period of 6-8 wk whilst awaiting elective surgery. ICU: Intensive care unit.

acute complication is associated with a very unfavorable outcome. Therefore the decision to proceed to early or delayed elective resection should still be made on a case-by-case basis. In patients who are scheduled for delayed elective resection, close clinical monitoring is recommended until the definitive surgical treatment, to allow for early management of any possible clinical deterioration.

The risk of recurrence

In uncomplicated diverticulitis, medical treatment, which consists of broad-spectrum antibiotic therapy for 5-10 d and resting of the bowel with parenteral nutrition, is usually successful. The indication for elective resection and its most appropriate timing in uncomplicated diverticulitis is the subject of controversial discussions. Surgery is primarily suggested after one to four episodes of diverticulitis[9,12-14]. There is little prospective data on the natural course of diverticulitis[15,16] and the risk of a recurrence after the first attack ranges from 5%-46%[4]. Various controversial risk factors for recurrence, such as age and numbers of acute attacks, or preoperative morbidity have been reported[4,13,17-20].

Therefore, in 2006, the American Society of Colon and Rectal Surgeons stated that the decision regarding elective resection should be made on a "case-by-case basis," no general recommendation was given for elective resection to prevent additional attacks[10]. The age of the patient, their medical condition, response to treatment and wishes should all be considered when making decisions regarding the indication for and timing of such treatment.

The main reason for performing elective resection is the prevention of serious complications, which include recurrent diverticulitis and the possible perforation that are associated with high morbidity and mortality. These factors must be estimated as part of the risk of an elective operation, which has a postoperative morbidity rate of 15%-20%[5].

Delayed or early elective resection for complicated non-perforated diverticulitis

There is a consensus of opinion that urgent surgery is indicated in perforated diverticulitis; whilst elective resection is the treatment of choice for complicated diverticulitis, there is limited data concerning the timing of elective resection.

It is most common for patients to be treated conservatively with parenteral nutrition and antibiotics for 5-10 d initially, followed by a period of recuperation and readmission to hospital, typically 6-8 wk later, for a delayed elective resection of the sigmoid colon to be performed. Alternatively patients may be treated for approximately 2-5 d with parenteral nutrition and antibiotics, with an early elective resection performed during the same hospital stay. A correlation between the risk of recurrence and the severity of the initial episode has been described. In complicated diverticulitis characterized by phlegmon or a pericolic abscess, a more aggressive approach is recommended because of the high risk of recurrence, and the high rate of severe complications, such as perforation, with a potentially fatal outcome[4,21,22].

In a comparison of these approaches in patients with complicated and non-complicated diverticulitis, Nataranjan found no relationship between outcome and timing[23], while Reissfelder *et al*[24] detected an advantage for delayed elective resection. Zingg reported a higher conversion rate in early elective resection compared with delayed elective resection, but the rate of complicated diverticulitis was higher in the early elective group (73% *vs* 13%); the outcome and major complications were similar[25]. Patients with inflammation were reported to have a significantly higher conversion rate (35.4% *vs* 13.5%)[25]. These results partially contradict our findings, but this may be explained by the selection of the patients as in previously reported studies, patients with both complicated and uncomplicated diverticulitis were included; only patients with complicated diverticulitis were included in our study.

The disadvantage of early elective surgery seems to be caused by the continuing presence of the acute inflammation with adhesions, which makes the procedure more technically demanding and therefore results in a higher conversion rate and longer operating time. In addition, the disadvantage of early elective resection detected in our trial may be caused by the selection bias in the other previously reported retrospective non-randomized trials[23-25] toward a higher severity of diverticulitis in the ear-

ly intervention group. The rate of patients with Hinchey Ⅱ stage disease was 15.4% in the early elective group, compared with 9.2% in the patients who actually underwent delayed elective resection, which may therefore explain the tendency towards higher rates of complications in this particular group.

Apart from the possibility of selection bias, delayed elective resection does have one major disadvantage that was identified in this trial; the period of 6-8 wk that is intended to allow the inflammation to settle can be associated with clinical deterioration or a recurrent attack; three patients underwent Hartman's procedure because of severe complications during this period.

Socioeconomic effects

To analyze the socioeconomic costs of both approaches, the total hospital costs that were billed to the insurance companies were compared. In the delayed elective group, the costs of the initial hospital stay for intravenous antibiotic treatment and the subsequent admission for surgical resection were both included. The analysis of the socioeconomic costs of the treatment was based on the diagnosis related groups in Germany during the relevant periods. It should also be mentioned that any costs of treatment by general practitioners between or after the hospitalizations were not included, nor were any indirect costs due to the inability of the patient to work.

There was no detailed analysis of the direct costs to the hospital for the hospitalization of each patient, so the financial profit for the hospital cannot be calculated from our data. Irrespective of these limitations, this trial suggested a socioeconomic advantage for early elective resection, which became even greater when the patients who required urgent surgery instead of their scheduled delayed elective resections were included in the analysis.

Limitations

Our trial is the first to analyze the impact of the timing of this operation in patients only with complicated diverticulitis; however, this study also has its limitations. The analysis is retrospective and observational, and the interventions were not randomized. Although the patients in the two groups showed largely comparable co-morbidities and disease stage according to Hinchey, a selection bias may be present since the decision to proceed with early or delayed elective surgery was based on clinical data, the preference of the surgeon and the medical condition of the patient.

Delayed elective resection showed a trend toward lower complication rates; compared with early elective resection, the operation appeared simpler to perform. Nevertheless, delayed elective resection carried a risk of complications occurring during the period of 6-8 wk that could necessitate an urgent resection with its consequent high morbidity which counterbalanced many of the advantages. Overall, early elective resection for complicated, but non-perforated diverticulitis was found to be a suitable alternative to delayed elective resection after 6-8 wk, with additional beneficial socioeconomic effects. Following the style of the recommendations made by the American Society of Colon and Rectal Surgeons regarding the indication for elective resection, we suggest a case-by-case decision with respect to early or delayed elective resection is similarly appropriate in patients with complicated diverticulitis.

COMMENTS

Background

The optimal management of complicated diverticulitis without perforation remains unclear. The trial, which was the first to include only patients with complicated diverticulitis, compares the outcomes of early elective resection with delayed elective resection after initial antibiotic treatment.

Research frontiers

It is most common for patients to be treated conservatively with parenteral nutrition and antibiotics for 5-10 d initially, followed by a period of recuperation and re-admission to hospital, typically 6-8 wk later, for a delayed elective resection of the sigmoid colon to be performed. Alternatively patients may be treated for approximately 2-5 d with parenteral nutrition and antibiotics, with an early elective resection performed during the same hospital stay. A correlation between the risk of recurrence and the severity of the initial episode has been described. In complicated diverticulitis characterized by phlegmon or a pericolic abscess, a more aggressive approach is recommended because of the high risk of recurrence, and the high rate of severe complications, such as perforation, with a potentially fatal outcome. There is limited data concerning the timing of elective resection.

Innovations and breakthroughs

This trial, which was the first to include only patients with complicated diverticulitis, compares the outcomes of early elective resection with delayed elective resection after initial antibiotic treatment. Overall, early elective resection for complicated, non-perforated diverticulitis is shown to be a suitable alternative to delayed elective resection after 6-8 wk, with additional beneficial socioeconomic effects.

Applications

To investigate the outcomes of early and delayed elective resection after initial antibiotic treatment in patients with complicated diverticulitis.

Peer review

The article reports interesting information on the surgical treatment of complicated diverticulitis. The authors report an adequate series and proper statistical analysis. The authors' comments are very interesting and of great help for the surgeons. The article deserves publication. It is an interesting topic with an analysis on the correct management of a very common disease.

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BRIEF ARTICLE

Serum Bcl-2 concentrations in overweight-obese subjects with nonalcoholic fatty liver disease

Giovanni Tarantino, Francesco Scopacasa, Annamaria Colao, Domenico Capone, Marianna Tarantino, Ernesto Grimaldi, Silvia Savastano

Giovanni Tarantino, Department of Clinical and Experimental Medicine, Federico Ⅱ University Medical School of Naples, 5, 80131 Naples, Italy
Francesco Scopacasa, Ernesto Grimaldi, Department of Biochemistry and Medical Biotechnology, Federico Ⅱ University Medical School of Naples, 5, 80131 Naples, Italy
Annamaria Colao, Silvia Savastano, Department of Molecular and Clinical Endocrinology and Oncology, Endocrinology Unit, Federico Ⅱ University Medical School of Naples, 5, 80131 Naples, Italy
Domenico Capone, Department of Neurosciences, Unit of Clinical Pharmacology, Federico Ⅱ University Medical School of Naples, 5, 80131 Naples, Italy
Marianna Tarantino, Department of Biomorphological and Functional Sciences, Federico Ⅱ University Medical School of Naples, 5, 80131 Naples, Italy
Author contributions: Tarantino G conceived the study, analyzed statistical data, and wrote the manuscript; Savastano S, Colao A and Capone D critically revised the paper; Tarantino M and Tarantino G performed ultrasonography; Grimaldi E and Scopacasa F performed laboratory analyses.
Correspondence to: Giovanni Tarantino, MD, Department of Clinical and Experimental Medicine, Federico Ⅱ University Medical School of Naples, Via Sergio Pansini, 5, 80131 Naples, Italy. tarantin@unina.it
Telephone: +39-81-7462024 Fax: +39-81-5466152
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Abstract

AIM: To shed some light on the relationship between anti-apoptotic serum Bcl-2 concentrations and metabolic status, anthropometric parameters, inflammation indices, and non-alcoholic fatty liver disease severity were investigated in 43 young individuals with fatty liver (FL) and 41 with nonalcoholic steatohepatitis (NASH).

METHODS: Circulating levels of Bcl-2 were detected in 84 patients with ultrasonographic findings of "bright liver" and/or hyper-transaminasemia of unknown origin and/or increase in γ-glutamyl-transpeptidase (γ-GT) strictly in the absence of other acute or chronic liver disease, whose age was not advanced, who gave consent to liver biopsy and were then divided on the basis of the histological results into two groups (43 with FL and 41 with NASH). Twenty lean subjects, apparently healthy and young, were chosen as controls.

RESULTS: Serum Bcl-2 concentrations were significantly higher in the FL group than in the NASH group. Insulin resistance and γ-GT activity were significantly higher in NASH subjects. Apoptotic hepatocytes were significantly more numerous in NASH patients. NASH patients presented with larger spleens and augmented C-reactive protein (CRP) concentrations than healthy subjects. Steatosis grade at histology was similar in both NASH and FL populations. The number of apoptotic cells was significantly related to anti-apoptotic Bcl-2 protein values in FL patients. Bcl-2 serum levels positively correlated to body mass index (BMI) values ($P \leqslant 0.0001$) but not to age of the population. Triglycerides/HDL ratio correlated well to waist circumference in males ($P = 0.0008$). γ-GT activity was associated with homeostatic metabolic assessment (HOMA) ($P = 0.0003$) and with serum ferritin ($P = 0.02$). Bcl-2 concentrations were not related to either spleen size or CRP values. NASH patients presented a weak negative correlation between lobular inflammation and Bcl-2 levels. A prediction by low values of serum Bcl-2 towards a greater presence of metabolically unhealthy overweight/obese patients (MUOs) was evidenced. HOMA, BMI and uric acid, in that sequence, best predicted serum Bcl-2 concentrations.

CONCLUSION: MUOs could be detected by Bcl-2 levels. By favoring the life span of hepatocytes, and enhancing triglyceride formation, the anti-apoptotic process inhibits free fatty acids toxicity in FL.

Key words: Bcl-2; Nonalcoholic fatty liver disease; Metabolically unhealthy overweight/obese

Peer reviewer: Andrew Seng Boon Chua, MD, Department of Gastroenterology, Gastro Centre Ipoh, 1, lorong Rani, 31, lebuhraya Tmn Ipoh, Ipoh Garden South, IPOH 30350, Malaysia

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INTRODUCTION

High serum levels of triglycerides/free fatty acids (FFA) and insulin resistance (IR) are features of non-alcoholic fatty liver disease (NAFLD), an additional manifestation of the metabolic syndrome[1]; the former is extremely common in patients suffering from overweight/obesity (O/O), and ranges from fatty liver (FL) to nonalcoholic steatohepatitis (NASH) and liver cirrhosis. In determining NASH, a role is thought to be played by FFAs that directly engage the core apoptotic machinery by activating the proapoptotic protein Bax, in a c-jun N-terminal kinase-dependent manner[2]. Moreover, increased apoptosis in liver specimens from NASH patients is associated with iron overload[3], which clearly correlates with IR and inflammation. Indeed, interesting approaches to study pro-apoptotic and anti-apoptotic processes at the level of the liver are the use of *in vitro* models to explore the molecular events, tissue expression and circulating levels of the single factors. The first procedure partially limits the translation to the human scenario, mainly when moving from FL to NASH, because NAFLD reflects the long-lasting, complex dynamics orchestrated by various cells, such as adipocytes, β cells, muscle cells, macrophages and natural killer T cells. Apoptosis being a composite system regulated in a complex fashion with the contribution of many different factors, the expression of a single protein in liver specimens gives only a snapshot of a process that is hardly static. In favor of the serum concentrations stands the fact that either the stimulatory or the counter-regulatory circuits might eventually determine the net global effect of a given component. In conditions of chronic inflammation or oxidative stress, as for metabolic syndrome (MS), it is conceivable that the entire network is in a long-term persistent state of activation leading to increased circulating levels. In the serum of NAFLD patients, cytochrome c, an indicator of apoptosis-related mitochondrial damage, has been scarcely detected[4]. Among the anti-apoptotic factors, circulating levels of soluble Fas have been studied in some acute and chronic liver diseases[5,6]. The expression of Bcl-2 in the liver tissue of NAFLD patients has been evaluated by real-time PCR or, alternatively, by immuno-histochemical staining, highlighting surprisingly different patterns. In fact, in patients with NASH the expression of the Bcl-2 protein in liver specimens is mildly increased[7]. In contrast, in rats with high-fat diet-induced NASH, the hepatic expression of Bcl-2 did not differ from that of animals on control diet[8]. Similarly, no significant difference in the expression of Bcl-2 in NASH and biopsy-proven FL patients was observed[2]. Other authors have shown that the tissue expression of this anti-apoptotic protein diminished with the advancement of liver steatosis[9]. Recent data have shown that NAFLD patients with elevated gamma glutamyl-transpeptidase (γ-GT) activity have significantly higher Bcl-2 staining levels compared to patients evidencing normal γ-GT[10]. We firstly hypothesized that serum Bcl-2 levels reflect the steady state of this anti-apoptotic protein. A recent study, showing that urinary Bcl-2 concentrations are elevated in females suffering from ovarian cancer regardless of their creatinine levels or age[11], supports this hypothesis. A further confirmation is given by the fact that decreased apoptosis is in part associated with increased serum Bcl-2 levels in patients with melanoma[12]. Secondly, novel evidence suggests that low-grade chronic inflammation, in which spleen volume plays a key role[13], is the cause of IR in O/O patients, whose serum levels of soluble Fas interfere with the apoptotic pathway[14]. Finally, serum levels of Bcl-2 and cellular oxidative stress have already been studied in patients with viral hepatitis, showing a close link[15]. On the other hand, a "metabolically benign obesity" which is not accompanied by IR has recently been postulated to exist in humans[16-18], although this hypothesis has been challenged by some authors[19]. As a consequence, some researchers have started considering NAFLD, independent of its severity, as a divide between metabolically healthy O/O and metabolically unhealthy O/O (MUO)[20] individuals. Against this background, we felt the need to shed some light on the relationship between anti-apoptotic serum Bcl-2 concentrations and metabolic status, anthropometric parameters, inflammation indices, such as C-reactive protein (CRP) and spleen volume[13], as well as NAFLD severity in the vast complex of O/O and MS, in young adults and middle aged individuals.

MATERIALS AND METHODS

This research was performed by initially screening 178 consecutive subjects referred to our out-patient Metabolic Unit with established (at least 4 years) O/O, from February 2006 to December 2009. The study was carried out according to the principles of the Declaration of Helsinki and informed written consent was obtained from each patient.

Enrollment criteria

Out of the 178 initial participants 15 were excluded due to marked intestinal meteorism which made it difficult to perform abdominal ultrasound (first step to screening NAFLD), 16 patients because they had undergone steroid therapy (seven for bronchial asthma, five for rheumatoid arthritis, two for neuritis and two for inflammatory bowel disease), and 28 because they had received one or more drugs (i.e., aspirin, metformin, statins and

Table 1 Clinical characteristics and laboratory data of the study population (mean ± SD)

	Diagnosis			
	H	FL	NASH	*P* value
Subjects (*n*)	21	43	41	
Females	11	30	24	0.95[1]
Age (yr)	19.1 ± 3.8	31.4 ± 13.9	35.6 ± 13	< 0.001[2]
Overweight	0	3	3	0.7[1]
Obesity 1st grade	0	13	9	0.5[1]
Obesity 2nd grade	0	9	6	0.6[1]
Obesity 3rd grade	0	18	24	0.85[1]
MS	0	24	36	0.001[5]
MUO	0	34	39	0.06[1]
BMI				
F	23.8 ± 1.13	38.8 ± 8.24	37.8 ± 6.6	< 0.001[2]
M	23.7 ± 0.6	38.6 ± 4.4	45.7 ± 10.5	0.01[3]
WC (cm)				
F	86.3 ± 1.4	114.8 ± 15.7	117.3 ± 17.7	0.001[2]
M	93.4 ± 2.3	121.2 ± 11.3	131.1 ± 15.1	< 0.001[3]
HOMA [median (range)]	1.77 (1.03-6)	2.3 (1.4-7.3)	4.1 (0.6-10.2)	< 0.0001[3]
γ-GT U/L [median (range)]	33.1 (23.4-44.8)	40.5 (26-80)	54 (22.5-397)	< 0.0001[9]
ALT U/L [median (range)]	27 (18-34)	36 (14-133)	40 (11-153)	0.004[6]
Uric acid	3.5 ± 0.4	5.1 ± 1.45	5.4 ± 1.4	< 0.001[2]
Ferritin				
F	84.6 ± 10.3	139.6 ± 76.	149.2 ± 69	0.001[2]
M	166.8 ± 37.4	218.5 ± 121.	280 ± 103	0.01[4]
Triglycerides	85 (66-121)	96 (51-290)	117 (51-386)	0.16[10]
HDL (mg/mL)				
F	63.6 ± 7.97	51.8 ± 9.38	50 ± 11.15	0.001[2]
M	55.3 ± 8.4	50.8 ± 7.9	44 ± 4.4	< 0.001[8]
Triglycerides/HDL [median (range)]				
F	1.32 (1.16-1.49)	2.19 (1.18-6.5)	1.51 (1.07-7.6)	0.004[6]
M	1.635 (1.39-1.75)	1.67 (1.25-2.7)	2.65 (1.25-8.8)	0.03[7]

[1]χ^2 test between fatty liver (FL) and non alcoholic steato hepatitis (NASH); [2]analysis of variance (ANOVA) [significance between healthy (H) and FL and between H and NASH]; [3]ANOVA (significance between H and FL, between H and NASH and also between FL and NASH); [4]ANOVA (significance between H and NASH); [5]Pearson χ^2 (significance between FL and NASH); [6]Kruskal-Wallis test (significance between H and FL and between H and NASH); [7]Kruskal-Wallis test (significance between NASH and FL and between NASH and H); [8]ANOVA (significance between NASH and FL and between NASH and H); [9]Kruskal-Wallis test (significance between H and FL, between H and NASH and also between FL and NASH); [10]Kruskal-Wallis test. F: Females; M: Males; *n*: Number; H: Healthy; FL: Fatty liver; NASH: Non alcoholic steato hepatitis; ALT: Alaninamino transferase; γ-GT: γ-glutamil transferase; HDL: High density lipoprotein; HOMA: Homeostatic metabolic assessment; BMI: Body mass index; MS: Metabolic syndrome; MUO: Metabolically unhealthy overweight/obese; WC: Waist circumference.

fibrates) that may have altered their laboratory data. Fourteen others were excluded because of the presence of hepatic co-morbidities in their medical history (HCV infection, alcohol abuse), and finally twenty subjects were considered drop-outs, because they refused to undergo full laboratory-instrumental examinations. Eighty-four of the 119 patients, with ultrasound findings of "bright liver" and/or hyper-transaminasemia of unknown origin and/or increase in γ-GT strictly in the absence of other acute or chronic liver disease, whose age was not advanced (Table 1), gave consent to liver biopsy and were then divided on the basis of the histological results (see below) into two groups (43 with FL and 41 with NASH) that formed the final study population. Twenty lean subjects, apparently healthy and young, were chosen as controls to avoid any confounding factor with circulating levels of Bcl-2 (see below).

Exclusion criteria

Any viral, autoimmune, metabolic liver disease (Wilson disease, hemochromatosis or antitrypsin deficiency) was ruled out by using appropriate testing, following the accepted diagnostic guidelines. Celiac disease was excluded by estimates of IgA anti-tissue transglutaminase antibodies. Alcohol abuse was ruled out, according to the DSM-IV diagnostic criteria, by means of screening tests such as MAST (Michigan alcohol screening test) and CAGE (Cut down, Annoyed, Guilty, and Eye opener), as well as random tests for blood alcohol concentration and the use of a surrogate marker, e.g., Mean Corpuscular Volume. Patients on antihypertensive drugs maintained a balanced therapeutic regimen throughout the study.

Metabolic profile

The degrees of obesity was established on the basis of BMI cut-off points of 25-29.9, 30-34.9, 35-39.9 and > 40 kg/m^2, respectively. Visceral obesity was identified by measuring waist circumference (WC) at the midpoint between the lower border of the rib cage and the iliac crest. MS was defined according to the revised Adults Treatment Panel Ⅲ (2001), and three or more criteria were considered: plasma glucose concentration of at least

100 mg/dL, WC > 88 cm, serum HDL concentration < 50 mg/dL for women and < 40 mg/dL for men, blood pressure of at least 130/85 mm Hg, and serum triglyceride concentration of at least 150 mg/dL. IR status was determined by the Homeostatic metabolic assessment (HOMA), which was assessed by the formula: fasting insulin (μU/mL) × fasting glucose (mg/dL)/405[21]. As a stringent measure of IR, a value of HOMA > 2 was introduced, according to the cut-off value of surrogate measures of IR for MS in non-diabetic adults[22]. Moreover, as the repeated HOMA measurements presented high within-person variability in O/O patients, HOMA values were averaged on the basis of at least five determinations to avoid misclassification. When reporting triglycerides values, only the data of examinees who had fasted a minimum of 9 h before the morning examination were included to avoid false increases, averaging the results of at least two determinations made on different days. To avoid the high intra-individual variability the triglyceride/HDL cholesterol ratio was evaluated (see below). MUO subjects were categorized in such a way in the presence of IR, MS, high levels of serum uric acid (UA),[23] and if their triglyceride/HDL cholesterol ratio was ⩾ 1.65 (men) or ⩾ 1.32 (women)[24].

Ultrasound evaluation

Ultrasonographic (US) measurements were performed by two operators, using a general electric vivid system. Spleen longitudinal diameter (SLD) was chosen to evaluate spleen volume and was carried out by postero-lateral scanning. Maximum length (the optically greatest overall longitudinal dimension obtained from one of the two poles) and cranio-caudal length (the optically maximal transversal dimension intercepting one of the two poles) were measured; the resulting values were then averaged, since the two measurements do not always coincide. The classification of "bright liver" or hepatic steatosis (HS) was based on the following scale of hyper-echogenicity: 0 = absent, 1 = light, 2 = moderate, 3 = severe, pointing out the difference between the densities of the liver and the right kidney. Technically, echo intensity can be influenced by many factors, particularly by gain intensity. To avoid confounding factors that could modify echo intensity and thus bias comparisons, the mean brightness levels of both the liver and the right kidney cortex were obtained on the same longitudinal sonographic plane. The levels of brightness of the liver and the right kidney were calculated three times directly from the frozen images.

Blood pressure measurements

Systolic/diastolic blood pressure was the average of three consecutive readings taken by the physician during the day, during the usual practice hours, after subjects had rested for 5 min in the sitting position.

Laboratory data

Serum triglycerides, HDL, basal insulin, ALT, γ-GT, UA, glycemia and ferritin were performed by in-house standard procedures. Hs-CRP values were determined by ELISA test, with reference values between 0.3 and 8.6 mg/L in healthy men and between 0.2 and 9.1 mg/L in healthy women (BioCheck, Inc CA, United States). Bcl-2 was determined using a Human Bcl-2 ELISA Kit (producer Bender MedSystems, Austria, EU) with a coefficient of variation ranging from 5.1 to 17.7, a sensitivity of 2.5 ng/mL, an overall intra-assay coefficient of 8.6 and a reference interval calculated on 21 healthy subjects (Table 1) by the non-parametric percentile method (CLSI C28-A3) of 7.4-22.6 ng/mL.

Liver histology

The diagnosis of NASH was made when three of the following five criteria were proven by liver biopsy: steatosis, hepatocyte ballooning, lobular inflammation, portal inflammation and mallory bodies. Data on Mallory bodies were collected as inclusion criteria to pinpoint the accuracy of diagnosis, but they were not used for evaluation. A 4-point scale for each of the four following criteria resulted in a sum score ranging from 0 to 12. Specifically, the scores were: Steatosis: 0 = None; 1 = Up to 33% of acini, mainly macrovesicular; 2 = 34%-66% of acini, commonly mixed steatosis; 3 = Over 66% of acini (panacinar), commonly mixed steatosis; Hepatocyte ballooning: 0 = None; 1 = Occasional in zone Ⅲ; 2 = Obvious in zone Ⅲ; 3 = Marked, predominantly in zone Ⅲ; Lobular inflammation: 0 = None; 1 = Scattered neutrophils, occasional mononuclear cells, 1 or 2 foci per 20 × objective; 2 = Neutrophils associated with ballooned hepatocytes, mild chronic inflammation, 3 or 4 foci per 20 × objective; 3 = Acute and chronic inflammation, neutrophils may concentrate in zone Ⅲ, over 4 foci per 20 × objective. Portal inflammation: 0 = None; 1 = Mild, some portal areas; 2 = Mild to moderate, most portal areas; 3 = Moderate to severe, most portal areas. Fibrosis was staged as follows: Stage 0 = None; Stage 1 = Zone Ⅲ perivenular, perisinusoidal (pericellular); Stage 2 = Stage 1 changes plus periportal fibrosis; Stage 3 = Bridging fibrosis; Stage 4 = Cirrhosis. Biopsy samples were taken within eight weeks prior to inclusion. FL was diagnosed when only steatosis was present, using the same grades previously reported[1-3]. The biopsy sample had to be at least 1.5 cm long with a minimum diameter of 0.8 mm. Inclusion of a NASH patient in the study required a sum score of at least 6 points.

Apoptosis detection

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. We used the Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay (Invitrogen). This method utilizes a dUTP modified with an alkyne, a small, bio-orthogonal functional group that enables the nucleotide to be more readily incorporated by TdT than other modified nucleotides, including BrdUTP, biotin-dUTP or fluo-

rescein-dUTP. Detection is based on a click reaction, i.e., a copper catalyzed reaction between an azide and alkyne.

Cells displaying TUNEL-labeled fluorescent nuclei were quantified by counting the number of positive cells per high-power field. A total of 10 high-power fields were analyzed for each patient with excitation and emission wavelengths of 380 and 430 nm, respectively, using an inverted laser scanning confocal microscope (Carl Zeiss) equipped with a ×40 NA 1.4 lens. Data are expressed as the number/mm^2 of TUNEL-positive cells.

Statistical analysis

Age data, derived from a normally distributed population [Kolmogorov-Smirnov test (K-S), $P = 0.15$], ferritin (K-S, $P = 0.6$), BMI (K-S, $P = 0.2$), WC (K-S, $P = 0.18$), SLD (K-S, $P = 0.52$), UA (K-S, $P = 0.105$) are given as mean plus SD. Variables not normally distributed, such as triglycerides (K-S, $P = 0.0013$), triglycerides/HDL (K-S, $P \leqslant 0.0001$), HOMA (K-S, $P = 0.0016$), ALT (K-S, $P = 0.0001$), CRP (S-W, $P = 0.001$) are expressed as median (range). Grades at histology and US were considered ordinals and managed in the same way. The difference in medians was assessed by the Mann-Whitney test for independent samples. One-way analysis of variance (ANOVA) was used to test the difference between the means of several subgroups of a variable (multiple testing). If the ANOVA test was positive ($P < 0.05$) then a Student-Newman-Keuls test for pairwise comparison of subgroups was performed. The ANOVA K-W test was used to calculate the differences between the medians of several subgroups of a variable. If K-W was positive ($P < 0.05$) then a test for pairwise comparisons of subgroups according to Conover was adopted. When adjusted for a covariate the ANOVA was transformed into ANCOVA and the significance was expressed as F-ratio. The Two-Way Tables cross-tabulated one categorical row variable with one categorical column variable and the significance was set by the Pearson χ^2. When cross-tabulation was stratified for another dichotomous variable, the Mantel-Haenszel χ^2 was carried out. Tracking the degree of association between single parameters, Spearman's coefficient of rank correlation (rho) for non uniform intervals was used. The Pearson's coefficient (r) was employed to analyze the correlation between data derived from a normally distributed population. To predict a binary variable, the logistic regression (Enter Method), with relative Odds ratios and 95% confidence intervals (CI), was employed utilizing data from an independent variable. To assess the independent effect of a quantitative variable on the prediction of the values of another variable, the linear regression analysis (least squares) was used, evaluating the standardized coefficient β (β) and t, which is a measure of the precision with which the regression coefficient is measured. A tolerance of less than 0.20 and/or a variance inflation factor of 5 and above indicated a multicolinearity problem. When confronted with the question of how accurate a parameter was in identifying MUO cases, the discrimination was evaluated using receiver operating characteristic curve analysis (ROC), expressed as area under the ROC (AUC). A criterion or cut-off was set and then sensitivity, specificity and positive likelihood ratio were estimated. Negative predictive value with the Bayes method was also calculated. The factor analysis was applied to detect the structure in the relationships among variables, selecting a subset of variables having the highest correlations with the principal component factors. The Cattell Scree plot, with relative eigen values, was performed to screen the real factors. Extraction of the main components amounted to a variance maximizing (varimax) the rotation of the original variable space. The critical value was calculated by doubling Pearson's correlation coefficient for 1% level of significance (5.152)/square root of patients (84) minus 2, i.e., 0.568. The concordance correlation coefficient (ρ_c), which measures precision and accuracy, was adopted to evaluate the degree of pair observations at US. Statistical analysis was performed operating on Systat 13 (Richmond, CA, United States) and MedCalc Version 11.4® (Frank Schoonjans) software packages.

RESULTS

Prevalence

The number of individuals affected by being overweight, or by 1st, 2nd and 3rd grade obesity was comparable in NASH and FL populations and was well-balanced for gender. NASH patients were characterized by older age and a larger percentage of MUO cases, having the largest number of MS among males (Mantel-Haenszel Chi-Square $P = 0.005$), a greater BMI and a larger WC in males (ANOVA, $F = 55.5$, $P = 0.0001$ and $F = 59.5$, $P = 0.0001$, respectively). Both insulin resistance and γ-GT activity were significantly higher in subjects diagnosed with NASH. Raised values of ALT activity were hardly detected in NASH and FL patients. Men with NASH were affected by hyperferritinemia and had the lowest levels of HDL and the highest triglycerides/HDL ratio. Serum uric acid was increased in FL and NASH patients without any difference between the two groups (Table 1). Apoptotic hepatocytes were significantly more numerous in NASH patients. NASH patients presented with larger spleens and augmented CRP concentrations compared with healthy subjects. In both populations of NASH and FL the steatosis grade at histology was similar, so was the steatosis severity at US. Serum concentrations of Bcl-2 were significantly higher in the FL group than in the NASH group and patients of both groups showed increased values compared to healthy subjects (Table 2 and Figure 1). The significance did not change after adjusting the values of Bcl-2 for γ-GT activity, with only the diagnosis being significant (ANCOVA, $F = 14.9$, $P = 0.0001$).

Associations and prediction

The number of apoptotic cells was significantly related to the anti-apoptotic Bcl-2 protein values (rh0 = 0.43, $P = 0.003$) in FL patients but not in NASH ones (rh0 = 0.17, $P = 0.27$). Bcl-2 serum levels correlated well with the BMI values of the whole population (rh0 = 0.55, $P = <$

Table 2 Serum concentrations of the anti-apoptotic protein Bcl-2, histology features and inflammation parameters of the patients enrolled

	Diagnosis			
	H	FL	NASH	*P* value
Subjects (*n*)	21	43	41	
Bcl-2 (ng/mL)	2.5 (1.4-13)	25 (2.5-168)	14 (2.5-89)	< 0.001[3]
Hepatocyte ballooning grade	NA	NA	2 (1-3)	NA
Steatosis score	NA	2 (1-3)	2 (1-3)	0.49[2]
Lobular inflammation grade	NA	NA	2 (1-3)	NA
Portal inflammation grade	NA	NA	1 (1-3)	NA
Fibrosis score	NA	NA	1 (1-2)	NA
Apoptotic cells (n/mm^2)	NA	290 (120-900)	780 (110-1650)	< 0.0001[2]
HS at US grade	NA	3 (0-3)	3 (0-3)	0.064[2]
CRP (mg/L)	0.9 (0.4-5.4)	3.9 (0.1-21.1)	5 (0.1-27)	0.0001[1]

[1]Kruskal-Wallis test [significance between healthy (H) and fatty liver (FL) and between H and non alcoholic steato hepatitis (NASH)]; [2]Mann-Whitney test; [3]Kruskal-Wallis test (significance between H and FL, between H and NASH and also between FL and NASH). *n*: Number; H: Healthy; FL: Fatty liver; NASH: Non alcoholic steato hepatitis; NA: Not applicable; HS: Hepatic steatosis at ultrasound; US: Ultrasound; SLD: Spleen longitudinal diameter at ultrasound; CRP: C-reactive protein.

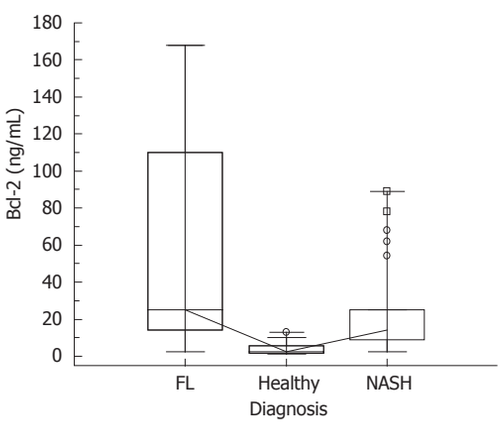

Figure 1 Serum concentrations of Bcl-2 in the whole population. Kruskal-Wallis test (significance between healthy and fatty liver (FL), between healthy and non alcoholic steato hepatitis (NASH) and also between FL and NASH); the highest median value was found in the FL group, $P \leqslant 0.001$.

0.0001). The correlation between serum concentrations of Bcl-2 and age was not significant (rh0 = 0.14, $P = 0.15$). Triglycerides/HDL ratio correlated well with WC in males (Pearson's $r = 0.51$, $P = 0.0008$). γ-GT activity was associated with HOMA values (rh0 = 0.345, $P = 0.0003$), as was γ-GT with serum ferritin (rh0 = 0.22, $P = 0.02$). Bcl-2 concentrations were not related with either SLD or CRP values (rh0 = 0.10 and 0.13 with $P = 0.3$ and $P = 0.2$, respectively). A non significant correlation was found between lobular inflammation and Bcl-2 levels in NASH patients (rh0 = -0.21, $P = 0.2$).

As to age, at linear regression, advancing years did not completely predict Bcl-2, β = -0.19, $t = -2$, $P = 0.053$ (Figure 2A). UA predicted Bcl-2 values well, β = 0.35, $t = 3.8$, $P = 0.0002$ (Figure 2B).

At multiple regression analysis, HOMA, BMI and UA, in this sequence, best predicted Bcl-2 serum concentrations, as higher IR corresponded to lower Bcl-2 values, whereas considerable BMI and elevated amount of UA matched with higher Bcl-2 serum concentrations (Table 3). A sufficient prediction by low serum Bcl-2 values towards a higher rate of MUOs was evidenced in logistic regression, OR = 0.98, CI 0.97-0.995, $P = 0.004$. Obviously, HOMA alone did not predict the status of MUO, OR 1.38, CI 0.87-2.2.

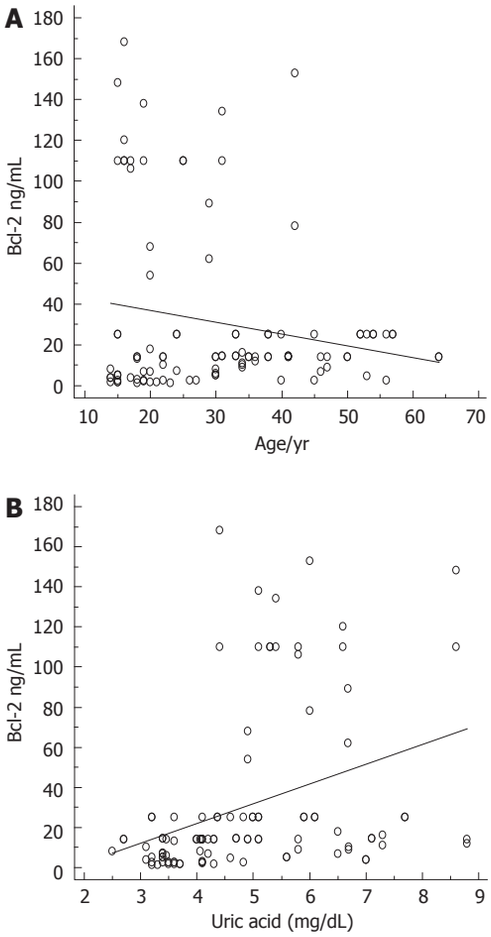

Figure 2 Prediction of Bcl-2 serum levels by age and uric acid. A: Prediction of antiapoptotic Bcl-2 protein serum concentrations by age, β = -0.19, $t = -2$, $P = 0.0527$; B: Prediction of antiapoptotic Bcl-2 protein serum concentrations by uric acid, β = 0.35, $t = 3.8$, $P = 0.0002$.

Insulin resistance was closely related to inflammation and lipid asset; apoptosis did not seem related to age (Table 4 and Figure 3). The concentrations of serum Bcl-2 were not predicted by the number/mm^2 of TUNEL-positive cells in NASH patients (β = 0.013, $t = 0.13$, $P = 0.9$, Figure 4).

Reliability

Grades of hepatic steatosis at US correlated with those at histology (rh0 = 0.45, $P \leqslant 0.0001$). The AUC of serum Bcl-2 only modestly predicted MUOs, i.e., 0.65%, CI 0.53 to 0.75, with a sensitivity of 87% and a specificity of 50%, a positive likehood ratio of 1.73 using as cut-off 106 ng/mL. The negative predictive value was 94% on the basis of a disease (MS) prevalence in the population

Table 3 Prediction of anthropometric parameters, apoptosis detection and laboratory data by Bcl-2 concentrations

Effect	β	*t*	*P* value	Tolerance
HOMA	-0.505	-4.27	0.000	0.440
HDL	0.22	1.4	0.16	0.242
WC	0.25	1.7	0.09	0.288
BMI	0.35	2.65	0.009	0.344
Triglycerides	-0.07	-0.17	0.86	0.040
Triglycerides/HDL	0.22	0.5	0.63	0.029
Uric acid	0.24	2.1	0.03	0.509
Ferritin	-0.15	-1.8	0.08	0.853
Apoptosis in NASH	0.013	0.13	0.9	1

NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; HOMA: Homeostatic metabolic assessment; HDL: High density lipoprotein; triglycerides and triglycerides/HDL were considered variables affected by collinearity; WC: Waist circumference; β: Beta, standardized coefficient; *t*: A measure of the precision with which the regression coefficient is measured.

Table 4 Hidden relationships highlighted by factor analysis

Factors	1	2
Bcl-2	-0.105	0.672
Age	-0.348	-0.424
Apoptosis	0.293	-0.425
MS	0.606	0.011
BMI	0.564	0.528
WC	0.604	0.467
HOMA	0.781	-0.211
HDL	-0.715	0.107
Triglycerides	0.521	-0.471
Triglycerides /HDL	0.698	-0.468
Uric Acid	0.554	0.520
Ferritin	0.279	0.064
ALT	0.545	0.097
Γ-GT	0.074	-0.384
CRP	0.717	-0.274
SLD	-0.202	0.282
HS at US	0.471	0.255
Steatosis	0.293	0.265

Insulin resistance is closely related to inflammation and lipid asset; apoptosis does not seem related to age. Percent of total variance explained for factor 1:26; for factor 2:14; the critical value is 0.568. HS: Hepatic steatosis; US: Ultrasound; SLD: Spleen longitudinal diameter at ultrasound; CRP: C-reactive protein; BMI: Body mass index; MS: Metabolic syndrome; ALT: Alaninamino transferase; γ-GT: γ-glutamyl transferase; HDL: High density lipoprotein; HOMA: Homeostatic metabolic assessment; WC: Waist circumference.

of 20%. The intra-inter-observational reproducibility of the sonographic estimations for bright liver was high, with a ρ_χ of 0.93 and 0.89, respectively.

DISCUSSION

Medical reports emphasize that MS, of which NAFLD is a further expression, is a major health problem in western countries. This research was conducted to establish whether serum Bcl-2 concentration could help clarify the role of the anti-apoptotic process in this common disease, not to detect a further marker to diagnose NASH.

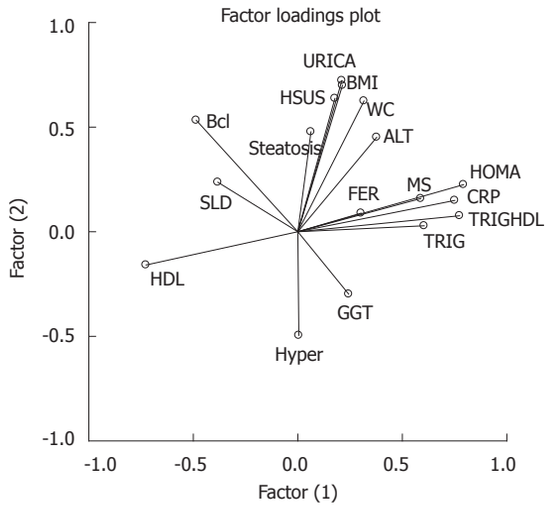

Figure 3 Variables clustered around factors. Homeostatic metabolic assessment, C-reactive protein, triglycerides, triglycerides/high density lipoprotein ratio, ferritin and metabolic syndrome clustered around factor 1. HDL: High density lipoprotein. WC: Waist circumference; BMI: Body mass index; HOMA: Homeostatic metabolic assessment; HDL: High density lipoprotein; GGT: γ-glutamyl-transpeptidase; TRIG:Triglycerides; TRIGHDL: Triglycerides/high density lipoprotein; CRP: C-reactive protein; MS: Metabolic syndrome; FER: Ferritin; ALT: Alaninamino transferase; BMI: Body mass index; URICA: Uric acid; HSUS: Hepatic steatosis at ultrasound; SLD: Spleen longitudinal diameter.

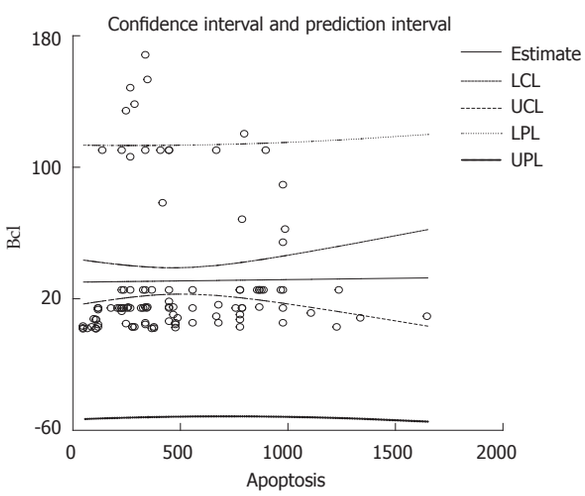

Figure 4 The concentration of serum Bcl-2 was not predicted by the number/mm2 of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-positive cells. LCL: Low confidence limit; UCL: Upper confidence limit; LPL: Low prediction limit; UPL: Upper prediction Limit.

The main finding was that low HOMA values predicted high Bcl-2 levels. Furthermore, high levels of this anti-apoptotic protein were found in patients with FL, which is also characterized by fewer apoptotic cells than the more severe form, i.e. NASH. First of all, to avoid a possible bias[25], the authors stress that the population's age did not affect anti-apoptotc protein Bcl-2 concentrations, as confirmed by regression equation and factor analysis. UA levels modestly prognosticate serum Bcl-2 concentrations according to previous research on serum soluble Fas concentrations, which correlated significantly with UA levels[26]. These findings indicate that the antioxidant properties of UA[27,28] are of biological importance *in vivo*.

Comparing the present results with relevant findings from other studies dealing with NASH, having found lower Bcl-2 concentrations, we support a low liver expression of Bcl-2[28]. To reinforce this finding, apoptosis is recognized as common in liver injury and may also contribute to tissue inflammation, fibrogenesis, and development of cirrhosis. The intensification of inflammation in NAFLD is accompanied by an inhibition of antiapoptotic Bcl-2[9]. Accordingly, in our study anti-apoptotic Bcl-2 protein concentrations showed an inverse trend towards lobular inflammation grades.

Contrary to current theory, hepatic steatosis appears to be a detoxification process, as FFAs are directly cytotoxic for the hepatocyte. The anti-apoptotic process, favoring the life span of hepatocytes, and enhancing triglyceride formation, inhibits FFAs toxicity. Probably this is the reason for the major concentrations of Bcl-2 in FL. On the other hand, our laboratory findings do not necessarily mirror the processes happening in liver due to the lack of correlation between apoptosis as well as inflammation and Bcl-2 levels in NASH patients. The strict association between IR, as well as BMI and Bcl-2 levels, could at least partially be explained as follows. All individuals possess a maximum capacity for adipose expansion which is determined by both genetic and environmental factors. Once the limit of adipose tissue expansion is reached, this ceases to store energy efficiently, with the subsequent accumulation of lipids in other tissues. Ectopic lipid accumulation in non-adipocyte cells causes lipotoxic insults including IR, apoptosis and inflammation. The adipose tissue expandability hypothesis states that a failure in the capacity of adipose tissue expansion, rather than obesity per se, is the key factor linking positive energy balance and MS[29]. MUOs could represent subjects who are unable to sustain the expandability of adipose tissue but are burdened by an abundant extra adipose tissue localization, for instance in liver. An alternative hypothesis suggests that a hyperleptinemic status, generally present in obese patients, might be involved. Now, since leptin reduces apoptosis, possibly via its ability to increase Bcl-2 and decrease Bax, altering the Bcl-2/Bax ratio, this status could explain the elevated concentrations of Bcl-2[30]. Some results show that IGF-1 increases mRNA levels and protein expression of antiapoptotic Bcl-2[31-33] even though, in obese subjects all the main components of the GH/IGF-1 axis might be widely variable[34]. In fact, serum IGF-1 levels inversely vary with severity of hepatic steatosis[35]. Coming back to serum concentrations of Bcl-2, a shift towards an antiapoptotic process could be protective in other areas[14]. By which mechanisms is Bcl-2 supposed to act? The endoplasmic reticulum (ER) is the main site for lipid biosynthesis in the cell. Disturbances of this critical cellular function lead to ER stress. Several recent observations suggest a role for Bcl-2 at the ER. Bcl-2 located at the ER was shown to interfere with apoptosis induction. In fact, Bcl-2 at the ER may regulate calcium flow between the ER and the mitochondria. In addition, Bcl-2 is able to interact with the endoplasmic protein Bap31, thus avoiding caspase activation at the ER. Bcl-2 may also hinder the function of ER located pro-apoptotic Bcl-2[36].

Limitations to this study are having detected a single protein in the apoptosis universe, and the use of US as first screening method for O/O. In fact, although US has acceptable sensitivity and specificity, nevertheless, it has drawbacks that include its inaccuracy in exact quantification of fat accumulation, possibly excluding patients with light hepatic steatosis. Furthermore, the sample size is apparently too small (the minimal required sample size per group with a type I error of 0.05 and a Type Ⅱ error of 0.05 for Bcl-2-analyzed as mean ± SD was calculated at 42 patients). Finally, in our study there is some overlap in the Bcl-2 values seen in NASH and FL patients, the minimum in both groups being 2.5 ng/mL(detection limit).

In conclusion, IR is strictly linked to serum Bcl-2 values. Those were higher in FL than in NASH patients suggesting a protective role of the anti-apoptotic process in liver and perhaps in other areas[37].

COMMENTS

Background

High serum levels of triglycerides/free fatty acids and insulin resistance are features of non-alcoholic fatty liver disease, an additional manifestation of the metabolic syndrome; the former is extremely common in patients suffering from overweight/obesity, and ranges from fatty liver to nonalcoholic steatohepatitis and liver cirrhosis. In determining nonalcoholic steatohepatitis, a role is thought to be played by free fatty acids that directly engage the core apoptotic machinery. Moreover, increased apoptosis in liver specimens from nonalcoholic steatohepatitis patients is associated with iron overload, which clearly correlates with insulin resistance and inflammation. Obviously, there is a certain balance between apoptosis and anti-apoptosis in determining the net effect on hepatocytes survival.

Research frontiers

The authors hypothesized that serum BCL-2 levels reflect the steady state of this anti-apoptotic protein.

Innovations and breakthroughs

The expression of Bcl-2 in the liver tissue of non-alcoholic fatty liver disease patients has previously been evaluated by real-time PCR or, alternatively, by immuno-histochemical staining, highlighting surprisingly different patterns. Serum Bcl-2 increase is a likely response to the apoptotic process to improve survival of hepatocytes. If the response to the metabolic injury was good (increased serum levels of Bcl-2) probably the more severe form. i.e., non-alcoholic steatohepatitis would not develop.

Applications

Metabolically unhealthy obese/overweight subjects could be further detected by Bcl-2 levels. By favoring the life span of hepatocytes, and enhancing triglyceride formation, the anti-apoptotic process probably inhibits free fatty acids toxicity in fatty liver.

Terminology

Bcl-2 (B-cell lymphoma 2) is the founding member of the Bcl-2 family of apoptosis regulator proteins encoded by the *BCL2* gene.

Peer review

This is a very well carried out study and well written paper. However it needs a few clarifications from the authors.

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BRIEF ARTICLE

Impact of changing our cannulation method on the incidence of post-endoscopic retrograde cholangiopancreatography pancreatitis after pancreatic guidewire placement

Takeshi Hisa, Ryusuke Matsumoto, Masato Takamatsu, Masayuki Furutake

Takeshi Hisa, Ryusuke Matsumoto, Masato Takamatsu, Masayuki Furutake, Department of Internal Medicine, Saku Central Hospital, Nagano 384-0301, Japan
Author contributions: Hisa T wrote the paper; Matsumoto R, Takamatsu M and Furutake M contributed equally to this work.
Correspondence to: Takeshi Hisa, MD, PhD, Department of Internal Medicine, Saku Central Hospital, Usuda 197, Saku, Nagano 384-0301, Japan. thisa@r8.dion.ne.jp
Telephone: +81-267-823131 Fax: +81-267-829638
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Abstract

AIM: To clarify whether the incidence of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) after pancreatic guidewire placement (PGW) can be reduced by using a different cannulation method.

METHODS: Between April 2001 and October 2009, PGW was performed in 142 patients with native papilla to overcome difficult biliary cannulation. Our cannulation method for ERCP was changed from contrast injection (CI) using a single-lumen catheter (April 2001-May 2008) to wire-guided cannulation (WGC) using a double-lumen catheter (June 2008-October 2009). The CI protocol was also changed during the study period: in the first period it was used for routine pancreatography for detecting small pancreatic cancer (April 2001-November 2002), whereas in the second period it was not (December 2002-May 2008). In PGW with CI using a single-lumen catheter, the contrast medium in the catheter lumen was injected into the pancreatic duct. The success rate of biliary cannulation, the incidence of PEP according to the cannulation method, and the impact of CI using a single-lumen catheter on PEP in comparison with WGC using a double-lumen catheter were investigated.

RESULTS: CI with routine pancreatography, CI without routine pancreatography, and WGC were performed in 27 patients, 77 patients and 38 patients, respectively. Routine pancreatography did not contribute to the early diagnosis of pancreatic cancer in our study period. In CI without routine pancreatography and WGC, diagnostic pancreatography was performed in 17 patients and no patients, respectively. The success rate of biliary cannulation by PGW alone was 69%, and the final success rate was increased to 80.3% by the addition of consecutive maneuvers or a second ERCP. PEP occurred in 22 patients (15.5%), and the severity was mild in all cases. When analyzed according to cannulation method, the incidence of PEP was 37.0% (10/27) in the patients who underwent CI with routine pancreatography, 14.3% (11/77) in those who underwent CI without routine pancreatography, and 2.6% (1/38) in those who underwent WGC. In all patients who underwent CI using a single-lumen catheter, the incidence of PEP was 20% (21/104), which was significantly higher than that in WGC using a double-lumen catheter. In univariate and multivariate analysis, CI using a single-lumen catheter showed a high, statistically significant, odds ratio for PEP after PGW.

CONCLUSION: The practice of a cannulation method involving the use of a double-lumen catheter minimizes the CI dose administered to the pancreatic duct and reduces the incidence of PEP after PGW.

Key words: Pancreatic guidewire placement; Wire-guided cannulation; Contrast injection; Difficult biliary cannulation; Post-endoscopic retrograde cholangiopancreatography pancreatitis

Peer reviewer: Dr. Kei Ito, Department of Gastroenterology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai City 983-0824, Japan

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INTRODUCTION

Recently, it has been emphasized that the use of wire-guided cannulation (WGC) in endoscopic retrograde cholangiopancreatography (ERCP) is associated with a reduced incidence of post-ERCP pancreatitis (PEP) compared with contrast injection (CI)[1-3]. In Japan, ERCP is usually performed under CI using a single-lumen catheter and a duodenoscope with a 15-degree backward-oblique angle because this technique allows selective biliary cannulation[4]. On the other hand, pancreatic guidewire placement (PGW) is one of the rescue maneuvers used to overcome difficult biliary cannulation[5-11]; however, it carries a risk of PEP[9-11]. We changed our cannulation method from CI using a single-lumen catheter to WGC using a double-lumen catheter and investigated whether the incidence of PEP after PGW was reduced by this change.

MATERIALS AND METHODS

Patients

Between April 2001 and October 2009, 2060 ERCP procedures were performed in patients with pancreatobiliary disease at Saku Central Hospital. PGW was performed in 142 patients with native papilla, in whom CI or guidewire insertion into the pancreatic duct *via* biliary cannulation was unintentional on at least three occasions, which was defined as difficult biliary cannulation. We did not establish a maximum number of cannulation attempts. Our strategy for difficult biliary cannulation is shown in Figure 1. When PGW did not successfully achieve biliary cannulation, pancreatic stenting, transpancreatic sphincterotomy, or another maneuver was attempted. In cases in which it was not possible to access the bile duct within 30 min, the procedure was stopped.

Endoscopic procedure

Our cannulation method for ERCP was changed from CI using a single-lumen catheter (April 2001-May 2008) to WGC using a double-lumen catheter (June 2008-October 2009). The CI protocol was also changed during the study period: in the first period it was used for routine pancreatography for detecting small pancreatic cancer (April 2001-November 2002), whereas in the second period it was not (December 2002-May 2008). The procedure was performed using a duodenoscope with a 15-degree backward-oblique angle (JF230, 260V: Olympus Optical Co., Tokyo, Japan). Biliary cannulation was attempted with a single-lumen catheter (Contour: Microinvasive, Boston Scientific Corp., Natrick, MA, United States; PR-4Q-1, PR-10Q-1: Olympus Optical Co., Tokyo, Japan)

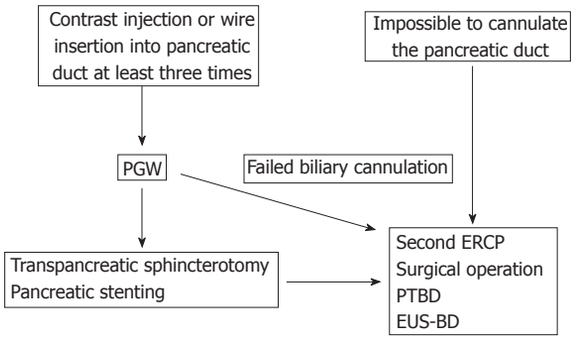

Figure 1 Our strategy for difficult biliary cannulation. PGW: Pancreatic guidewire placement; ERCP: Endoscopic retrograde cholangiopancreatography; PTBD: Percutaneous transhepatic biliary drainage; EUS-BD: Endoscopic ultrasound-guided biliary drainage.

under CI, a double-lumen catheter (MTW Endoskopie, Wesel, Germany), or a sphincterotome (CleverCut: Olympus Optical Co., Tokyo, Japan) for WGC.

In PGW, the tip of a 0.025 or 0.035 inch-guidewire (Revowave: Olympus Optical Co., Tokyo, Japan; Jagwire: Microinvasive, Boston Scientific Corp., Natrick, MA, United States) was placed between the pancreatic body and tail. In CI using a single-lumen catheter, the contrast medium in the catheter lumen was injected into the pancreatic duct during guidewire insertion, as shown in Figure 2. After PGW, biliary cannulation was attempted in the region towards the upper left of the pancreatic guidewire.

In WGC, the tip of the guidewire was passed through the catheter lumen and exposed/fixed at 2 mm from the tip of the catheter. The operator controlled the catheter and used it to adjust the axis between bile duct terminal and the catheter. In principle, the use of CI into the pancreatic duct was limited except during diagnostic pancreatography in WGC. When guidewire resistance arose during PGW, the minimum amount of contrast medium was used to identify the running direction of the main pancreatic duct to prevent the branch ducts from being injured by the tip of the guidewire.

All patients were administered a protease inhibitor drip infusion (ulinastatin, 100 000 units for 1 d) during and after the ERCP, and their serum amylase levels were measured before the ERCP and at 3 h, 6 h, and 18-24 h after. The diagnosis and grade of PEP were defined according to the international consensus criteria[12].

All procedures were performed by one operator (Hisa T), who experienced about 250-300 ERCPs per year.

Outcome

In the 142 patients subjected to PGW, the success rate of biliary cannulation, the incidence of PEP according to the cannulation method, and the impact of CI using a single-lumen catheter on PEP in comparison with WGC using a double-lumen catheter were investigated.

Statistical analysis

The patients' basic characteristics and the outcome of PGW were compared between CI using a single-lumen catheter and WGC using a double-lumen catheter using the χ^2 test. Logistic regression modeling was used to eval-

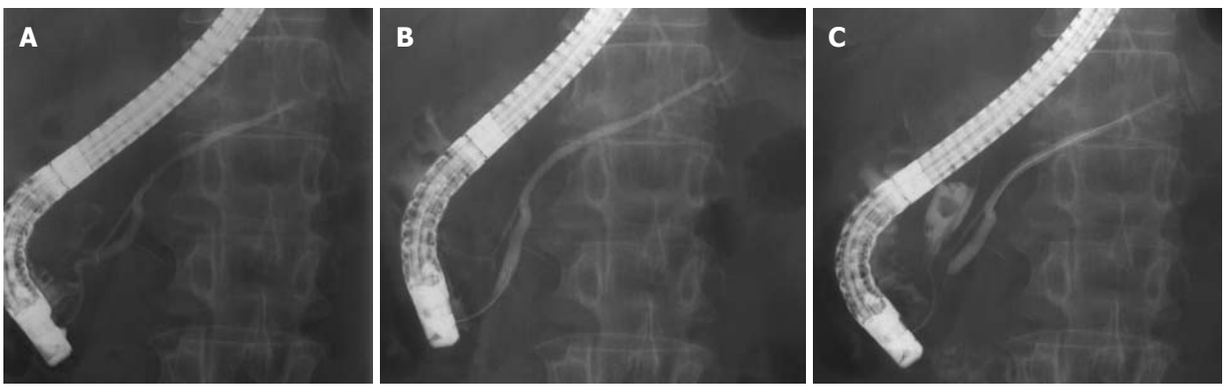

Figure 2 Fluoroscopic image taken during pancreatic guidewire placement using a single-lumen catheter. A: The injection of contrast medium into the pancreatic duct was repeated for difficult biliary cannulation; B: During pancreatic guidewire placement (PGW), the guidewire pushes contrast medium from the catheter lumen and into the pancreatic ductal system; C: PGW straightens the terminal bile duct and achieves successful biliary cannulation.

Table 1 Patient characteristics and endoscopic procedures used in pancreatic guidewire placement

	Total (n = 142)	Cannulation method		
		CI using a single-lumen catheter (n = 104)	WGC using a double-lumen catheter (n = 38)	P value
Mean age (mean ± SD)	72.7 (11.5)	68.5 (10.9)	75.3 (13.2)	
Male sex (%)	71 (50)	48 (46)	23 (61)	0.18
Periampullary diverticulum (%)	28 (20)	21 (20)	7 (18)	1.00
Diagnosis (%)				
Choledocholithiasis	55 (39)	33 (32)	22 (58)	0.01
Biliary cancer	33 (23)	25 (24)	8 (21)	0.90
Pancreatic cancer	24 (17)	20 (19)	4 (11)	0.33
Ampullary cancer	4 (2.8)	4 (3.8)	0 (0)	0.57
ERCP maneuvers (%)				
Biliary sphincterotomy	69 (49)	50 (48)	19 (50)	0.99
Transpancreatic sphincterotomy	2 (1.4)	1 (0.96)	1 (2.6)	0.87
Papillary ballon dilatation	4 (2.8)	4 (4)	0 (0)	0.63
Bile duct stone removal	20 (14)	14 (13)	6 (16)	0.91
Biliary stenting	55 (39)	36 (35)	19 (50)	0.14
Pancreatic stenting	9 (6.3)	8 (7.7)	1 (2.6)	0.50

ERCP: Endoscopic retrograde cholangiopancreatography; CI: Contrast injection; WGC: Wire-guided cannulation.

uate the impact of CI using a single-lumen catheter on the incidence of PEP after PGW, compared with that of WGC using a double-lumen catheter. First, a crude odds ratio was estimated by univariate analysis in Model 1, and then it was adjusted for basic characteristics such as sex and age by multivariate analysis in Model 2. Finally, it was also adjusted for potential confounding variables including failed cannulation, bile duct stone removal, pancreatic brush cytology, and biliary sphincterotomy by multivariate analysis in Model 3. These potential confounders were selected as risk factors for PEP.

Statistical analysis was performed using SPSS software, version 14.0J (SPSS Inc, Japan). Odds ratios and 95% confidence intervals were used, and two-sided P values of less than 0.05 were considered statistically significant.

RESULTS

The patients' characteristics and the endoscopic procedures used for PGW was illustrated in Table 1. There were no significant differences in these parameters, except for the incidence of choledocholithiasis, between CI using a single-lumen catheter and WGC using a double-lumen catheter.

CI with routine pancreatography, CI without routine pancreatography, and WGC were performed in 27 patients, 77 patients and 38 patients, respectively. Routine pancreatography did not contribute to the early diagnosis of pancreatic cancer in our study period. In CI without routine pancreatography and WGC, diagnostic pancreatography was performed in 17 patients and no patients, respectively.

The success rate of biliary cannulation by PGW alone was 69% (98/142), and the final success rate increased to 80.3% (114/142) after the addition of consecutive maneuvers or a second ERCP. In the 28 patients for whom biliary cannulation failed, a surgical operation was performed in 6 patients, percutaneous transhepatic biliary drainage was performed in 12 patients, endoscopic ultrasound-guided biliary drainage was performed in 2 patients, and an alternative examination or a follow-up

Table 2 Outcome of pancreatic guidewire placement *n* (%)

	Total (*n* = 142)	Cannulation method		
		CI using a single-lumen catheter (*n* = 104)	WGC using a double-lumen catheter (*n* = 38)	*P* value
Successful biliary cannulation	98 (69)	69 (66)	29 (76)	0.35
Post-ERCP pancreatitis	22 (16)	21 (20)	1 (2.6)	0.012

ERCP: Endoscopic retrograde cholangiopancreatography; CI: Contrast injection; WGC: Wire-guided cannulation.

Table 3 Impact of contrast injection using a single-lumen on post-endoscopic retrograde cholangiopancreatography pancreatitis after pancreatic guidewire placement according to multivariate analysis

	Model 1 crude OR (95% C.I.)	Model 2 adjusted OR (95% C.I.)	Model 3 adjusted OR (95% C.I.)
CI using a single-lumen catheter	9.4 (1.2-72)[a]	10.2 (1.3-83)[a]	10.8 (1.3-88)[a]
Female		1.5 (0.56-3.9)	1.4 (0.50-3.7)
< 50 yr		6.8 (0.79-59)	6.1 (0.67-56)
Failed cannulation			1.2 (0.30-4.9)
Bile duct stone removal			1.9 (0.46-7.5)
Pancreatic brush cytology			0.55 (0.061-5.0)
Biliary sphincterotomy			1.3 (0.36-4.7)

[a]*P* < 0.05. CI: Contrast injection; OR: Odds ratio; C.I.: Confidence interval.

study was performed in 8 patients. When analyzed according to the cannulation method, the success rate of biliary cannulation by PGW alone was 66% (69/104) in the patients who underwent CI using a single-lumen catheter and 76% (29/38) in the patients who underwent WGC using a double-lumen catheter, as shown in Table 2. There were no significant differences between the two cannulation methods with regard to the success rate of biliary cannulation.

PEP occurred in 22 patients (15.5%), and the severity was mild in all cases. When analyzed according to the cannulation method, the incidence of PEP was 37.0% (10/27) in the patients who underwent CI with routine pancreatography, 14.3% (11/77) in those who underwent CI without routine pancreatography, and 2.6% (1/38) in those who underwent WGC. In all patients who underwent CI using a single-lumen catheter, the incidence of PEP was 20% (21/104), and it was significantly higher than that in the patients who underwent WGC using a double-lumen catheter, as shown in Table 2. In univariate and multivariate analysis, CI using a single-lumen catheter showed a high, statistically significant, odds ratio for PEP after PGW, as shown in Table 3. PEP did not occur in any patient who underwent pancreatic stenting.

DISCUSSION

In the present study, the incidence of PEP in the patients treated with PGW was 15.5%. When analyzed according to cannulation method, it was 37.0% in the patients who underwent CI with routine pancreatography, 14.5% in those who underwent CI without routine pancreatography, and 2.6% in those who underwent WGC. When CI using a single-lumen catheter is followed by PGW, the contrast medium in the catheter lumen is injected into the pancreatic duct and causes opacification of the entire pancreatic duct, as shown in Figure 2. Adding routine pancreatography to PGW is equal to performing two pancreatic ductal opacification procedures. Our data indicate that contrast injection into the entire pancreatic duct strongly correlates with the incidence of PEP after PGW. Cheon *et al*[13] also emphasized that a progressively higher frequency of PEP was detected as the extent of pancreatic ductal system opacification increased. In univariate and multivariate analysis, CI using a single-lumen catheter showed a high, statistically significant, odds ratio for PEP after PGW. Hence, a cannulation method involving the use of a double-lumen catheter to minimize the CI dose delivered into the pancreatic duct would be expected to reduce the incidence of PEP after PGW compared with CI using a single lumen catheter.

Herreros de Tejada *et al*[10] performed a multicenter, randomized, controlled trial involving 188 patients who had been subjected to 5 unsuccessful biliary cannulation attempts by WGC alone and divided them into the standard cannulation group (*n* = 87) and the attempted PGW group (*n* = 76). They emphasized that the incidence of PEP in the attempted PGW group was 17% (13/76), compared with 8% (7/87) in the standard cannulation group. However, as their attempted PGW group included 19 patients for whom PGW was unsuccessful and they did not explain in which cases PEP occurred, the incidence of PEP in the patients who successfully underwent PGW might have been lower. Ito *et al*[11] performed a prospective, randomized, controlled trial involving 70 patients who underwent PGW with CI, and they divided their subjects into the pancreatic stent group (*n* = 35) and the no-pancreatic stent group (*n* = 35). They concluded that the frequency of PEP in the pancreatic stent group was significantly lower than that in the no-pancreatic stent

Table 4 Study on pancreatic guidewire placement

Primary author	Study design	No. of patients	Cannulation method	Success rate fo biliary cannulation	Incidence fo post-ERCP pancreatitis
Maeda[8]	RCT	Control (n = 26), attempted PGW (n = 27)	CI	54%, 93%	0%, 0%
Ito[9]	OS	PGW (n = 113)	CI	73%	12%
Herreros de Tejada[10]	RCT	Control (n = 87), attempted PGW (n = 76)	WGC	56%, 47%	8%, 17%
Ito[11]	RCT	PGW with no-PS (n = 35), PGW with PS (n = 35)	CI	94%, 80%	23%, 2.9%
Present study	OS	PGW (n = 142)	CI/WGC (104/38)	69% (CI 66%, WGC 76%)	16% (CI 20%, WGC 2.6%)

RCT: Randomized controlled trial; OS: Observational study; PGW: Pancreatic guidewire placement; PS: Pancreatic stent; CI: Contrast injection; WGC: Wire-guided cannulation.

group (2.9% *vs* 23%). Hence, this suggests that papillary edema and/or CI into the pancreatic duct are strongly associated with the risk of PEP after PGW combined with CI. If PEP occurs due to pancreatic branch duct injuries caused by the PGW, pancreatic stenting will not be useful for preventing PEP. One concern about WGC without CI, especially in cases involving tortuous pancreatic ducts, is the risk of duct injury due to accidental guidewire insertion into a pancreatic branch duct. When guidewire resistance arises during PGW, we inject the minimal dose of contrast medium into the pancreatic duct in order to adjust the direction of the GW. It is interesting that the frequency of PEP in the pancreatic stent group in Ito's study[11] was similar to that in the WGC group treated with a double-lumen catheter in our study (2.9% *vs* 2.6%). Nevertheless, pancreatic branch duct injury caused by PGW can lead to PEP. Therefore, it is necessary to investigate PGW cases in combination with information about accidental guidewire insertion/placement into the branch pancreatic duct in order to clarify whether PGW has a powerful effect on PEP.

Our study has the following limitations: it is not a prospective controlled study; the routine administration of protease inhibitors or improvements in the operator's technique might have influenced the reduction in the incidence of PEP; information regarding the extent of pancreatic ductal system opacification is not investigated. However, our results suggest that the incidence of PEP after PGW is reduced by the use of a double-lumen catheter as it minimizes the CI dose delivered into the pancreatic duct.

In our study, the success rate of biliary cannulation by PGW alone was 69%. There have been 3 previous studies on PGW[8-11], as shown in Table 4, and the success rate of biliary cannulation by PGW alone ranged from 47% to 93%. The lowest success rate, which was found in Tejada's study[10], can be attributed to the setting of a maximum number of cannulation attempts (15 attempts), since there was no limitation on the number of cannulation attempts in other studies. In contrast, the highest success rate, which was found in Maeda's study[8], may have arisen from the small study population and the use of the vasodilator isosorbide dinitrate to improve the biliary cannulation rate. Our study is similar to Ito's study[9] as it had a large study population; there were no limitations on the number of cannulation attempts; and it obtained a success rate of approximately 70% for PGW alone, which is acceptable. If PGW alone cannot successfully achieve biliary cannulation, PGW permits the use of another rescue technique such as transpancreatic sphincterotomy, another attempt at biliary cannulation, or precut sphincterotomy under pancreatic stenting. PGW is useful as a rescue maneuver for patients in whom biliary cannulation is difficult, providing the pancreatic duct is accessible.

In conclusion, when PGW is used as the initial rescue technique for difficult biliary cannulation, a cannulation method involving the use of a double-lumen catheter is recommended to minimize the dose of contrast medium injected into the pancreatic duct and hence reduce the incidence of PEP after PGW.

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COMMENTS

Background

In Japan, endoscopic retrograde cholangiopancreatography (ERCP) is usually performed under contrast injection (CI) using a single-lumen catheter and a duodenoscope with 15-degree backward-oblique angle. Pancreatic guidewire placement (PGW) is one of the rescue maneuvers used to overcome difficult biliary cannulation. In CI using a single-lumen catheter, the contrast medium in the catheter lumen is injected into the pancreatic duct during PGW, and it carries a risk of post-ERCP pancreatitis (PEP).

Research frontiers

It has been emphasized that the use of wire-guided cannulation (WGC) is associated with a reduced incidence of PEP compared with CI. WGC using a double-lumen catheter can minimize the dose of contrast medium injected into the pancreatic duct during PGW.

Innovations and breakthroughs

The authors changed their cannulation method from CI using a single-lumen catheter to WGC using a double-lumen catheter. In the patients who underwent CI using a single-lumen catheter, the incidence of PEP after PGW was significantly higher than that in WGC using a double-lumen catheter (20% *vs* 2.6%). In univariate and multivariate analysis, CI using a single-lumen catheter showed a high, statistically significant, odds ratio for PEP after PGW.

Applications

When PGW is used as the initial rescue technique for difficult biliary cannulation, a cannulation method involving the use of a double-lumen catheter is recommended to reduce the incidence of PEP after PGW.

Terminology

PGW is used to overcome difficult biliary cannulation during ERCP. After PGW,

biliary cannulation **is attempted in the region towards the upper left of the pan**creatic guidewire. **WGC is a cannulation method in ERCP using a guidewire** passed through the catheter lumen under fluoroscopic guidance.

Peer review

This is an interesting article describing the utility of PGW without injection of the contrast for achieving biliary cannulation.

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BRIEF ARTICLE

Human papillomavirus in upper digestive tract tumors from three countries

Andres Castillo, Chihaya Koriyama, Michiyo Higashi, Muhammad Anwar, Mulazim Hussain Bukhari, Edwin Carrascal, Lida Mancilla, Hiroshi Okumura, Masataka Matsumoto, Kazumasa Sugihara, Shoji Natsugoe, Yoshito Eizuru, Suminori Akiba

Andres Castillo, Chihaya Koriyama, Muhammad Anwar, Suminori Akiba, Department of Epidemiology and Preventive Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
Michiyo Higashi, Department of Human Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
Mulazim Hussain Bukhari, King Edward Medical University, Lahole 54000, Pakistan
Edwin Carrascal, Department of Pathology, Universidad del Valle, Cali 760043, Colombia
Lida Mancilla, Universidad Santiago de Cali, Cali 760035, Colombia
Hiroshi Okumura, Masataka Matsumoto, Shoji Natsugoe, Department of Surgical Oncology and Digestive Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
Kazumasa Sugihara, Department of Maxillofacial Diagnostic and Surgical Science, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
Yoshito Eizuru, Division of Oncogenic and Persistent Viruses, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
Author contributions: Castillo A, Koriyama C and Akiba S conceived the study, analyzed the data and participated in editing of the manuscript; Castillo A performed human papillomavirus analysis; Higashi M, Bukhari MH and Carrascal E conducted pathological analyses; Anwar M, Mancilla L, Okumura H, Matsumoto M, Sugihara K and Natsugoe S retrieved clinical information and obtained a collection of tissue specimens for analysis; Eizuru Y and Akiba S gave analytical support for all the analyses.
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Correspondence to: Dr. Chihaya Koriyama, Department of Epidemiology and Preventive Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544,Japan. fiy@m.kufm.kagoshima-u.ac.jp
Telephone: +81-99-2755298 Fax: +81-99-2755299
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Abstract

AIM: To clarify human papillomavirus (HPV) involvement in carcinogenesis of the upper digestive tract of virological and pathological analyses.

METHODS: The present study examined the presence of HPV in squamous cell carcinomas of the oral cavity (n = 71), and esophagus (n = 166) collected from Japan, Pakistan and Colombia, with different HPV exposure risk and genetic backgrounds. The viral load and physical status of HPV16 and HPV16-E6 variants were examined. Comparison of *p53* and $p16^{INK4a}$ expression in HPV-positive and HPV-negative cases was also made.

RESULTS: HPV16 was found in 39 (55%) oral carcinomas (OCs) and 24 (14%) esophageal carcinomas (ECs). This site-specific difference in HPV detection between OCs and ECs was statistically significant ($P < 0.001$). There was a significant difference in the geographical distribution of HPV16-E6 variants. Multiple infections of different HPV types were found in 13 ECs, but multiple infections were not found in OCs. This difference was statistically significant ($P = 0.001$). The geometric means (95% confidence interval) of HPV16 viral load in OCs and ECs were 0.06 (0.02-0.18) and 0.12 (0.05-0.27) copies per cell, respectively. The expression of $p16^{INK4a}$ proteins was increased by the presence of HPV in ECs (53% and 33% in HPV-positive and -negative ECs, respectively; $P = 0.036$), and the high-risk type of the HPV genome was not detected in surrounding normal esophageal mucosa of HPV-positive ECs.

CONCLUSION: Based on our results, we cannot deny the possibility of HPV16 involvement in the carcinogenesis of the esophagus.

Key words: Human papillomavirus; Viral load; Physical status; E6; p53; $p16^{INK4a}$

Peer reviewers: Tamara M Alempijevic, MD, PhD, Assistant Professor, Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia, 2 Dr Koste Todorovica St., 11000 Belgrade, Serbia; Liang-Shun Wang, MD, Professor, Vice-superintendent, Shuang-Ho Hospital, Taipei Medical University, No. 291, Jhongjheng Rd., Jhonghe City, New Taipei City 237, Taiwan, China

Castillo A, Koriyama C, Higashi M, Anwar M, Bukhari MH, Carrascal E, Mancilla L, Okumura H, Matsumoto M, Sugihara K, Natsugoe S, Eizuru Y, Akiba S. Human papillomavirus in upper digestive tract tumors from three countries. *World J Gastroenterol* 2011; 17(48): 5295-5304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i48/5295.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i48.5295

INTRODUCTION

Human papillomaviruses (HPVs) belong to the *Papillomaviridae* family and are non-enveloped icosahedral viruses with a diameter of 55 nm and have more than 100 types[1]. The International Agency for Research on Cancer considers that there is convincing evidence indicating that infection with *HPV16*, *18*, *31*, *33*, *35*, *39*, *45*, *51*, *52*, *56*, *58*, *59 or 66* can lead to cervical cancer[2]. To date, approximately 20 types have been identified as high-risk HPVs that increase the risk of cervical cancer. Among them, HPV16 and HPV18 are considered to be associated with 70% of all cervical cancer cases[2]. In contrast, low-risk HPV types such as *HPV6* and *HPV11* cause genital warts but not cancer.

The association of HPV with cancers of the upper digestive tract (UDT) is also suspected. Major malignancies observed in the UDT include cancers of the oral cavity, oropharynx, larynx and esophagus. Meta-analysis of 4680 samples from 94 reports published between 1982 and 1997 has shown that HPV was 2-3 times more likely to be detected in precancerous oral mucosa and approximately five times more likely to be detected in oral carcinoma (OC) than in normal mucosa[3]. Among the studies used in this meta-analysis, the largest and best-designed study was that by Maden *et al*[4]. They examined 112 normal mucosal specimens and 118 OCs and detected HPV16 in six (5%) cases of OC but in only one (0.9%) normal mucosal specimen. In contrast, *HPV6* was detected in 12 OCs and 10 normal mucosal specimens. High-risk HPV has also been detected in esophageal carcinomas (ECs). A review of studies published between 1982 and 2001 has shown that 15.2% of the 2020 squamous cell carcinomas (SCCs) of the esophagus tested using polymerase chain reaction (PCR) were *HPV* positive[5]. However, previous studies have shown various *HPV*-positive rates in non-genital cancers worldwide. One argument is that this difference was caused by different HPV-detection methods with different sensitivity and specificity among studies. Another possible explanation is different HPV exposure risk and/or susceptibility of disease/infection across study populations.

Furthermore, the role of *HPV* in UDT carcinomas, particularly ECs, remains unclear and controversial[6]. Two European prospective serological studies that used stored serum specimens[7,8] and a Chinese case-control study[9] have found a strong association between the risk of ECs and seropositivity for *HPV16*. In contrast, two retrospective studies conducted in Europe[10,11] and a large prospective serological study in China[12] have found no significant association of *HPV16* or *HPV18* with ECs.

In the present study, cases of oral cavity and esophageal cancer were examined for concomitant HPV infection, the type of HPV involved, and multiple infection with different types of *HPV* in Japan, Pakistan and Colombia, with different *HPV* exposure risks and genetic backgrounds, using the same methods. In order to shed light on the etiological significance of *HPV* in the development of OCs and ECs, the viral load and physical status of *HPV16* (which is the most commonly found HPV type worldwide) and HPV16-E6 variants were examined. Comparison of *p53* and $p16^{INK4a}$ expression in HPV-positive and HPV-negative OCs and ECs was also made.

MATERIALS AND METHODS

Ethics

Institutional Review Board of the Faculty of Medicine, Kagoshima University, Japan, approved the present study.

Subjects

This study examined 261 formalin-fixed and paraffin-embedded tissues of SCC of the UDT: 92 cases (17 OCs and 75 ECs) diagnosed at Kagoshima University Hospital, Kagoshima, Japan during 1987-2005; 90 cases (48 OCs and 42 ECs) diagnosed at King Edward Medical University, Lahore, Pakistan during the period 1996-2002; and 55 cases (6 OCs and 49 ECs) diagnosed at Hospital Universitario del Valle in Cali, Colombia during 1996-2001. For 11 HPV-positive EC cases from Japan, additional formalin-fixed and paraffin-embedded tissues of the esophagus, with or without cancer cells, were analyzed. The histological classifications for OCs and ECs were made using the guidelines determined by Japan Society for Head and Neck Cancer[13] and Japanese Society for Esophageal Diseases[14], respectively. These Japanese classifications follow their corresponding WHO classifications.

DNA extraction

Five-micrometer-thick sections of each tissue, containing a minimum of 60% (typically 70%-90%) tumors cells, were prepared. In each tissue sample, 0.8 mL lemosol and 0.2 mL ethanol were added. Subsequently, the samples were washed with 1 mL ethanol. After centrifugation, the pellet was resuspended in digestion buffer (50 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, pH 8.0, 0.5% Tween 20) containing 200 μg Proteinase K (Invitrogen, Carlsbad CA, United States) and incubated at 56 ℃ for 24 h.

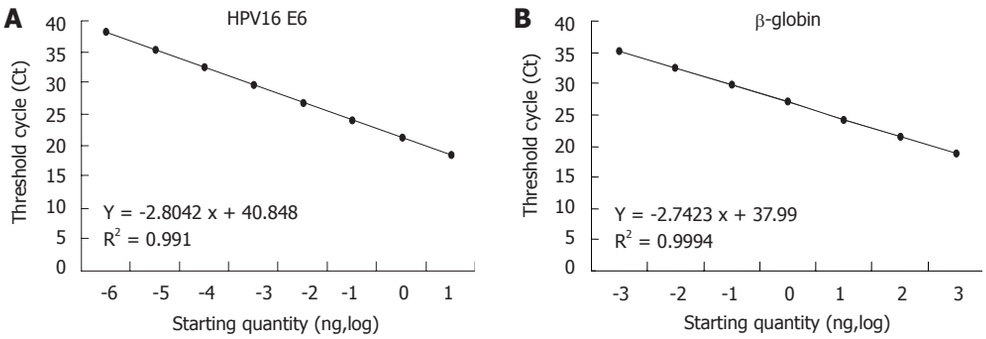

Figure 1 Real-time polymerase chain reaction standard curves. A: Human papillomavirus (HPV)-16 E6 DNA standard calibration curve was generated automatically by plotting Ct values against the logarithm of the copy numbers of eightfold serially diluted of HPV-16 cloned in pUC19 plasmid; B: A seven fold dilution series of a human DNA control (Dynal UK) was used to generate the standard curve for β-globin.

After incubation, the solution was heated at 100 ℃ for 10 min and centrifuged. Phenol-chloroform and DNA ethanol precipitation was made in all HPV16-positive samples in order to determine the DNA amount by using an ND-1000 spectrophotometer (Nano Drop Products, Wilmington, DE, United States). Since the quantity of tissue embedded in the paraffin blocks varied between samples, *β-globin* gene amplification was made for all the samples to check the presence of PCR amplification inhibitors and of amplifiable DNAs. The *β-globin* gene amplification with a set of PCO3/PCO4 primers[15] was conducted under the following PCR conditions: initial denaturation at 95 ℃ for 4 min, 40 cycles with the cycling profile of 95 ℃ for 1 min, 52 ℃ for 1 min and 72 ℃ for 2 min, and final extension for 5 min at 72 ℃.

HPV detection and genotyping

The prevalence of *HPV* DNA was analyzed with the broad-spectrum SPF1/2 *HPV* primers PCR method as described previously[16]. The reaction was performed in a final volume of 25 μL containing 3 μL DNA template and 1.5 U AmpliTaq gold (PerkinElmer, Waltham, MA, United States). The mixture was incubated for 15 min at 95 ℃, followed by 40 cycles of 1 min at 94 ℃, 1 min at 45 ℃, and 1 min at 72 ℃, and a final extension of 5 min at 72 ℃. The PCR products were run on a 3% agarose gel and the 65-bp product was visualized with ethidium bromide staining. The *HPV* types were determined using the INNO-LiPA *HPV* genotyping v2, which is based on the reverse hybridization principle. Part of the *L1* gene region of the *HPV* genome was amplified using SPF10 forward and reverse primers tagged with a biotin at the 5' end, and denatured[17]. Biotinylated amplicons were hybridized with specific oligonucleotide probes immobilized on the strip. In total, there were 25 genotypes (HPV6, 11, 16, 18, 31 33, 35, 39, 40, 42-45, 51-54, 56, 58, 59, 66, 68, 70, 73 and 74). After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with 5-bromo-4-choro-3-indolyl phosphate/nitro blue tetrazolium chromogen gave a purple/brown precipitate and results could be interpreted visually.

HPV16 viral load

The quantitative real-time PCR analysis was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States). Each HPV16 DNA positive sample was amplified for 76 bp of the *E2* gene using the following primers: forward 5'-AACGAAGTATCCTCTCCTGAAATTATTAG-3' (3361-3389 nt); reverse 5'-CCAAGGCGACGGCTTTG-3' (3427-3443 nt), as well as 81 bp of the *E6* gene, primers forward 5'-GAGAACTGCAATGTTTCAGGACC-3' (94-116 nt); reverse 5'-TGTATAAGTTGTTTGCAGCTCTGTGC-3' (150-169 nt), in the presence of specific hybridization probes for *E2*-(FAM-CACCCCGCCGCGACCCATA-TAMRA) (3406-3424 nt) and *E6*-(FAM-CAGGAGCGACCCAGAAAGTTACCACAGTT-TAMRA) (119-147 nt). The reaction was performed in a 25 μL mixture containing 1 × TaqMan Master Mix (Applied Biosystems), 300 nmol primers, 100 nM dual-labeled *E2* or *E6* fluorogenic hybridization probe, and 1-2 μL DNA template. Incubation for 10 min at 95 ℃ allowed activation of the AmpliTaq Gold DNA polymerase and denaturation of nucleic acids; 40 cycles of denaturation at 95 ℃ for 15 s and annealing-extension at 60 ℃ for 1 min were then carried out to amplify the *E2* and *E6* genes. Serial dilutions of full-length HPV16 genome cloned in *pUC19* plasmid (kindly given by Dr. Massimo Tommasino, IARC, France) containing equivalent amounts of *E2* and *E6* genes from 86 to 862 million copies per reaction served as a standard control (Figure 1A). Each sample was assayed two or three times. DNAs extracted from SiHa cell SiHa cells was used as control for *E2* (negative) and *E6* (positive) amplification. This cell line derived from a cervical carcinoma is known to harbor one HPV16 genome or two per cell[18]. Since this cell has only an integrated viral genome, its *E2* gene is disrupted. To adjust for the differences in the amount of input genomic DNA between samples, quantitative real-time PCR for human *β-globin* gene was performed by 2× QuantiTect SYBR Green PCR kit (QIAGEN, Hilden, Germany) using the PC03/PC04 primers set[15]. A sevenfold dilution series of a human DNA control (Dynal UK, Bromborough, Wirral, United Kingdom) was used to generate the

standard curve (Figure 1B). The amount of *β-globin* DNA present in each sample was divided by the weight of one genome equivalent (i.e. 6.6 pg/cell) and a factor of two (because there are two copies of *β-globin* DNA/genome equivalent or cell) to obtain the number of genome equivalents or cells in the sample. The viral load of each sample was expressed as the number of *HPV16* copies per cell.

HPV16 physical status

The HPV16 physical status was determined on the assumption that the *E2* gene is disrupted in integrated viral genome, and therefore, the expected ratio of *E2* to *E6* copy numbers was zero. On the other hand, episomal viral genome had equivalent copy numbers of the *E2* and *E6* genes (an *E2*/*E6* ratio was nearly equal to unity) and mixed presence of integrated and episomal forms of *HPV16* had an *E2*/*E6* ratio between 0 and 1[19].

HPV16 E6 sequencing and variant analysis

HPV16 E6 gene was divided into two fragments and amplified by two semi-nested PCRs, using outer primers 5' -TTGAACCGAAACCGGTTAGT-3' (forward, 46-66 nt) and 5'-GCATAAATCCCGAAAAGCAA-3'(reverse,236-256nt), and inner primers 5'-GCACCAAAAGAGAACTGCAA-3'(forward, 85-105 nt) for the first half. The outer primers of the second half were 5'-GGGATTTATGCATAGTATATAGAGA-3'(forward, 246-270 nt) and 5'-CTTTGCTTTTTGTCCAGATGTC-3' (reverse, 453-474 nt), and inner primers 5'-CAGGACACAGTGGCTTTTGA-3' (reverse, 421-440 nt). The primers were designed using the web-based tool Primer3[20]. Each reaction mix for *E6* amplification contained 5 μL template DNA, 200 μmol/L dNTP, 0.5 μmol/L each primer, and 1 U Hot star Taq DNA polymerase (QIAGEN) in a total volume of 25 μL reaction buffer (50 mmol/L KCl, 20 mmol/L Tris-HCl, pH 8.3). The first-round PCR condition was 95 ℃ for 15 min, followed by 40 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min, and 74 ℃ for 1 min, and a final cycle of 74 ℃ for 10 min. The second-round PCR condition was essentially the same as the first round, except that 1 μL of the first-round PCR products was used as template and that the number of PCR cycles was 35. The amplified products were confirmed through electrophoresis with 2% agarose gels at 100 V for 25 min. The positive amplicon was purified using the QIAGEN PCR purification kit and directly sequenced by fluorescent dye-labeled dideoxynucleotides and cycle sequencing methods using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, NJ, United States). The HPV16 *E6* sequences were aligned by CLUSTAL W multiple alignments package[21], and compared with sequences of HPV16 variants that have been published elsewhere. HPV16 variants Genbank accession numbers were as follows: *K02718*, E-*350T* prototype; *AF536179*, *E-350G* variant; *AF402678*, Asian-American variant; *AF534061*, Asian variant and *AF536180*, African variant 1.

Immunohistochemistry for $p16^{INK4a}$ and p53

The paraffin-embedded samples were cut in 2-3-μm-thick slices, deposited on coated glass slides, and dewaxed using xylene. After rinsing with ethanol, the slides were incubated for 30 min in 0.3% H_2O_2/methanol and for 5 min in a microwave oven at 95 ℃ in 0.01 mol sodium phosphate/citrate buffer (pH 8.0). In order to block the nonspecific binding of the antibody, the slides were incubated for 30 min with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at room temperature. A 1:200 dilution in 5% BSA-PBS of monoclonal anti-$p16^{INK4a}$ antibody was used (BD PharMingen, San Jose, CA, United States). The slides were incubated overnight at 4 ℃, washed with PBS, incubated with biotinylated horse anti-mouse IgG for 30 min, and then washed with PBS and incubated with 1:50 dilution of the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, United States) for 30 min at room temperature. The reaction was visualized by adding diaminobenzidine (Dako, Carpinteria, CA, United States) for 10 min. The sections were counterstained with hematoxylin and visualized. Immunostaining was considered negative when 0%-9% of the carcinoma cells were stained, and was considered positive when 10%-100% of the cells were stained, according to criteria reported previously[22]. For p53, the procedure was the same as for $p16^{INK4a}$, but primary antibodies of p53 (1:50 dilution) was used (DO-7; Dako Japan, Kyoto, Japan). The interpretation of the positive signal was the same as that used for $p16^{INK4a}$ immunostaining.

Statistical analysis

The χ^2 test, Fisher's exact test, Kruskal-Wallis test, and calculations of geometric mean of viral load and corresponding 95% confidence intervals were calculated with STATA software, version 9.2. (STATA Corp., College Station, TX, United States). All the *P* values presented were two sided.

RESULTS

The present study examined cases of SCC of the oral cavity (n = 71) and esophagus (n = 166) collected from Japan, Pakistan and Colombia. Sex distribution and mean ages by country are shown in Table 1. Although there was no sex difference in OCs among the countries, the proportion of male Japanese EC cases was higher than those of other countries. Pakistani OC and EC cases were younger than those in other countries.

The results of HPV detection using PCR with SPF1/2 consensus HPV primers are shown in Table 2. Although Colombian and Pakistani cases showed relatively higher HPV-positive rates in OCs and ECs, respectively, these differences were not statistically significant even after adjusting for sex and age distributions. In total, HPV was detected in 56% and 19% of SCCs of the oral cavity and the esophagus, respectively. The prevalence of HPV in

Table 1 Sex and age distribution of oral carcinoma and esophageal cancer cases

	Cancer site	
	Oral	Esophagus
Male/total (%)		
Japan	11/17 (65)	66/75 (88)
Pakistan	30/48 (63)	25/42 (59)
Colombia	4/6 (67)	27/49 (55)
[1]*P* value	0.972	< 0.001
Mean age in yr (95% confidence interval)		
Japan	68 (64, 73)	64 (62, 65)
Pakistan	49 (45, 53)	54 (50, 57)
Colombia	67 (61, 73)	64 (60, 67)
[2]*P* value	0.013	< 0.001

[1]*P* values were obtained by χ^2 test. [2]*P* values were obtained by one-way analysis of variance.

Table 2 Frequency of human papillomavirus DNA in tumors by cancer site and country *n* (%)

Cancer site country	Total	HPV-positive	HPV16-positive	Multiple HPV
Oral cavity	71	40 (56)	39 (55)	0
Japan	17	8 (47)	7 (41)	0
Pakistan	48	27 (56)	27 (56)	0
Colombia	6	5 (83)	5 (83)	0
[1]*P* value		0.305	0.193	-
Esophageal	166	31 (19)	24 (14)	13 (8)
Japan	75	11 (15)	9 (12)	5 (7)
Pakistan	42	11 (26)	9 (21)	5 (12)
Colombia	49	9 (18)	6 (12)	3 (6)
[1]*P* value		0.308	0.331	0.521

[1]Comparison among the three countries by χ^2 test. HPV: Human papillomavirus.

carcinomas was significantly higher in OCs than in ECs ($P < 0.001$), and similar trends were observed in all countries.

HPV16 was by far the most frequently detected HPV type. Except for one case, all HPV-positive SCC cases of the oral cavity harbored HPV16 only (39/40); the exception was an OC case in which HPV6 was detected. There were no multiple infections of different HPV types in a single carcinoma specimen among OCs (Table 2). Among 31 HPV-positive ECs, 24 were HPV16-positive. Table 3 lists 19 EC cases with HPV of a type other than HPV16; of these, 13 involved multiple infections of different HPV types. All multiple-infection cases except one involved combinations of HPV16 and other HPV genotypes (12/13). The exceptional case involved multiple infection with HPV51 and HPV68.

The physical status of HPV16 was determined by the ratio between copy numbers of the viral *E2* and *E6* genes, which were measured by real-time PCR. Among the 63 HPV16-positive cases examined, only four had HPV16 in the episomal form (two OCs and two ECs), as shown in Table 4. In eight carcinomas (three OCs and five ECs), the HPV16 genome was present in both the episomal and integrated forms. In the remaining cases, the viral

Table 3 List of esophageal carcinomas with human papillomavirus genome other than human papillomavirus 16

Country	Sex	Age (yr)	*HPV16*	Other *HPV* type
Japan	Male	49	-	6
	Male	66	-	51/68
	Female	64	+	18
	Male	67	+	51
	Female	75	+	51
	Male	65	+	51
Pakistan	Female	39	-	18
	Male	43	-	45
	Male	48	+	6
	Male	34	+	6
	Male	54	+	35
	Male	55	+	45
	Male	58	+	45
Colombia	Male	59	-	18
	Male	65	-	18
	Female	68	-	18
	Female	74	+	6
	Female	72	+	18
	Male	67	+	18

HPV: Human papillomavirus.

Table 4 Physical status and E6 variants of human papillomavirus16 in Japan, Pakistan and Colombia

		n (%)				
		All	Japan	Pakistan	Colombia	[1]*P* value
Oral cavity		39	7	27	5	
Physical status	Episomal	2	0	2 (7)	0	0.147
	Mixed	3	0	1 (4)	2 (40)	
	Integrated	34	7 (100)	24 (89)	3 (60)	
HPV16 E6 variant	Prototype	6	3 (75)	3 (21)	0	0.048
	E-350G	13	1 (25)	9 (64)	3 (60)	
	Asian	2	0	2 (14)	0	
	Asian-American	2	0	0	2 (40)	
Esophagus		24	9	9	6	
Physical status	Episomal	2	0	0	2 (33)	0.017
	Mixed	5	0	4 (44)	1 (17)	
	Integrated	17	9 (100)	5 (56)	3 (50)	
HPV16 E6 variant	Prototype	2	2 (40)	0	0	< 0.001
	E-350G	8	3 (60)	5 (100)	0	
	Asian	0	0	0	0	
	Asian-American	6	0	0	6 (100)	

[1]*P* values were obtained by Fisher's exact test. HPV: Human papillomavirus.

genome was considered to be present in the integrated form only. The frequency of HPV16 genome integration into the host genome was not related to sex, age or cancer site. However, the distribution of HPV16 physical status in ECs significantly differed among the three countries. All Japanese HPV16-positive cases showed an integrated form, but the frequency of integrated HPV16 was low in Pakistani and Colombian ECs.

HPV16 E6 variant analysis was also conducted (Table 4). The specimens in some cases had insufficient DNA, therefore, only 39 HPV16-positive cases were analyzed.

Table 5 Viral load of human papillomavirus 16 by country, physical status, and human papillomavirus 16 E6 variants

		n	Viral load/cell		
			GM	95% CI	[1]*P* value
Oral cavity	All	39	0.064	0.022, 0.185	
Country	Japan	7	0.047	0.028, 0.079	0.033
	Pakistan	27	0.037	0.010, 0.144	
	Colombia	5	1.883	0.058, 61.21	
Integrated form	Absent	2	0.001	-	0.048
	Present	37	0.081	0.028, 0.233	
E6 variant	*E-350G*	13	1.081	0.249, 4.705	0.038
	Others	10	0.127	0.063, 0.256	
Esophagus	All	24	0.121	0.053, 0.274	
Country	Japan	9	0.072	0.023, 0.222	0.476
	Pakistan	9	0.124	0.019, 0.799	
	Colombia	6	0.251	0.036, 1.737	
Integrated form	Absent	2	0.161	-	0.601
	Present	22	0.118	0.049, 0.285	
E6 variant	*E-350G*	8	0.444	0.101, 1.957	0.291
	Others	8	0.215	0.055, 0.847	

[1]*P* values were obtained by Kruskal-Wallis test or Mann-Whitney *U* test. CI: Confidence interval; GM: Geometric means.

Table 6 Clinicopathological features of oral and esophageal carcinomas *n* (%)

Cancer site	Pathological features	HPV-negative	HPV-positive	[1]*P* value
Oral cavity	[2]Histological grading			
	Well-differentiated	17 (81)	20 (77)	0.899
	Moderate differentiation	3 (14)	5 (19)	
	Poor differentiation	1 (5)	1 (4)	
	[3]*p53*			
	Positive	13 (45)	16 (48)	0.773
	Negative	16 (55)	17 (52)	
	[4]$p16^{INK4a}$			
	Positive	11 (38)	10 (29)	0.427
	Negative	18 (62)	25 (71)	
Esophagus	[2]Histological grading			
	Well-differentiated	42 (31)	10 (32)	0.831
	Moderate differentiation	64 (48)	16 (52)	
	Poor differentiation	28 (21)	5 (16)	
	[3]*p53*			
	Positive	59 (44)	11 (35)	0.368
	Negative	74 (56)	20 (65)	
	[4]$p16^{INK4a}$			
	Positive	42 (33)	16 (53)	0.036
	Negative	86 (67)	14 (47)	

[1]*P* values were obtained by χ^2 test. [2]Information of histological grading was missing for 10 human papillomavirus (HPV)-negative and 14 HPV-positive oral carcinomas (OCs), and two HPV-negative esophageal carcinomas (ECs). [3]Tissue specimens were not enough to examine *p53* expression in two HPV-negative and seven HPV-positive OCs, and two HPV-negative ECs. [4]Tissue specimens were not enough to examine $p16^{INK4a}$ expression in two HPV-negative and five HPV-positive OCs, and seven HPV-negative and one HPV-positive ECs.

In Japan, only the *E-350T* prototype and the *E-350G* variant were detected. The predominant HPV16 variant was E-350G in Pakistani cases. In contrast, the Asian-American variant was more frequently found in Colombia, and these differences were statistically significant for both OCs and ECs ($P = 0.048$ and $P < 0.001$, respectively).

The geometric means of HPV16 were 0.064 and 0.121 per cell for OCs and ECs, respectively, and this difference was not statistically significant ($P = 0.552$). The HPV16 viral loads were also compared by country and the presence of the HPV16 integrated form and E-350G variant (Table 5). HPV16 in Colombian cases or cases with the E-350G variant tended to show higher viral loads in both OCs and ECs. The geometric means of the virus in OCs were 1.081, 0.147, 0.138 and 0.075 per cell for the E-350G variant, E-350T prototype, Asian-American variant, and Asian variant, respectively.

Comparison of $p16^{INK4a}$ and p53 protein expression in HPV-positive and HPV-negative OCs and ECs suggested that the $p16^{INK4a}$ expression was affected by the presence of the HPV genome in ECs (Table 6). However, the expression of these tumor suppressor genes was not related to HPV status in OCs.

It is difficult to deny the possibility that high-risk HPV was harbored in non-cancerous tissue adjacent to HPV-positive carcinoma, therefore, additional paraffin-embedded tissues of the 11 HPV-positive ECs from Japan were examined (Table 7). None of the normal esophageal epithelia adjacent to HPV-positive EC harbored a high-risk type of HPV genome.

DISCUSSION

The HPV genome was detected in 56% and 19% of SCCs of the oral cavity and esophagus, respectively, in cases collected from Japan, Pakistan and Colombia. The HPV prevalence in both OCs and ECs did not significantly differ by country (Table 2). On the other hand, there was a significant geographical difference in the distribution of HPV16 E6 variants, which was also related to the viral load (Table 5). HPV16-positive OC cases with the E-350G variant showed a higher viral load than those with non-E-350G variants. Similar trends were observed in ECs although the difference was not statistically significant. One of the reasons for this difference is nucleotide alterations in primers and probes sequences. Among HPV16 intratypes, there is one polymorphism in the sequence of the E6 probe at nucleotide 145, and the Asian-American variant harbors this nucleotide substitution (C to T). However, this polymorphism is unlikely to cause a difference in viral load because the copy number of HPV16 in the Asian-American variant was similar to other intratypes except E-350G (data not shown). The HPV16 E-350G variant contains a polymorphism at residue 83, leucine to valine (L83V), which is associated with the risk of invasive cancers of the cervix in European studies[23,24]. Yamada *et al*[25] have identified five phylogenetic clusters of HPV16 with distinct geographic distributions by analyzing sequences of *E6, L1* and *LCR* regions isolated from cervical samples collected worldwide. HPV16 is the most prevalent HPV type detected in UDT cancer, therefore, different geographic distribution patterns of HPV16-E6 variants with differing copy num-

Table 7 Detection of human papillomavirus genome in adjacent normal epithelium of human papillomavirus 16-positive Japanese esophageal carcinomas

Case ID/sex/age	ID # of block	Histology	HPV	HPV type
EC4/M/48	39	SCC	+	16
	[1]32	SCC	-	
	[1]40	SCC	+	16
	14	Normal	-	
	37	Normal	-	
EC5/M/67	20	SCC	+	16, 51
	[1]8	SCC	+	16
	[1]14	SCC	+	6
	[1]15	SCC	-	
	41	Normal	+	6
EC7/F/75	35	SCC	+	16, 51
	[1]5	SCC	-	
	[1]13	SCC	-	
	24	Normal	+	6
EC10/M/66	12	SCC	+	16
	[1]6	SCC	-	
	20	Normal	-	
	22	Normal	-	
EC11/F/47	26	SCC	+	16
	[1]18	SCC	-	
	[1]10	SCC	+	6
	41	Normal	-	
EC16/M/65	55	SCC	+	16, 51
	55	Normal	+	6
EC28/M/61	12	SCC	+	16
	20	Normal	-	
EC35/F/64	25	SCC	+	16, 18
	28	Normal	-	
	33	Normal	-	
EC61/M/64	12	SCC	+	16
	28	Normal	-	
EC63/M/66	9	SCC	+	51, 68
	3	Dysplasia	-	
	11	Normal	-	
EC67/M/49	16	SCC	+	6
	[1]5	SCC	-	
	1	Normal	-	

[1]Additional esophageal carcinoma specimens analyzed for each patient. ID: Identification; SCC: Squamous cell carcinoma; EC: Esophageal carcinomal; HPV: Human papillomavirus.

bers might affect the detection rate of HPV genome if different methods for HPV detection were applied.

HPV involvement in UDT carcinogenesis is unclear. In the present study, a significant positive association between the presence of the HPV genome and $p16^{INK4a}$ expression was observed in ECs but not in OCs (Table 6). In cervical carcinomas, $p16^{INK4a}$ protein expression is known to be upregulated by HPV[26], where retinoblastoma protein is inhibited by HPV E7 protein, causing the release of E2F protein, which in turn leads to $p16^{INK4a}$ upregulation. Although the results of the present study suggest an etiological involvement of HPV in EC development, this association should be confirmed.

HPV detected in normal specimens or low-grade squamous intraepithelial lesions has been found not to be integrated into the host genome[27]. However, it has been reported in many studies[19,28] that HPV16 is often integrated into the host genome in cervical carcinomas and is frequently accompanied by episomal HPV. These findings suggest an etiological involvement of HPV when integrated into the host genome. In most HPV16-positive cases in the present study, the viral DNA was integrated into the host genome. This finding is compatible with studies conducted in areas of high EC incidence in China, in which > 90% of high-risk HPV detected in ECs was found to be integrated into the host genome[29,30].

In Japanese ECs, high-risk type HPV genomes were not detected in surrounding normal epithelial tissues of HPV-positive ECs (Table 7). This result is similar to a recent study from Australia[31], showing that none of the 55 samples of normal esophageal squamous epithelium were HPV-positive. Although we cannot deny the possibility that tumor cells are more susceptible to the infection with high-risk type of HPV, these observations indicate a possible association between HPV infection and carcinogenesis in some ECs.

The geometric mean HPV16 load in ECs was 0.121 per cell in the current study. It is unlikely, however, that the low viral load was a consequence of formalin fixation because the parallel amplification of a housekeeping gene (*β-globin*) gave an estimate of the amount of amplifiable genomic DNA in individual samples. Our findings are compatible with Chinese studies on ECs that have reported a viral load of < 1 to 157 copies per cell[32].

The prevalence of HPV was evidently higher in OCs than in ECs ($P < 0.001$). HPV typing analysis established that HPV16 was most common among SCCs with concomitant HPV infection. In fact, HPV16 was the only high-risk type detected in OCs. In ECs, although HPV16 was the most frequently detected, other high-risk HPV types such as HPV18, 45 and 51 were also detected. In this study, multiple infection with different HPV types was observed in 37% of HPV-positive ECs, but multiple infection was not found in OCs. Double infection with different HPV types in ECs has also been reported in a study from China[33]. These findings are in contrast to those reported in a Japanese study that showed multiple-type HPV infection in 17/30 (56.7%) specimens of the oral cavity mucosa[34].

Infection with multiple HPV types is not rare in cervical samples. Studies have shown that 10% or more of clinical lesions contain at least two different HPV types[35,36]. Interestingly, the prevalence of multiple infections has been reported to decrease with increasing severity of cervical neoplasia[37,38]. A study has shown that the frequency of multiple-type HPV infection is related to many factors, such as age and sexual behavior, as well as to variables affecting immune response, e.g., immunosuppressive conditions and *HLA* genotypes[38]. In the present study, ECs with multiple-type HPV infection did not exhibit different clinicopathological features from ECs with single HPV infection. Silins *et al*[39] have suggested that infection with HPV6 might interfere with HPV16 in terms of cervical carcinogenesis. In the present study, there were three ECs with co-infection of HPV16 and HPV6. These cases did not have any common clinical features.

In a previous study of HPV16 variants using cervical specimens[25], the Asian-American variant was isolated mainly from Central and South America and Spain. African variants 1 and 2, and the Asian variant were present mainly in samples from Africa and Southeast Asia, respectively. In all regions other than Africa, the E-350T prototype and the *E-350G* variant were detected. In the present study, only the *E-350T* prototype and the *E-350G* variant were detected in Japan. On the other hand, in Pakistan, *E-350G* was the predominant *HPV16* variant. In Colombia, the Asian-American variant was the most commonly found type, but this variant was not found at all in Japan and Pakistan. Our findings are similar to those of Yamada *et al*[25], who detected the *E-350G*, *E-350T*, and Asian-American variants in 52%, 25%, and 20%, respectively, of 228 HPV16-positive cervical cancer specimens from Central and South America. No particular *HPV16 E6* variant predisposed those infected to OCs or ECs.

One important question regarding the presence of HPV in UDT carcinomas is the route of HPV infection. HPV is known to be sexually transmitted in the case of the anogenital organs[40], as well as in some cases of HPV infection of the oral cavity[41]. In addition, several other possible routes of infection have been proposed for HPV infection of the oral and pharyngeal cavities. These include intrapartum infection during passage through an infected birth canal, transplacental infection *in utero* prior to birth, and postnatal infection by contact[42,34]. For instance, HPV can be transmitted from a mother to her newborn baby during vaginal delivery, and this can result in recurrent respiratory papillomatosis. In addition, HPV DNA has been detected in the foreskin of normal newborns[43], in a high percentage of neonates vaginally delivered by HPV-infected mothers, and in the amniotic fluid[44]. These findings favor the mechanisms for HPV transmission at birth.

In the present study, HPV16 was frequently integrated into the host genome in patients with OCs and ECs. However, the viral loads in these malignancies were much lower than those found in cancer of the cervix. It should be noted, however, that human cancer can be regarded as a stem cell disease originating from a small fraction of cancer cells that show self-renewal and pluripotency and are capable of initiating and sustaining tumor growth[45]. HPV may be present in only a small fraction of cancer cells with a stem cell-like nature present even in advanced tumors.

In conclusion, there was no significant difference of HPV prevalence in SCC of the UDT among populations at different risk of HPV exposure. The present study cannot deny a possibility of HPV16 involvement in the development of EC.

COMMENTS

Background

Human papilloma virus (HPV) is suspected to play causal roles in a variety of human malignancies, mainly in cervical cancer. Recently, scientific studies have suggested an etiologic role HPV in a subset of cancers in the upper digestive tract (UDT), such as cancers of the oral cavity, oropharynx, larynx and esophagus. These studies have now recognized that HPV infection in oral cavity is a strong risk factor for head and neck squamous cell carcinoma; mostly for oropharyngeal cancer, but also for esophageal carcinoma, but this remains unclear and controversial.

Research frontiers

The evaluation of causality for the infectious agents as human carcinogens is difficult given their ubiquitous nature, the substantial length of time between infection and the cancer event, the nature of cofactors, and the rarity of malignancy among those infected. Thus, a central problem for the epidemiologist is to define the natural history of infection and to identify those factors that are related to the development of cancer. Hence, informative biomarkers of the agent (such as viral load), of the host (such as abnormal antibody pattern), and of other oncogenic exposures (such as tobacco use) are required for understanding the virus-human interactions and for developing interventions.

Innovations and breakthroughs

In the present study, cases of oral cavity and esophageal cancer were examined for concomitant HPV infection, the type of HPV involved, and multiple infection with different types of HPV in three countries, Japan, Pakistan and Colombia, with different risks of HPV exposure and genetic backgrounds, using the same methods. To shed light on the etiological significance of HPV in the development of oral cancer (OC) and esophageal cancer (EC), the viral load and physical status of HPV16 (which is the most commonly found HPV type worldwide) and HPV16-E6 variants were examined. Comparison of p53 and p16INK4a expression in HPV-positive and HPV-negative OCs and ECs was also made.

Applications

The prophylactic vaccine against HPV16 could prevent HPV16-associated malignancies if the vaccine were demonstrated to be capable of preventing oral HPV16 infection. Thus, these findings have created new potential opportunities for the primary prevention of other HPV-related malignancies.

Terminology

HPV is a member of the papillomavirus family, which is capable of infecting humans. Viral load is a measure of the severity of viral infection that can be calculated by estimating the amount of virus in a cell or tissue. Viral physical status: the HPV genome can be found in two physical states: integrated into the host genome or not integrated as an episomal molecule. E6 is the HPV protein associated with cancer development. p53 protein prevents cell growth and stimulates apoptosis in the presence of DNA damage. p16 protein is involved in tumor suppression.

Peer review

Currently, the HPV vaccines potentially hold promise for the prevention of a greater majority of HPV-positive cervical cancers in woman. Thus, studies that attempt to clarify the association between HPV with cancers of the oral cavity and esophagus are important. They give us reason to be optimistic that HPV vaccines may be protective against UDT HPV infection, and consequently, effective in preventing HPV-associated UDT cancers in both men and women.

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BRIEF ARTICLE

Constant serum levels of secreted asialoglycoprotein receptor sH2a and decrease with cirrhosis

Ron Benyair, Maria Kondratyev, Elena Veselkin, Sandra Tolchinsky, Marina Shenkman, Yoav Lurie, Gerardo Z Lederkremer

Ron Benyair, Maria Kondratyev, Elena Veselkin, Sandra Tolchinsky, Marina Shenkman, Gerardo Z Lederkremer, Department of Cell Research and Immunology, George Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
Yoav Lurie, Liver Unit, Department of Gastroenterology, Tel Aviv Sourasky Medical Center, Tel Aviv 69978, Israel
Author contributions: Benyair R and Kondratyev M contributed equally to this work; Lurie Y and Lederkremer GZ designed the research; Benyair R, Kondratyev M, Veselkin E, Tolchinsky S and Shenkman M performed the research; Lurie Y contributed study samples; Benyair R, Kondratyev M and Lederkremer GZ analyzed the data; and Lederkremer GZ wrote the manuscript.
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Correspondence to: Dr. Gerardo Z Lederkremer, Departments of Cell Research and Immunology, George Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. gerardo@post.tau.ac.il
Telephone: +972-3-6409239 Fax: +972-3-6422046
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Abstract

AIM: To investigate the existence and levels of sH2a, a soluble secreted form of the asialoglycoprotein receptor in human serum.

METHODS: Production of recombinant sH2a and development of a monoclonal antibody and an enzyme-linked immunosorbent assay (ELISA). This assay was used to determine the presence and concentration of sH2a in human sera of individuals of both sexes and a wide range of ages.

RESULTS: The recombinant protein was produced successfully and a specific ELISA assay was developed. The levels of sH2a in sera from 62 healthy individuals varied minimally (147 ± 19 ng/mL). In contrast, 5 hepatitis C patients with cirrhosis showed much decreased sH2a levels (50 ± 9 ng/mL).

CONCLUSION: Constant sH2a levels suggest constitutive secretion from hepatocytes in healthy individuals. This constant level and the decrease with cirrhosis suggest a diagnostic potential.

Key words: Asialoglycoprotein receptor; Hepatitis C virus; Liver function; Cirrhosis; Liver diagnosis

Peer reviewer: Jeff Butterworth, MB, FRCP, Department of Gastroenterology, Shrewsbury and Telford Hospital NHS Trust, Mytton Oak Road, Shrewsbury, Shropshire SY3 8XQ, United Kingdom

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INTRODUCTION

The human asialoglycoprotein receptor (ASGPR) is expressed on the sinusoidal membrane of hepatocytes and serves in the clearance of asialoglycoproteins from the plasma[1]. As evaluated through whole-body scintigraphy, using a radioactive ASGPR ligand, a technetium-99m-labeled asialoglycoprotein analog, only the liver shows any significant expression of the ASGPR[2]. The levels of the ASGPR are much reduced upon hepatocyte dedifferentiation[3,4], upon chronic alcohol consumption[5] and in liver fibrosis and cirrhosis[6,7]. We had previously described a soluble form of the ASGPR, termed sH2a, se-

creted from the human hepatoma cell line HepG2. SH2a is formed by cleavage in the endoplasmic reticulum of its precursor[8], encoded by an alternatively spliced variant of the ASGPR H2 subunit mRNA[9], and does not arise by shedding at the cell surface. Here we show for a group of healthy individuals that ASGPR sH2a is secreted to the serum at surprisingly constant levels, and that these are much reduced in hepatitis C virus (HCV) patients with liver cirrhosis.

MATERIALS AND METHODS

Materials

Immobilon-P paper was purchased from Millipore Corp (Bedford, MA). Protein A-sepharose was from Repligen (Cambridge, MA). N-glycosydase-F was obtained from Roche Applied Science. Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and dimethylpimelidate were from Pierce (Rockford, IL). A solution of 3,3',5,5' -tetramethylbenzidine (TMB) was purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). Alkaline phosphatase (ALP) substrate p-nitrophenylphosphate was from Chemicon International (Temecula, CA). Imperial protein stain was from Pierce. Common reagents were from Sigma.

Antibodies

Polyclonal antibodies specific for a peptide corresponding to the carboxyterminus of sH2a or to a peptide unique to sH2a (169 antibody) were described in earlier studies[8].

A monoclonal antibody was prepared by intraperitoneal immunization of BALB/c mice with a conjugate of keyhole limpet hemocyanin (KLH) with the carboxyterminal peptide of H2 and complete Freunds adjuvant. Conjugation was performed using succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate. Hybridoma cells, resulting from fusion of the mouse splenocytes with NS/O myeloma cells, were screened by ELISA, selecting a clone (B9) that reacted strongly with the peptide but not with KLH. The isotype of B9 was IgG3, as analyzed using an Isostrip kit (Roche). Ascitic fluid, obtained by injection of hybridoma cells to mice was used in all experiments due to difficulties in IgG3 purification by standard methods. It did not show any significant background. Goat anti-mouse IgG conjugated to agarose was from Sigma. ALP- or HRP-conjugated goat anti-mouse or anti-rabbit IgG antibodies were from Jackson Laboratories (West Grove, PA).

Recombinant sH2a

Recombinant sH2a was generated by PCR using the primers GAACCATCAGGAGGATCCCAAAGTGAGGGTC and GGAATTCTCAGGCCACCTCGCC and cloned into pET28a (Novagen), which adds an N-terminal 6XHis tag, using *Bam*HI and *Eco*RI. Expression was in *Escherichia coli* Rosetta DE3 pLysS at 37 ℃ until A_{600} = 0.6 and then with isopropyl β-D-1-thiogalactopyranosid induction for 2 h at 32 ℃. After cell sonication and addition of 6 mol guanidinium-hydrochloride, tagged sH2a was purified on Ni^{2+}-NTA-agarose (Qiagen) followed by dialysis. Protein concentration was determined by the bicinchoninic acid assay (Pierce).

Cell culture

NIH 3T3 cells and a stable transfectant expressing H2a (2-18 cell line)[8] were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% newborn calf serum. HepG2 cells were grown in MEM plus 10% fetal calf serum.

Immunoprecipitation and immunoblotting

Immunoprecipitations from cell supernatants (1.2-1.5 mL from 90 mm plates), using rabbit anti-H2a carboxyterminal or 169 antibodies were done as described before[8]. Immunoprecipitations from serum samples were done in a similar manner or using anti-H2a antibody crosslinked to protein A-sepharose with dimethylpimelidate where indicated. Immunoprecipitation with B9 was followed by goat anti-mouse IgG bound to agarose. Treatment of immunoprecipitates with N-glycosidase-F and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were done as described before[8].

Immunoblotting was done as described before[10] using anti-H2a carboxyterminal antibody and detection was performed with TMB solution or using the electrochemiluminescence procedure and a Bio-Rad ChemiDoc XRS system.

Competitive enzyme-linked immunosorbent assay

Corning ELISA plate wells were coated with the carboxyterminal peptide of sH2a (5 μg/mL) and blocked with 3% bovine serum albumin in Tris-buffered saline (TBS), pH 7.5. B9-containing ascitic fluid (1:1500) was preincubated overnight at 4 ℃ with serial dilutions of the serum sample and then incubated on the coated ELISA plate wells for 1 h at room temperature (RT). After TBS wash wells were reacted with goat anti-mouse IgG conjugated to ALP for 1 h at RT. P-nitrophenyl phosphate was added and *A* quantified at 405 nm.

Study subjects

Retrospective samples were from a group of healthy blood donors and a group of HCV-infected patients with cirrhosis, at the Liver Unit, Tel Aviv Sourasky Medical Center. Cirrhosis was determined by percutaneous liver biopsy, performed using a Tru-Core Ⅱ [R] biopsy instrument under ultrasound guidance. The study had a priori approval by the hospital ethical committee according to the Helsinki Declaration and written informed consent was obtained from all participants.

RESULTS

sH2a in human sera

We had seen that sH2a is normally secreted from the human hepatoma cell line HepG2[8]. To analyze whether

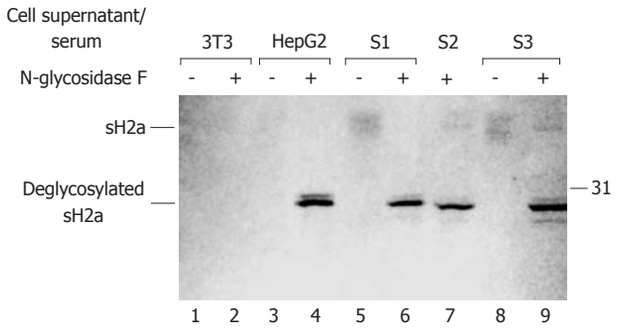

Figure 1 sH2a is detected in normal human sera. Cell supernatants from 90 mm petri-dishes of NIH 3T3 (lanes 1-2) or HepG2 cells (lanes 3-4) or 0.3 mL of normal human sera from 3 donors (S1, lanes 5 and 6; S2, lane 7; S3, lanes 8 and 9) were immunoprecipitated with polyclonal anti-H2a carboxyterminal antibodies that were crosslinked to protein A-agarose, and the immunoprecipitates were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was then done with anti-H2a carboxyterminal antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and color development with 3,3',5,5'-tetramethylbenzidine. Samples on lanes 2, 4, 6, 7 and 9 were treated with N-glycosidase-F after immunoprecipitation. On the right is the molecular weight of a protein standard in kilodaltons. On the left are the migrations of sH2a before or after deglycosylation.

sH2a is present in human serum, we subjected samples of normal human sera to immunoprecipitation with anti-H2a carboxyterminal antibody, treatment with N-glycosidase-F, SDS-PAGE and western blotting with the same antibody (Figure 1, lanes 6, 7 and 9). We detected a band of about 28 kDa, similar in size to the one observed for sH2a in media from HepG2 cells (Figure 1, lane 4). Media from a control cell line that does not express sH2a (NIH 3T3 cells) showed no signal. Without the N-glycosidase-F treatment a disperse band of glycosylated sH2a of about 40 kDa is seen (lanes 3, 5 and 8), which probably represents heterogeneously glycosylated species. We also analyzed for the presence of ASGPR H1 in normal human serum and none was detected (data not shown).

Production of recombinant sH2a and development of an enzyme-linked immunosorbent assay to quantify the serum levels of sH2a

We produced a recombinant 6xHis-tagged sH2a (Figure 2A and B), which allowed us to estimate the level of sH2a in serum. sH2a contained in a sample of normal human serum was compared with recombinant sH2a by immunoprecipitation followed by immunoblot, giving an estimated sH2a concentration of 148 ± 22 ng/mL of serum (Figure 2C and D).

For comparative analysis of the concentration of sH2a in serum we developed a new specific mouse monoclonal anti-peptide H2a antibody. The B9 hybridoma produced a monoclonal antibody that recognized specifically sH2a secreted from transfected NIH 3T3 cells (2-18 cell line) or present in human serum (Figure 3A). The B9 antibody was used to develop an ELISA assay based on the binding of this antibody to its peptide. The binding could be competed by 81% after preincubation of the antibody with a solution containing 0.5 μg/mL of the same peptide, but not of a control peptide (Figure 3B). The bind-

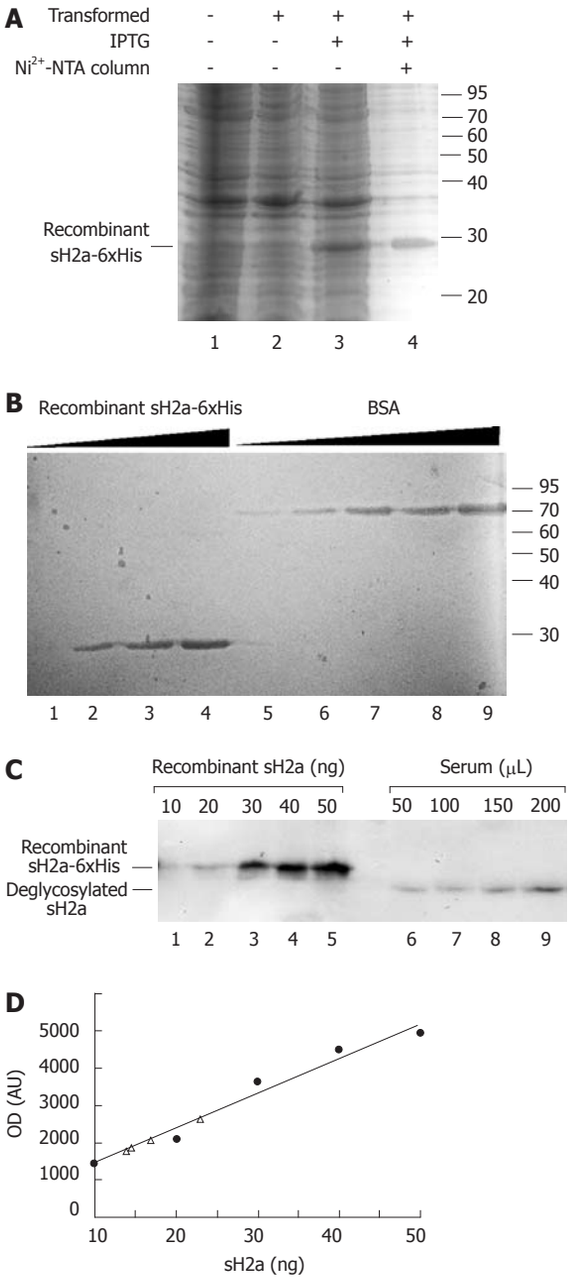

Figure 2 Recombinant sH2a concentration in serum. A: *Escherichia coli* Rosetta DE3 pLysS were either left untransformed or transformed by heat shock with a plasmid carrying 6xHis-tagged sH2a and induced with 0.3 mmol isopropyl β-D-1-thiogalactopyranosid as explained in Materials and Methods. Cell lysates were treated with 6 mol guanidinium-hydrochloride, dyalized and run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), comparing with an aliquot of tagged sH2a purified on a Ni2+-NTA-agarose column (lane 4). The gel was stained with imperial blue; B: Increasing amounts of recombinant affinity purified 6xHis-tagged sH2a (lanes 1-4 correspond to 127, 635, 1270 and 1905 ng) were compared with increasing amounts of bovine serum albumin (lanes 5-9 are 100, 200, 500, 1000 and 2000 ng) on 12% SDS-PAGE, stained with Imperial blue; C and D: The indicated increasing concentrations of recombinant 6xHis-tagged sH2a were run on SDS-PAGE after immunoprecipitation with B9 antibody (lanes 1-5) and compared with B9-immunoprecipitates from increasing volumes of normal human serum treated with N-glycosidase-F (lanes 6-9) and analyzed by immunoblot as in Figure 1, except that detection was done using the electrochemiluminescence procedure. The immunoblot was quantified and the graph (D) shows a curve of recombinant sH2a (full circles) and extrapolation of the concentration of sH2a in 50, 100, 150 and 200 μL of serum (triangles). Shown is an immunoblot representative of three repeat experiments.

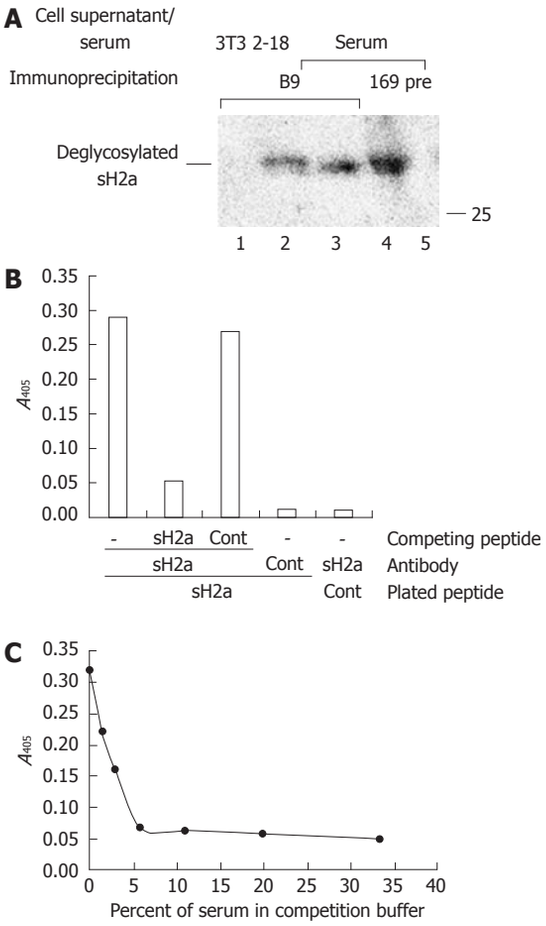

Figure 3 Monoclonal antibody and enzyme-linked immunosorbent assay for detection of sH2a in human sera. A: Medium from two 90 mm dishes of control NIH 3T3 cells (lane 1) or of the same cells stably expressing sH2a (2-18 cell line, lane 2) or 0.5 mL of normal human serum (lanes 3-5) were immunoprecipitated with B9 monoclonal antibody (lanes 1-3), or with a mixture of polyclonal anti-H2a 169 and anti-carboxyterminal antibodies (lane 4) or with control preimmune rabbit serum (lane 5). All immunoprecipitates were treated with N-glycosidase-F and analyzed as in Figure 2C. Shown is an immunoblot representative of three repeat experiments; B: Competitive enzyme-linked immunosorbent assay (ELISA) was performed as described in Materials and Methods, binding the anti-sH2a monoclonal B9 antibody (sH2a) or a control antibody (cont) to the specific sH2a or control (cont) peptides plated on a 96 well plate, after competition with the same sH2a or control peptides (0.5 μg/mL). Values are averages of quadruplicates; C: A similar ELISA assay was done with the B9 antibody and plating the sH2a peptide, but competing the antibody with different dilutions of human serum from a healthy individual (indicated as percent serum of the total volume).

ing could also be competed in a concentration-dependent manner by preincubation of the antibody with normal human serum; a 1:32 dilution of serum resulted in 50% reduction in the binding (Figure 3C).

Serum levels of sH2a in healthy individuals and in hepatitis C patients with cirrhosis

Using the ELISA assay described above we analyzed samples of serum obtained from 62 healthy individuals, with both male and female individuals with a wide range of ages. The levels of sH2a were very constant, 147 ± 19 ng/mL, giving a median of 146.5, with an interquartile range of 137-158 ng/mL (Figure 4A).

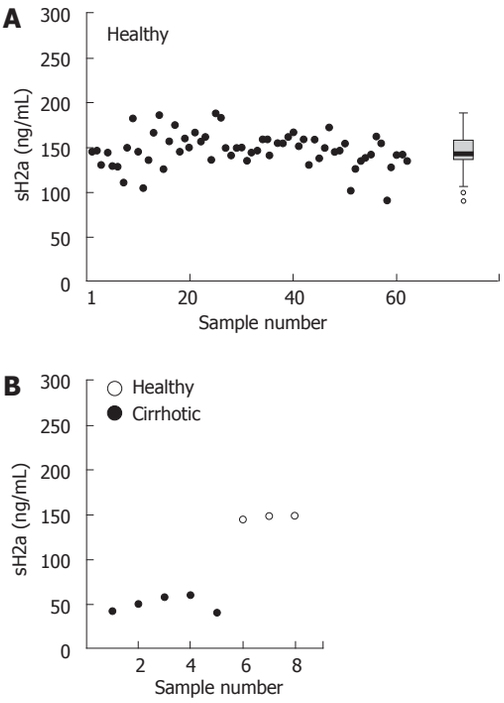

Figure 4 Levels of sH2a in healthy individuals and in hepatitis C virus patients with cirrhosis. A: The enzyme-linked immunosorbent assay (ELISA) described in Figure 3 was used to measure the concentration of sH2a in 62 healthy individuals, male and female, with a wide range of ages. The box height indicates the interquartile range (IQR). The thick horizontal bar is the median. The whiskers extend to the farthest non-outlier value smaller than 1.5 x IQR. Empty circles indicate "mild" outliers (< 3 x IQR); B: The concentration of sH2a in sera was measured blindly by ELISA, from 5 hepatitis C virus patients with cirrhosis compared to 3 healthy individuals.

We then analyzed sH2a levels blindly in sera from a group that included 3 healthy subjects and 5 hepatitis C patients with cirrhosis, as determined by biopsy. All patients had a very low level of sH2a, 50 ± 9 ng/mL, about a third of that in the healthy individuals (Figure 4B).

DISCUSSION

Our results show for the first time the presence of ASGPR sH2a in human serum, with surprisingly constant levels in healthy individuals and a striking decrease in cirrhosis. Although the number of samples from cirrhotic patients that were available to us is very small, the fact that in healthy individuals sH2a is so constant and in all patients analyzed it is dramatically reduced suggests that this might be a general phenomenon.

Hepatocytes are targets for most insults to the liver, including hepatitis viruses and alcohol[11]. The damage caused abrogates the function of the hepatocytes and leads to a fibrogenic process that further impairs liver function and develops to cirrhosis[12,13]. In this context we suggest that sH2a, secreted to constant levels in healthy individuals, is a marker of liver function, correlating to the mass of functional hepatocytes. Reduction of the levels of sH2a could reflect early events in the fibrogenic process, affecting hepatocyte function.

Release of soluble ASGPR fragments to human se-

rum had been described previously[14,15]. These fragments were recognized by polyclonal anti-ASGPR antibodies or by a mixture of monoclonal ones, for which it was not elucidated if they detect H1, H2a or H2b. They probably recognize mainly the H1 subunit (the most abundant) after release from damaged hepatocytes. Indeed it was shown in mice that ASGPR H1 subunit is released increasingly upon hepatocyte damage[16]. This is different from the case of sH2a, which arises from an mRNA that is made specifically for the production of a constitutively secreted protein, the levels of which are reduced by impaired function of the hepatocytes that secrete it. Because of the presence and constancy of sH2a in serum of healthy individuals, it is probably not a favored antigen for autoantibodies that appear in autoimmune hepatitis against the membrane ASGPR, which were found to recognize mainly the H1 subunit[17,18].

The levels of sH2a in serum are reduced in a manner that resembles the reduction in hepatocyte membrane ASGPR in cirrhosis[6,7]. Therefore, sH2a could have a potential for noninvasive diagnosis of liver disease.

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COMMENTS

Background

A specific and sensitive non-invasive indicator of liver function has long been sought after, as the classical markers like albumin levels and prothrombin time can reveal only severe liver disease. The asialoglycoprotein receptor is specifically expressed in the liver and sH2a is a soluble secreted form.

Research frontiers

Several proposed experimental markers or combinations of routine tests are being evaluated, to measure the degree of liver fibrosis in the pathway to cirrhosis. Some of these markers are already commercially available. Nevertheless, the gold standard continues to be the invasive and risky procedure of biopsy.

Innovations and breakthroughs

This study describes for the first time that sH2a is present in human serum. The levels of sH2a were found to be surprisingly constant in a healthy group and dramatically reduced in liver cirrhosis.

Applications

SH2a could be a potential non-invasive diagnostic reporter that reflects the functional mass of the major liver cell type, the hepatocytes.

Terminology

The asialoglycoprotein receptor serves in the clearance of asialoglycoproteins from the plasma. Asialoglycoproteins derive from many glycoproteins in circulation (hormones, growth factors, *etc.*) that at some point have lost the terminal sialic acids in their glycans, by the action of sialidases. Cirrhosis is the end stage of liver disease caused by a wide range of factors like infectious hepatitis, alcoholism, obesity, *etc.*

Peer review

The authors present an important step in the potential development of more sensitive and accurate non-invasive markers to assess functional liver mass.

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BRIEF ARTICLE

Predicting tumor response after preoperative chemoradiation using clinical parameters in rectal cancer

Chan Ho Park, Hee Cheol Kim, Yong Beom Cho, Seong Hyeon Yun, Woo Yong Lee, Young Suk Park, Doo Ho Choi, Ho-Kyung Chun

Chan Ho Park, Hee Cheol Kim, Yong Beom Cho, Seong Hyeon Yun, Woo Yong Lee, Ho-Kyung Chun, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-dong 50, Gangnam-gu, Seoul 135-710, South Korea
Young Suk Park, Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-dong 50, Gangnam-gu, Seoul 135-710, South Korea
Doo Ho Choi, Department of Radiation Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-dong 50, Gangnam-gu, Seoul 135-710, South Korea
Author contributions: Park CH, Kim HC and Chun HK designed research; Park CH, Cho YB, Yun SH, Park YS, Choi DH and Lee WY analyzed data; Park CH and Kim HC wrote the paper.
Correspondence to: Hee Cheol Kim, MD, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-dong 50, Gangnam-gu, Seoul, 135-710, South Korea. hckim@skku.edu
Telephone: +82-2-34101655 Fax: +82-2-34106980
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Abstract

AIM: To evaluate the clinical parameters and identify a better method of predicting pathological complete response (pCR).

METHODS: We enrolled 249 patients from a database of 544 consecutive rectal cancer patients who underwent surgical resection after preoperative chemoradiation therapy (PCRT). A retrospective review of morphological characteristics was then performed to collect data regarding rectal examination findings. A scoring model to predict pCR was then created. To validate the ability of the scoring model to predict complete regression.

RESULTS: Seventy patients (12.9%) achieved a pCR. A multivariate analysis found that pre-CRT movability (P = 0.024), post-CRT size (P = 0.018), post-CRT morphology (P = 0.023), and gross change (P = 0.009) were independent predictors of pCR. The accuracy of the scoring model was 76.8% for predicting pCR with the threshold set at 4.5. In the validation set, the accuracy was 86.7%.

CONCLUSION: Gross changes and morphological findings are important predictors of pathological response. Accordingly, PCRT response is best predicted by a combination of clinical, laboratory and metabolic information.

Key words: Rectal cancer; Preoperative chemoradiotherapy; Downstaging; Tumor regression; Validation

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INTRODUCTION

The treatment strategy of rectal cancer has substantially changed in recent decades. Historically, postoperative

chemoradiation was considered to be the first-line therapy for stage Ⅱ and Ⅲ rectal cancers[1,2]; however, preoperative chemoradiation therapy (PCRT) is now considered to be the optimal therapy regimen for locally advanced distal rectal cancer due to its improved local control, reduced toxicity, and increased rate of sphincter preservation[3-6].

Notably, most evidence suggests that patient response to PCRT is largely variable, as pathological complete response (pCR) occurs in approximately 15% to 30% of all individuals who are treated with PCRT[7,8]. As an outcome measure, the rate of pCR is significant at multiple levels: it not only represents a surrogate endpoint for comparisons of treatment regimen efficacy, but it also may affect the actual course of treatment. Furthermore, pCR has been associated with improved local control, increased recurrence-free survival rates, and better sphincter preservation[9-14]. A study by Habr-Gama *et al*[15] has reported excellent long-term results in the non-operative treatment of patients with clinical evidence of complete response after PCRT. Nevertheless, even though surgery is recommended after PCRT regardless of tumor response, the ability to predict an individual's chance of achieving pCR before commencing therapy would probably enable a more tailored treatment plan[16,17].

To date, although the abilities of many techniques to predict treatment response after PCRT have been evaluated, including endorectal ultrasound (ERUS), computed tomography (CT), ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) and magnetic resonance imaging (MRI), no single modality has been proven to be efficacious, and while several molecular markers, including epidermal growth factor expression, have been associated with PCRT response[18,19], the current data are not definitive enough to support the clinical use of any of these biomarkers.

Accordingly, to identify a better method of predicting pCR, this study evaluated several clinical parameters that have previously been shown to influence pCR.

MATERIALS AND METHODS

Patients

Of the 3194 consecutive patients who underwent surgical resection for rectal cancer at the Samsung Medical Center, Korea between October 1998 and May 2009, 544 underwent surgical resection after PCRT. For this study, the inclusion criteria included: (1) histologically confirmed rectal adenocarcinoma; (2) tumors located within 10 cm of the anal verge; (3) locally advanced (cT3-4 or N1) tumors; (4) curatively resected tumors; and (5) no evidence of distant metastatic disease. Additionally, individuals were excluded according to the following criteria: (1) history of any other malignancy or hereditary colon cancer syndrome; (2) history of rectal cancer requiring emergency surgery; and (3) multiple missing data points in the database or an inability to evaluate pathological tumor response.

Of the initial 544 patients with rectal cancer who underwent PCRT, 70 (12.9%) patients achieved a complete pathological response, whereas the remaining 474 did not. Of these 474 patients, 295 patients were excluded from the study for the following reasons: the presence of another malignancy (one patient); a history of hereditary colon cancer syndrome (three patients); transanal local excision including transanal endoscopic microsurgery (nine patients); a lack of sufficient clinical information in the database (14 patients); and an inability to evaluate tumor regression grade (TRG) after repeated pathological examinations (268 patients). Thus, 249 individuals with rectal cancer were included in the present study; all of whom had undergone PCRT and curative surgery.

Treatment

All of the subjects underwent PCRT. Radiation therapy was administered using a three-field technique within 6-wk periods, delivering 40.4-50.4 Gy. Chemotherapy was also initiated on the first day of pelvic radiotherapy and was delivered concurrently using two chemotherapeutic regimens: (1) 5-fluorouracil (500 mg/m^2 per day) for 3 d during the first and last weeks of radiotherapy; and (2) oral capecitabine (825 mg/m^2) given twice daily during radiotherapy without weekend breaks. The median interval between PCRT and surgery was 55 d (range: 26-120 d).

All of the subjects also underwent radical surgery, including total mesorectal excision, high vascular ligation and *en bloc* resection of any adjacent involved organ after complete PCRT. The operative techniques that were employed in this study included abdominal perineal resection and low anterior resection with colorectal or coloanal anastomosis.

Evaluation

Before PCRT, clinical staging was determined by CT, MRI and/or endorectal ultrasound. Clinical restaging was then conducted 6 wk after the completion of PCRT *via* CT, MRI and ^{18}F-FDG-PET/CT using the protocols described in our previous study[20]. Therapeutic responses with ^{18}F-FDG-PET/CT were evaluated *via* the maximal standard uptake value (SUV). In 66 patients, the SUV could not be evaluated, either because ^{18}F-FDG-PET/CT was not ordered (50 patients) or was performed at a different hospital (16 patients).

After radical surgery, the final tumor pathological staging was evaluated by experienced pathologists. Tumors were classified according to the TNM grading system, 6th edition. Responses to treatment were evaluated according to the TRG, as described by Mandard *et al*[21]. Specifically, the TRG classification system was defined as follows: grade 0, no regression; grade 1, dominant tumor mass with obvious fibrosis and/or vasculopathy (minimal regression); grade 2, dominant fibrotic changes with few tumor cells or groups (moderate regression); grade 3, very few tumor cells in the fibrotic tissue with or without mucous (near total regression); grade 4, no observed tumor cells with only fibrotic masses or acellular mucin pools present (complete regression).

Table 1 Patient demographics and tumor characteristics in the test and validation sets *n* (%)

Characteristics	Test set	Validation set
Total number of patients	249 (100)	15 (100)
Median age (yr, ranges)	55, 24-81	56, 33-74
Sex		
Male	177 (71.1)	11 (73.3)
Female	72 (28.9)	4 (26.7)
Low margin from anal verge (cm)		
≤ 5	187 (75.1)	12 (80)
> 5	62 (24.9)	3 (20)
Histological type		
Adenocarcinoma	238 (95.6)	14 (93.3)
Mucinous	9 (3.6)	1 (6.7)
Signet ring cell	2 (0.8)	0 (0)
Surgery		
Abdominal-perineal resection	37 (14.9)	0 (0)
Low anterior resection	208 (83.5)	14 (93.3)
Hartmann's operation	4 (1.6)	1 (6.7)
Chemotherapy regimen[1]		
FL group	200 (80.6)	9 (60)
Capecitabine group	48 (19.4)	6 (40)
Median interval to surgery (d, ranges)	55, 26-120	54, 41-78

[1]Missing data: n = 1, test set; FL: 5-fluorouracil and leucovorin.

We evaluated several variables to estimate the relationship between gross findings and tumor response. Prior to PCRT (pre-CRT) tumor location, the movability, size, morphology and involved bowel circumference were recorded. Next, after the PCRT (post-CRT), the tumor size and morphology were reassessed at a follow-up hospital visit 6 wk after completion of PCRT. The involved bowel circumference was subdivided into "encircling" and "unidirectional" categories by digital rectal examination (DRE), wherein encircling was defined as occupying more than half of the lumen. Tumor movability was also subcategorized into "movable," "tethered," and "fixed." The pre-CRT tumor size was assessed using DRE in combination with any available colonoscopic or radiologic imaging results. The post-CRT tumor size was measured according to the pathological tumor or scar size. The tumor morphology was classified as being either "benign-like" or "malignant-like" in shape. By definition, benign-like shapes were observed to only consist of scar tissue, discolored lesions, erosions and shallow ulcers, whereas, in malignant-like shapes, infiltrative, fungating and ulcerofungating lesions were present. Pre-CRT tumor morphology was assessed using DRE and colonoscopic results. The resected specimens were used to classify the post-CRT morphology. Gross changes were determined to be present if malignant-like specimens had transitioned into benign-like specimens after PCRT.

Statistical analysis

All statistical analyses were performed using either the χ^2 or Fisher's exact test in addition to logistic regression modeling. Significant differences were defined as $P < 0.05$. To predict complete regression, we used scoring models with parameters that revealed statistical significance, wherein scoring model performance was assessed by receiver operating characteristic (ROC) curve plots for risk scoring and area under the curve (AUC) estimation. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each variable were calculated.

Validation

An independent sample set of patients with primary rectal cancer who underwent PCRT was used to evaluate the performance of the scoring model in predicting complete regression. For this validation, 15 patients were enrolled between May 2009 and July 2009. Scores from this preliminary analysis were tested for sensitivity, specificity, PPV and NPV.

RESULTS

Patient demographics

The demographics of the included patients are described in Table 1. In total, 177 men (71.1%) and 72 women (22.9%) were enrolled with a median age of 55 years (range: 24-81 years). Of these individuals, 210 presented with T3 grade tumors, whereas 39 had T4 grade disease. The demographics of subjects who were enrolled in the separate validation set are also described in Table 1.

Treatment response

After PCRT, the following pathological tumor staging distribution was observed: ypT0, n = 74 (including ypTis in four patients); ypT1, n = 11; ypT2, n = 51; ypT3, n = 105; and ypT4, n = 8. At this point in time, 189 patients were found to be node-negative, whereas 60 patients were node-positive, as per the pathological N staging criteria (including ypT0N1 in three patients). TRG grading was as follows: grade 0 in two patients, grade 1 in 49, grade 2 in 86, grade 3 in 42, and grade 4 in 70.

Clinicopathological factors predicting pathological tumor response

The pre-CRT size (P = 0.001), pre-CRT movability (P < 0.001), pre-CRT carcinoembryonic antigen (CEA) (P = 0.021), ycT (P < 0.001), ycN (P < 0.001), post-CRT size (P < 0.001), post-CRT morphology (P = 0.004), gross change (P < 0.001), and post-CRT SUV (P < 0.001) were all identified to be univariate predictors of complete regression (Table 2). Multivariate predictors for complete regression included pre-CRT movability (P = 0.024), post-CRT size (P = 0.018), post-CRT morphology (P = 0.023) and gross change (P = 0.009). The data from pre-CRT CEA and post-CRT SUV were incomplete, therefore, we did not include these variables in the multivariate analysis (Table 3).

Prediction of complete regression

Scoring models were established to predict pCR using pre-CRT CEA and post-CRT SUV, which identified four statistically significant variables: pre-CRT movability, post-CRT tumor size, post-CRT morphology, and gross change. Each parameter was scored as either 0 or 1 according to

Table 2 Univariate analysis to identify predictors of tumor and complete regression

Variable	Tumor regression grade		*P* value
	0-3	4	
Sex			0.940
Male	127	50	
Female	52	20	
Age (yr)			0.926
≤ 56	96	38	
> 56	83	32	
Low margin from AV (cm)			0.641
≤ 5	133	54	
> 5	46	16	
Pre-CRT size (cm)			0.001
≤ 4	73	44	
> 4	102	23	
cT classification			0.054
cT2, 3	146	64	
cT4	33	6	
cN classification			0.073
cN -	37	22	
cN +	142	48	
Pre-CRT involved bowel circumference			0.365
Encircling	65	21	
One direction	114	49	
Pre-CRT movability			< 0.001
Movable	13	20	
Tethered/fixed	166	50	
Pre-CRT CEA (ng/mL)[1]			0.021
≤ 5	97	52	
> 5	48	11	
ycT classification			< 0.001
ycT 1,2	42	38	
ycT 3,4	137	32	
ycN classification			< 0.001
ycN -	57	44	
ycN +	122	26	
Post-CRT size (cm)			< 0.001
≤ 3	115	64	
> 3	64	6	
Post-CRT morphology			0.004
Benign-like shape	154	69	
Malignancy-like shape	25	1	
Cell type			0.141
High grade	161	67	
Low grade	18	3	
Gross Change			< 0.001
Yes	129	67	
No	50	3	
Post-CRT SUV[2]			< 0.001
≤ 5	63	29	
> 5	86	5	

Low grade: Poorly differentiated or mucinous; High grade: Well or moderately differentiated; [1]Missing data: n = 41; [2]Missing data: n = 66. AV: Anal verge; CRT: Chemoradiation therapy; CEA: Carcinoembryonic antigen; SUV: Standard uptake value.

each criterion. Although the pre-CRT CEA and post-CRT SUV were not included in the multivariate analyses, they were included in the risk score calculation, due to their assumed clinical importance. The scoring model was calculated by summing these six scores, defining the minimum scoring model as 0 and the maximum as 6 (Table 4). Next, a risk score was calculated for each subject, given

Table 3 Multivariate analysis to identify predictors of tumor and complete regression

	Tumor regression grade (0-3 *vs* 4)		
	OR	95% CI	*P* value
Pre-CRT size	1.265	0.622-2.569	0.516
Pre-CRT movability	2.780	1.141-6.777	0.024
Post-CRT size	3.473	1.235-9.767	0.018
Post-CRT morphology	11.100	1.389-88.673	0.023
Gross Change	5.847	1.519-19.814	0.009
ycT classification	2.073	0.968-4.440	0.061
ycN classification	1.887	0.917-3.883	0.085

CRT: Chemoradiation therapy; OR: Odd ratio; CI: Confidence interval.

Table 4 Scoring model

Parameters	No. of points	
	0	1
Pre-CRT movability	Tethered/fixed	Movable
Pre-CRT CEA (ng/mL)	> 5	≤ 5
Post-CRT morphology	Malignancy-like shape	Benign-like shape
Post-CRT SUV	> 5	≤ 5
Post-CRT size (cm)	> 3	≤ 3
Gross change	No	Yes

CRT: Chemoradiation therapy; CEA: Carcinoembryonic antigen; SUV: Standard uptake value .

that no missing data points existed for that selected risk factor. After excluding for the 98 subjects for whom ^{18}F-FDG-PET/CT and pre-CRT CEA were not measured, scoring was performed in a total of 151 individuals. The AUC of the scoring model was 80.5%, suggesting that this was a reasonable predictor of complete regression (Figure 1). When the cutoff point was set at 4.5, the sensitivity and specificity rates for predicting complete regression were 64.5% and 80%, respectively, with a PPV of 45.5%, an NPV of 89.7% and an accuracy of 76.8% (Table 5).

Validation of risk score

The ROC curve for the risk score performance that was obtained from the validation set had an AUC = 0.875, which can be seen in Figure 1. These results indicate that this scoring model performed well in an independent sample. Using a cutoff point of 4.5, the prediction of complete regression had a sensitivity of 75%, specificity of 90%, PPV of 75%, NPV of 90.91%, and accuracy of 86.7%.

DISCUSSION

Even though the response to PCRT is believed to be important in predicting prognosis and treatment strategy decisions, no reliable technique has been shown to predict accurately pCR after PCRT, and only limited data exist for each modality[22]. Various imaging modalities, including ERUS, CT, MRI and PET, have been evaluated in terms of their ability to predict the response to treat-

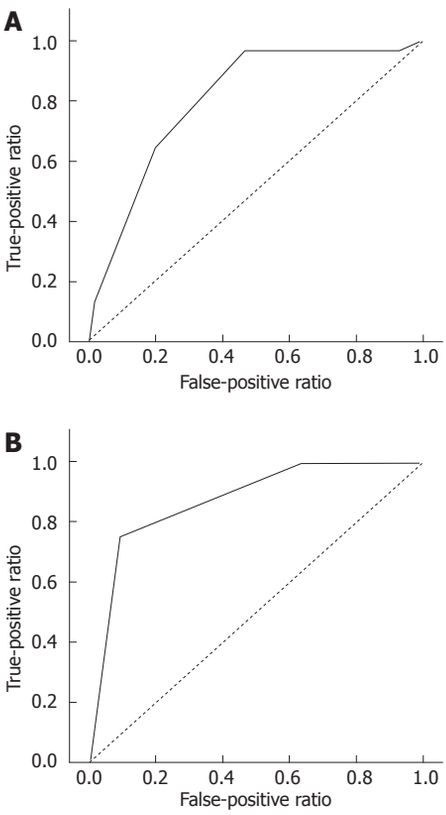

Figure 1 Area under the receiver operating characteristic curve. A: Risk score in the test set was 80.5%, suggesting it was a reasonable predictor of complete regression ($P < 0.001$, 95% confidence interval = 0.723-0.886, $n = 151$); B: In the validation set, risk score was 87.5%. ($P < 0.031$, 95% confidence interval = 0.672-1.078, $n = 151$).

ment after PCRT. Recent studies have reported a limited accuracy for ERUS (48%-72%)[23,24], and the accuracy of CT and MRI in assessing the depth of tumor infiltration after PCRT has also been shown to be limited at approximately 50%[25].

Our retrospective analysis evaluated the influence of certain pretreatment parameters on pCR after PCRT, and identified an accurate method for predicting pCR. Specifically, we hypothesized that a combination of clinical, laboratory and metabolic information would best predict pCR. Particular attention was given to gross changes and morphological evaluations by surgeons or other clinicians. These data were then classified and analyzed to verify the predictive value of radiosensitivity. Previously, some reports have indicated that gross assessments by DRE had a low PPV for assessing complete response. In one retrospective review of 488 rectal cancer patients who underwent PCRT and subsequently were followed by clinical re-evaluation (DRE and sigmoidoscopic examination under anesthesia), the clinical CR rate was 19%, with only 25% of the subjects achieving pCR (10%)[26]. In another study from Guillem and colleagues, DRE was found to underestimate the response of rectal cancer to PCRT[27]. Furthermore, other evidence indicates that DRE is not a reliable technique for distinguishing between post-radiation fibrosis and residual cancer[28]. To date, the majority

Table 5 Predicting score accuracy

Measure of accuracy	Cut off value, 4.5	
	n	%
Sensitivity	20/31	64.5
Specificity	96/120	80
Positive predictive value	30/44	45.5
Negative predictive value	96/107	89.7

of studies have evaluated the efficacy of DRE as a single modality for predicting pCR through predictive value calculations. Although we concluded that DRE alone may be able to distinguish pCR, we hypothesized that the supplementation of DRE with other clinical findings would increase the accuracy of pCR predictions.

A German study has definitively shown that FDG-PET metabolic imaging is superior to CT and MRI morphological imaging in predicting response to PCRT[29]. In this particular study, the therapeutic response was evaluated through comparisons of FDG-PET imaging that was conducted pre- and post-treatment with the SUV, resulting in a sensitivity of 100% and a specificity of 60% (the PPV and NPV were 77% and 100%, respectively). Notably, another study has reported the overall accuracy of FDG-PET to be 60%, although imaging was only performed after PCRT[20]. The primary limitation of FDG-PET in predicting tumor response is the relatively low specificity, therefore, it is viewed as somewhat risky to base treatment decisions on FDG-PET exclusively. Furthermore, to achieve the best predictive rates for CR with FDG-PET, imaging must be performed twice.

To improve the accuracy of pCR predictions, we designed a scoring model using important clinical and gross parameters. The pre-CRT CEA and post-CRT SUV were identified to be significant in a univariate analysis in our study, therefore, in addition to prior reports that these variables affect pCR, we included these parameters in the scoring model[8,22,29]. In fact, the scoring model facilitated the identification of four statistically significant variables in the multivariate analysis, including pre-CRT movability, post-CRT size, post-CRT morphology and gross change, and the AUC of the scoring model was 76.5% in the test set. After the pre-CRT CEA and post-CRT SUV were added to the scoring model, the accuracy of the pCR prediction increased (AUC of the risk score was 80.5%). In addition, these parameters are objective, easily applied, and already widely used. In this respect, we included the pre-CRT CEA and post-CRT SUV in the scoring model, although these parameters were not revealed to be significant in the multivariate analysis.

After the data analysis, our scoring model was found to be significantly correlated with pCR in both the test and validation sets. At a cutoff point of 4.5, the overall accuracy of our scoring model was 76.8% for predicting pCR in the test set; however, the accuracy of the model in predicting pCR decreased to 62.85% in the test set when ^{18}F-FDG-PET/CT (post-CRT SUV) was the only variable used. This secondary finding suggests that the

predictive ability of the scoring model probably requires both ^{18}F-FDG-PET/CT and DRE or colonoscopy morphological assessments. Furthermore, when compared with the aforementioned German study that evaluated the predictive ability of FDG-PET, our model had a superior specificity, a similar NPV, and an inferior sensitivity and PPV[29]; however, although our scoring model only used the post-CRT SUV, the German FDG-PET study required pre- and post-treatment SUV measurements. Moreover, the accuracy of our model was substantially improved in the validation set, suggesting that the ability to predict CR can be improved by combining clinical and radiological findings, including metabolic information obtained through DRE, colonoscopy and ^{18}F-FDG-PET/CT.

Our study had some limitations that warrant discussion. First, our results may have been affected by external bias, because the investigation was not prospective. Specifically, although all data were mined from a prospective database that adopted clear definitions for gross changes, the actual classification of gross change could easily be affected by surgeons' subjective determinations. Second, the exclusion of some individuals could have resulted in a sampling bias, although our model was vetted by a separate validation set. Despite these drawbacks, our model had significant value because it allowed clinicians to predict accurately pCR without performing any additional examinations.

In conclusion, our results indicate that gross tumor change and other associated morphological findings may represent important predictors for pathological tumor response, wherein both clinical parameters are easily estimated *via* physical examination. Moreover, because our scoring model includes both gross findings and clinical variables, we contend that PCRT response is best predicted by a combination of clinical, laboratory and metabolic findings.

COMMENTS

Background

Pathological complete response (pCR) has been associated with improved oncologic outcome. Although surgery is recommended after preoperative chemoradiation therapy (PCRT), regardless of tumor response, the ability to predict an individual's chance of achieving pCR before commencing therapy would probably allow for a more tailored treatment.

Research frontiers

Although it is important to predict therapeutic responses, there is no reliable technique to accurately predict pCR after PCRT. This study demonstrates that a combination of clinical, laboratory and metabolic information would best predict pCR.

Innovations and breakthroughs

Recent reports have highlighted the importance of tailored treatment in rectal cancer, including "wait and watch" after chemoradiation. In this study, the authors designed a scoring model using clinical and gross parameters for predicting pCR. The developed scoring model was found to be significantly predictive in both test and validation sets.

Applications

This study demonstrates that gross tumor changes and other associated morphological findings may represent important predictors of pathological tumor responses with both clinical parameters being easily estimated *via* physical examination.

Peer review

The study aimed to evaluate a number of clinical parameters and identify a method of predicting pCR after CRT in patients with advanced rectal cancer. It is an interesting study, meticulously carried out and reported.

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BRIEF ARTICLE

Mutations around interferon sensitivity-determining region: A pilot resistance report of hepatitis C virus 1b in a Hong Kong population

Xiao-Ming Zhou, Paul KS Chan, John S Tam

Xiao-Ming Zhou, Department of Epidemiology, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China
Paul KS Chan, Department of Microbiology, The Chinese University of Hong Kong, Shatin, Hong Kong, China
John S Tam, World Health Organization, Geneva 1200, Switzerland
Author contributions: Zhou XM performed the majority of experiments and wrote the manuscript; Chan PKS and Tam JS provided financial support, vital reagents and analytical tools, and conception of this manuscript.
Supported by The National Key Technology Research and Development Program of China, No. 2009ZX10004-104
Correspondence to: Dr. Xiao-Ming Zhou, Department of Epidemiology, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China. xmzhou@shmu.edu.cn
Telephone: +86-21-37990333-7309 Fax: +86-21-57248768
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Abstract

AIM: To explore mutations around the interferon sensitivity-determining region (ISDR) which are associated with the resistance of hepatitis C virus 1b (HCV-1b) to interferon-α treatment.

METHODS: Thirty-seven HCV-1b samples were obtained from Hong Kong patients who had completed the combined interferon-α/ribavirin treatment for more than one year with available response data. Nineteen of them were sustained virological responders, while 18 were non-responders. The amino acid sequences of the extended ISDR (eISDR) covering 64 amino acids upstream and 67 amino acids downstream from the previously reported ISDR were analyzed.

RESULTS: One amino acid variation (I2268V, $P = 0.023$) was significantly correlated with treatment outcome in this pilot study with a limited number of patients, while two amino acid variations (R2260H, $P = 0.05$ and S2278T, $P = 0.05$) were weakly associated with treatment outcome. The extent of amino acid variations within the ISDR or eISDR was not correlated with treatment outcome as previously reported.

CONCLUSION: Three amino acid mutations near but outside of ISDR may associate with interferon treatment resistance of HCV-1b patients in Hong Kong.

Key words: Hepatitis C virus 1b; Extended interferon sensitivity-determining region; Interferon-α; Resistance; Hong Kong; Mutation

Peer reviewer: Dr. Hisato Nakajima, The Jikei University School of Medicine, Department of Gastroenterology and Hepatology, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan

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INTRODUCTION

The interferon (IFN)-α/ribavirin combination treatment for hepatitis C virus (HCV) can achieve a sustained virological response rate of 20%-50%. The effectiveness of the combination treatment is partly correlated with HCV genotypes[1]. HCV genotype 6a shows a more favorable response with a sustained virological response rate around 50%-70%[2,3], while HCV genotype 1 is hard to be treated with a low sustained virological response

rate between 20% and 40%[4]. Pegylated (PEG)-IFN-α can improve the sustained virological response. But HCV genotype 1 is still more difficult to treat than other HCV genotypes, i.e., 2 and 3 by PEG-IFN-α[4,5].

The interferon sensitivity-determine region (ISDR, amino acid position 2209-2248, refers to HCV-J, GenBank accession no: D90208) for HCV 1b was previously identified to be associated with resistance to IFN-α-based treatment[6,7]. However, this observation was not reproducible in the studies in Europe, China and United States[8-12]. The host genetic background may be one of the reasons accounting for the discrepancies[13]. Furthermore, isolates of HCV 1b circulated in Japan may have substantial genetic difference compared with those circulating in Europe or United States[14]. The genetic difference of virus strains may be another reason for the discrepancies[15].

Several functional domains were identified within NS5A encoding region[16]. One of them can bind to Protein Kinase-R (PKR)[17,18]. This domain encloses the entire ISDR, with additional 26 amino acids extended downstream. PKR can be activated by IFN-α, then phosphorylates elongation-initiation-factor-2, which is an essential component of cellular protein translation complex, resulting in stopping the synthesis of intracellular proteins[19]. When NS5A binds to PKR, the synthesis of proteins will be restored, then the replication of HCV within host cells can be rescued[18]. This hypothesis suggests that the PKR binding domain is still a good candidate to look for variations that are associated with outcomes of IFN-α-based treatment[20].

No study on the IFN resistance-associated mutation in HCV-1b has yet been carried out in the Hong Kong population[21]. Although result from this population may conform to other geographic regions, HCV-1b strains circulated in Hong Kong had a more conservative genomic pattern than its counterpart in any other regions due to its specific geographic location and political history. Thus we carried out this study to explore amino acid variations within extended ISDR (eISDR) associated with the IFN-α/ribavirin combination treatment[22].

MATERIALS AND METHODS

Ethics

This work was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Recruited patients all submitted written consent to take part in the study. The study was approved by the local Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (ref. CRE-2006.405).

Study population

Samples were collected from Hong Kong patients between 1998 and 2004. Their HCV genotypes were determined by the type-primer-specific reverse transcription-polymerase chain reaction (RT-PCR) genotyping method as described before.

All patients included in this study were previously naive to HCV treatment. After HCV-1b genotyping determination, they received 5 million international units IFN-α three times per week and ribavirin (1000 mg for those weighing less than 75 kg and 1200 mg for those weighing more than 75 kg) for one year. If HCV-6a genotyping was determined, they would receive a short-term treatment for 6 mo. The HCV RNA load was measured once every six months during treatment and then every year after the completion of treatment. Patients with HCV RNA detected in the serum six months after treatment were defined as non-response/resistant (NR). Patients with negative HCV RNA results for at least one year after treatment were defined as having sustained virological response (SVR).

Altogether 19 NR and 18 SVR patients with determined endpoint of treatment were recruited in this pilot study. These patients had no recorded history of injecting drug use or liver transplantation.

Polymerase chain reaction primers for extended interferon sensitivity-determining region

The eISDR within HCV 1b genome starts from amino acid loci 2145 to 2315 (refers to HCV-J, GenBank accession no: D90208). This region extends 64 amino acids upstream and 67 amino acids downstream to the ISDR (2209-2248 in HCV-J). With the downstream extension, this eISDR includes the entire PKR-binding domain.

This region was amplified by a nested RT-PCR reaction. Primers used were: outer forward primer (NS5A-1b-1F): 5'-TGGATGGAGTGCGGTTGCACAGGTA-3'; outer reverse primer (NS5A-1b-1R): 5'-TCT TTC TCC GTG GAG GTG GTA TTG G-3'; inner forward primer (NS5A-1b-2F): 5'-TGTAAAACGACGGCCAGTCAGGTACGCTCCGGCGTGCA-3'; inner reverse primer (NS5A-1b-2R): 5'-CAGGAAACAGCTATGACCGGGGCCTTGGTAGGTGGCAA-3'. The underlined inner-primer sequences represent the M13 sequencing primers.

Amplification of extended interferon sensitivity-determining region

HCV RNA was extracted from each serum sample using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol. Five microliters of viral RNA were used in a final 50-μL single-tube single-step RT-PCR reaction using SuperScript™ One-Step RT-PCR system with Platinum® *Taq* kit (Invitrogen, Life Technologies, Carlsbad, CA). Cycling conditions were: reverse transcription at 50 ℃ for 30 min, 94 ℃ for 2 min followed by 30 cycles of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, extension at 68 ℃ for 1 min. Second round nested PCR reactions were carried out using 1 μL of the first round RT-PCR product as template and using HotStar *Taq* polymerase (Qiagen, Hilden, Germany) in a final 50-μL reaction with 1 × Q-solution. The HotStar *Taq* polymerase was used at a working concentration of 5 U

per 50-μL reaction. Cycling conditions were: 95 ℃ for 15 min to activate the HotStar *Taq* polymerase followed by 30 cycles of denaturation at 95 ℃ for 1 min, annealing at 55 ℃ for 1 min, extension at 72 ℃ for 1 min, and a final extension cycle at 72 ℃ for 5 min.

Sequencing for extended interferon sensitivity-determining region

The 609-bp PCR amplicons were purified from agarose gel using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Bioscience UK limited, Little Chalfont, United Kingdom), and directly used for BigDye® Terminator v3.1 Cycle Sequencing reaction (Applied Biosystems, Foster City, CA). The nucleotide sequences were translated to amino acid sequences following the right open-reading-frame. All the sequences were deposited into GenBank.

Alignment of amino acid sequences

The amino acid sequences were aligned by the multiple-alignment algorithm under Clustal-X (version 1.83) using a protein weight matrix of BLOSUM 62.

Calculation of amino acid variations

Consensus amino acid sequences were deduced from all the eISDR sequences. The number of amino acids that were different between each query sequence and the consensus eISDR sequence was added up as the degree of amino acid variations for this query sequence in the eISDR (1b-VAR2). The number of amino acids that were different within the ISDR was added up as the degree of amino acid variations for this query sequence in the ISDR (1b-VAR1).

Statistical analysis

The difference in patient age between the IFN-NR group and the IFN-SVR group was assessed by two-tailed Student's *t* test. The sex distribution between patients of the IFN-NR group and the IFN-SVR group was assessed by two-tailed Fisher's exact test (mid-*P* approach). The association of the amino acid variations within eISDR with treatment outcomes was evaluated by two-tailed Fisher's exact test. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 11.1.0, SPSS Inc., Chicago, IL) and Two-by-two (http://www.med. uio.no/imb/stat/two-by-two/manual.html) for two-tailed Fisher's exact test.

RESULTS

Patient information

The mean age of the 37 patients was 48.4 years (SD = 9.3 years, range 28-67 years). There was no significant difference in age between the IFN-NR and the IFN-SVR patients (47.9 years *vs* 49.0 years, $P = 0.73$). There was no significant difference in sex distribution between the IFN-NR (male/female: 11/7) and the IFN-SVR (male/female: 11/8) patients ($P = 1.00$).

Analysis of interferon sensitivity-determining region and extended interferon sensitivity-determining region

The sequence differences between the IFN-SVR and the IFN-NR patient groups are illustrated in Figure 1. Amino acid variations were found distributed over 56 different loci. Six loci, at which more variations existed between the two groups, were examined by two-tailed Fisher's exact test (Table 1). One amino acid variation (I2268V, $P = 0.023$) was significantly correlated with the treatment outcome, while the two amino acid variations (R2260H, $P = 0.05$ and S2278T, $P = 0.05$) were weakly associated with treatment outcomes.

Prediction of interferon treatment outcomes by individual variation

Prediction of the treatment outcome by I2268V has a specificity of 100%, but a low sensitivity of 22.2%. Prediction by R2260H or by S2278T has a sensitivity of 100%, but a low specificity 21.1%.

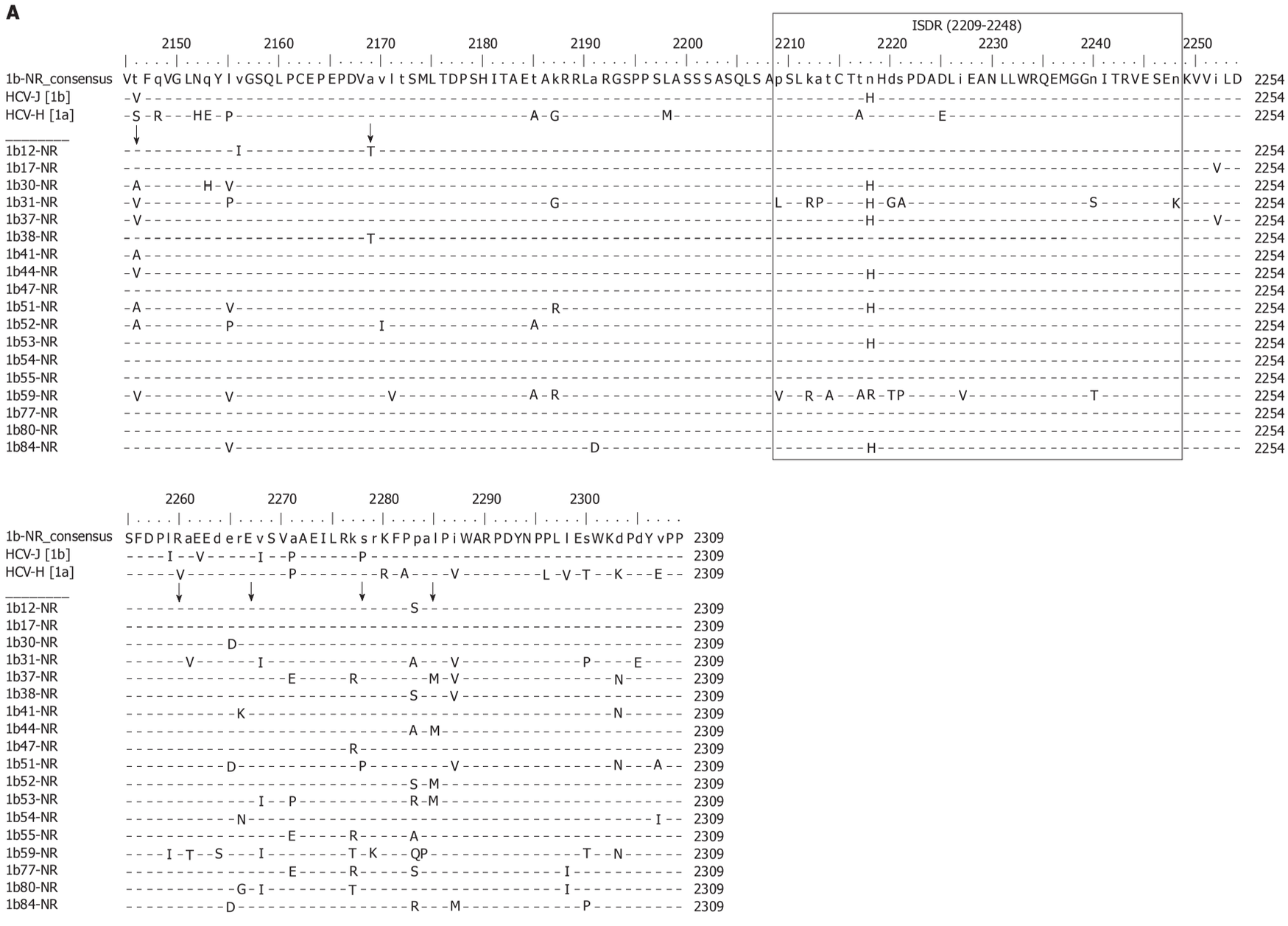
Association of treatment outcome with degree of amino acid variations within interferon sensitivity-determining region or extended interferon sensitivity-determining region

Previous studies have suggested that the degree of amino acid variations within the ISDR was associated with resistance to IFN-α treatment. We examined the correlation between the number of amino acid variations within the ISDR and the eISDR and the treatment outcome. A consensus resistant amino acid sequence was deduced from the conserved sequence of the IFN-NR group. This sequence has one amino acid different from reference strain HCV-J within the ISDR and seven amino acids within the entire eISDR. The degree of amino acid variations within the ISDR (1b-VAR1) and the eISDR (1b-VAR2) was calculated and compared. 1b-VAR1 ranged from 0 to 9 and 1b-VAR2 ranged from 1 to 24, both showing no correlation with the treatment outcome ($P = 0.958$, $P = 0.563$) with a previously defined cutoff of four amino acid variations.

DISCUSSION

In this pilot study with a limited number of cases, amino acid variations in three loci within the eISDR but outside the ISDR were identified to be possibly correlated with the resistance to the IFN-α/ribavirin combination treatment. R2260H ($P = 0.05$) and I2268V ($P = 0.023$) were located within PKR-binding domain[23,24]. S2278T ($P = 0.05$) was located within a hyperphosphorylation signal motif "PPALP"[25]. All these three loci were located within the putative NS5A transcriptional activation domain[16].

Based on these preliminary results, R2260H and I2268V suggested that PKR binding ability of NS5A may still play a major role in determining the IFN-α resistance of HCV-1b[20]. S2278T may imply that modification of the NS5A protein, which eventually alters the interaction of NS5A with other proteins like PKR, can contribute to the resistance to treatment[26,27].


A
ISDR (2209-2248)
2150 2160 2170 2180 2190 2200 2210 2220 2230 2240 2250
1b-NR_consensus Vt F qVG LNq Y l vGSQL PCE P EPDVa v l t SML TDP SH I TA Et AkRRLa RGSPP SLA SS S ASQLS Ap SL kat C Tt n Hds PDADL i EAN LLWRQEMGGn I TRVE SEn KVV i LD 2254
HCV-J [1b] 2254
HCV-H [1a] 2254
1b12-NR 2254
1b17-NR 2254
1b30-NR 2254
1b31-NR 2254
1b37-NR 2254
1b38-NR 2254
1b41-NR 2254
1b44-NR 2254
1b47-NR 2254
1b51-NR 2254
1b52-NR 2254
1b53-NR 2254
1b54-NR 2254
1b55-NR 2254
1b59-NR 2254
1b77-NR 2254
1b80-NR 2254
1b84-NR 2254
2260 2270 2280 2290 2300
1b-NR_consensus SFD P l R aEE d erE v S V aA E I L Rk s r K F P pa l P i WAR P DYN PPL l EsWKd PdY vPP 2309
HCV-J [1b] 2309
HCV-H [1a] 2309
1b12-NR 2309
1b17-NR 2309
1b30-NR 2309
1b31-NR 2309
1b37-NR 2309
1b38-NR 2309
1b41-NR 2309
1b44-NR 2309
1b47-NR 2309
1b51-NR 2309
1b52-NR 2309
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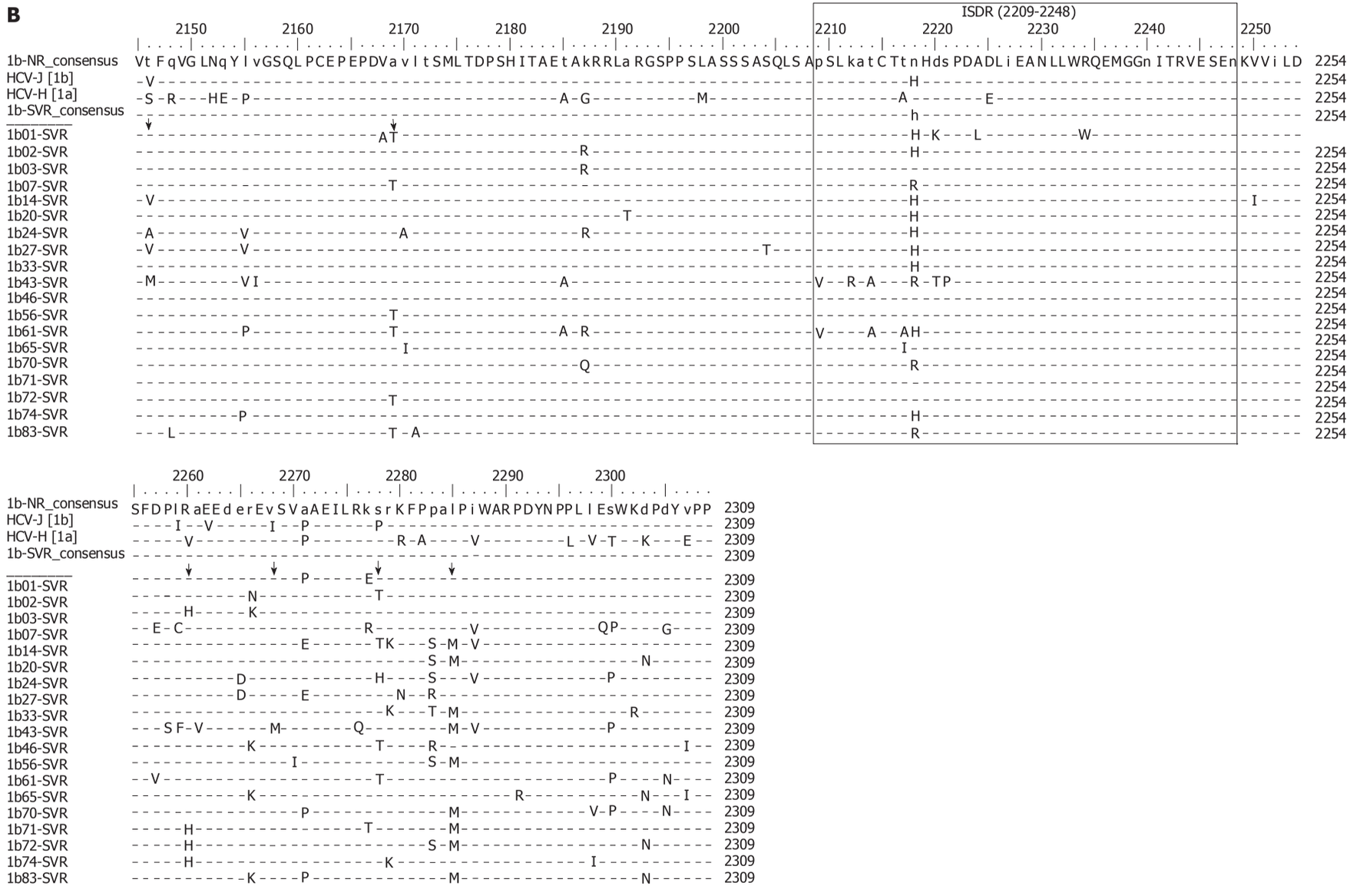

Figure 1 Amino acid variations within the extended interferon sensitivity-determining region for interferon-non-response group (A) and interferon-sustained virological response group (B). In the line of "1b-NR_consensus", capital letters denote the conserved amino acids for all sequences while lower-case letters denote the most frequent amino acid in that position. In other lines, letters denote amino acids different from IFN-NR (non-response) consensus sequence, while "-" means the same amino acid as in IFN-NR consensus sequence. Suffix "NR" follows the hepatitis C virus (HCV) strains which were resistant to IFN-α treatment. Suffix "SVR (sustained virological response)" follows the HCV strains which responded to IFN-α treatment. The previously defined interferon sensitivity-determining region was labeled by a square-line box. Arrows indicate the loci where amino acid variations were potentially associated with outcomes of IFN-α treatment and were examined in this study.

Table 1 Amino acid variations within extended interferon sensitivity-determining region of hepatitis C virus 1b

Locus	Amino acid variations		mid-*P*	Prediction	
	IFN-NR (*n* = 18)	IFN-SVR (*n* = 19)		Sensitivity (%)	Specificity (%)
2146	T (10/18, 55.6%)	T (15/19, 78.9%)	0.085		
	V (4/18, 22.2%)	V (2/19, 10.5%)	0.20		
	A (4/18, 22.2%)	A (1/19, 5.3%)	0.09		
		M (1/19, 5.3%)	-		
2169	A (16/18, 88.9%)	A (13/19, 68.9%)	0.12		
	T (2/18, 11.1%)	T (6/19, 31.6%)	0.12		
2260	R (18/18, 100%)	R (15/19, 78.9%)	0.05		
	H (0/18, 0%)	H (4/19, 21.1%)	0.05	100	21.1
2268	V (14/18, 77.8%)	V (18/19, 100%)	0.09		
	I (4/18, 22.2%)	I (0/19, 0%)	0.023	22.2	100
		M (1/19, 5.3%)	-		
2278	S (17/18, 94.4%)	S (14/19, 73.7%)	0.09		
	T (0/18, 0%)	T (4/19, 21.1%)	0.05	100	21.1
	P (1/18, 5.6%)	H (1/19, 5.3%)	-		
2285	L (14/18, 77.8%)	L (10/19, 52.6%)	0.085		
	M (4/18, 22.2%)	M(9/19, 47.4%)	0.085		

Locus position refers to the amino acid position in hepatitis C virus-J. *P* values were calculated by two-tailed Fisher's exact test (mid-P). "-" in the column of "*P*" means no *P* value calculated for this rare amino acid in that position. Sensitivity and specificity were calculated for the amino acid variations associated with treatment outcome. IFN: Interferon; NR: Non-response; SVR: Sustained virological response.

This trans-activating activity of the NS5A transcriptional activation domain was identified in an *in vitro* GAL4 system, and had not been demonstrated *in vivo*[28,29]. Some studies have shown that this NS5A trans-activating activity can stimulate the expression of IL8, an antagonist of IFN-α *in vivo*[30]. Whether the variations in these three loci can affect the expression of IL8 remains to be studied.

In contrast to previous reports, no correlation was found in the current study between the degree of amino acid variations within the ISDR or eISDR and IFN-α resistance of HCV-1b.

The association of IFN-α resistance and the amino acid variations was different from the reports from Europe and United States[15]. Previous reports did not find any amino acid variations within the ISDR that were correlated with treatment resistance. This difference may be due to the concurrent circulation of multiple subtypes of HCV-1b. The similarities between HCV-1b strains in Europe or United States were 90.3%, while in Hong Kong were 94.3% in average. Thus, HCV-1b strains distributed in Europe or United states were more diversified than those in Hong Kong. The convergency of HCV-1b population in Hong Kong introduces less genomic confounding factor. This may lead to different observations among studies involving a small number of samples.

Therefore, an expanded study is needed to further clarify the observations acquired from the Hong Kong population.

The association can be further ascertained in future studies by a larger group of registered HCV-1b patients, for those patients infected with IFN-resistant type of HCV-1b would be pursuable to abandon IFN treatment as early as possible. Alternative treatment, i.e., traditional Chinese medicine may be used instead, which may improve patient living status without the side-effects caused by IFN simultaneously. For the patients infected with IFN-sensitive type of HCV-1b, less IFN dosage should be administered to achieve the same efficiency of treatment.

COMMENTS

Background

Currently, an interferon-α (IFN-α) based regimen is the most effective and widely accepted therapy for hepatitis C virus (HCV) infections. When combined with ribavirin, IFN-α typically can achieve a 20% to 50% sustained virological response (SVR) in HCV-infected patients. The effect of HCV sequence variation in response to therapy is less clear.

Research frontiers

The association of a region of 40 amino acids in length within HCV 1b genome, termed as the interferon sensitivity-determining region (ISDR). However, similar studies performed with cohorts of American, European and Chinese patients reached discrepancy results. This discrepancy may resulted from high divergence of HCV 1b strains distributed worldwide.

Innovations and breakthroughs

The authors had early found that mutations within eISDR but outside of ISDR are in association with interferon treatment failure for HCV genotype 6a. This exploration were carried out in scope of full genomewide analysis. Thus, the authors explored mutations within eISDR in HCV-1b patients from Hong Kong, wish to reach a relative firmly report.

Applications

The study results suggest that we also can predict the prognosis of interferon treatment to HCV-1b patients. Interferon dosage can be regulated based on HCV-1b sequences, so reach to a balance between treatment effectiveness and tolerance of the side-effect of this drug.

Terminology

ISDR: Interferon sensitive-determine region, located within HCV NS5A. HCV NS5A had been firmly demonstrated that it can interact with various signal proteins induced by interferon. The relation of NS5A with interferon resistance were firmly established in HCV patients. But certain mutations had not ever been determined for predicting the prognosis of interferon treatment.

Peer review

This is a good study in which authors analyze the association of mutations within eISDR of HCV-1b with the resistance of treatment by IFN-α in Hong Kong. The results are interesting and suggest that three mutations may associated with treatment success/failure. These mutations could be used for predicting prognosis before the start of treatment. Thus, prescription of IFN-α may be improved for future treatments to HCV-1b patients.

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CASE REPORT

Ischemic colitis masquerading as colonic tumor: Case report with review of literature

Parakkal Deepak, Radha Devi

Parakkal Deepak, Internal Medicine, University of Chicago (North Shore University Health System), 2650 Ridge Avenue, Evanston, IL 60201, United States
Radha Devi, Internal Medicine, University of Illinois at Chicago (St Francis Hospital), Evanston, IL 60201, United States
Author contributions: Deepak P and Devi R contributed to collecting patient data, writing the manuscript and submission process.
Correspondence to: Parakkal Deepak, MD, Internal Medicine, University of Chicago (North Shore University Health System), 2650 Ridge Avenue, Evanston, IL 60201, United States. dparakkal@yahoo.co.in
Telephone: +1-847-6305398 Fax: +1-847-6305398
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Abstract

Ischemic colitis can mimic a carcinoma on computed tomographic (CT) imaging or endoscopic examination. A coexisting colonic carcinoma or another potentially obstructing lesion has also been described in 20% of the cases of ischemic colitis. CT scan can differentiate it from colon cancer in 75% of cases. However, colonoscopy is the preferred method for diagnosing ischemic colitis as it allows for direct visualization with tissue sampling. Varied presentations of ischemic colitis have been described as an ulcerated or submucosal mass or as a narrowed segment of colon with ulcerated mucosa on colonoscopy. Awareness and early recognition of such varied presentations of a common condition is necessary to differentiate from a colonic carcinoma, and to avoid unnecessary surgery and related complications.

Key words: Colon pathology; Colitis; Ischemic pathology; Colonic neoplasms/diagnosis; Differential Diagnosis; Biopsy; X-Ray computed tomography

Peer reviewer: Yuji Naito, MD, PhD, Professor, Department of Gastroenterology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

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INTRODUCTION

Ischemic colitis is the most common cause of ischemic injury in the colon with an estimated incidence of 4.5-44 cases per 100 000 person-years[1]. This commonly presents with moderately severe, crampy abdominal pain, mostly localized to the left lower quadrant, sometimes associated with diarrhea or rectal bleeding[2]. Rarely, ischemic colitis can mimic a carcinoma on computed tomographic (CT) imaging or endoscopic examination. This case report highlights this rare presentation along with a practical approach to the management based on a review of literature.

CASE REPORT

A 92 year old man presented to the hospital with complaints of fatigue, past medical history significant for hypertension and congestive heart failure. Physical examination revealed an irregularly irregular pulse with electrocardiogram demonstrating atrial fibrillation with rapid ventricular rate. He was started on a therapeutic dosage of low molecular weight heparin, however had five episodes of bright red blood per rectum the next day requiring transfusion of 2 units of blood with mild abdominal pain. He had no prior history of weight loss, loss of appetite, change in bowel habits or gastrointestinal bleeding. Physical exam at this time revealed stable vital signs with an unremarkable abdominal exam and bright red blood

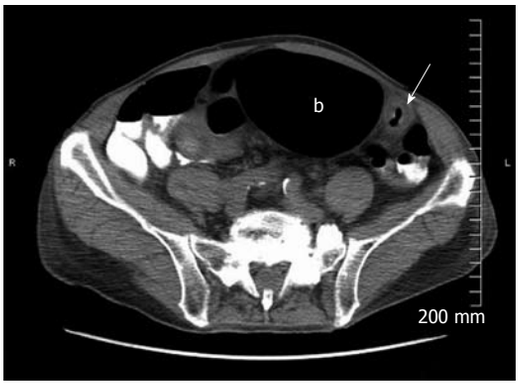

Figure 1 Computed tomography of the abdomen demonstrating circumferential thickening of a long segment of the distal colon (marked with white arrow) with a proximal dilated segment (marked with "b" inside the lumen of the dilated segment).

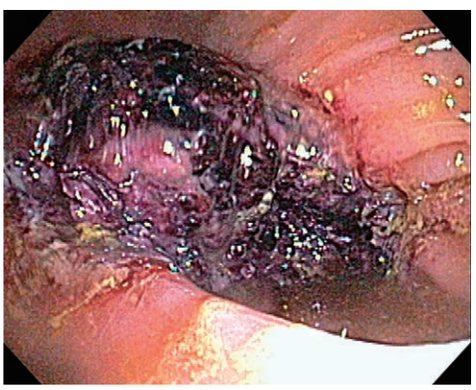

Figure 2 Colonoscopy image demonstrating friable mass occupying up to half the circumference of the splenic flexure.

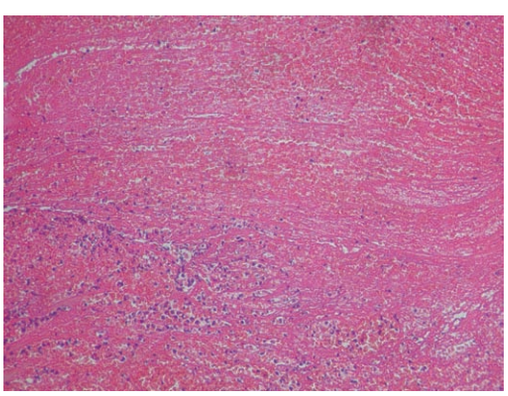

Figure 3 Hematoxylin and eosin high power microscopic image demonstrating acute colitis with marked fibrinopurulent exudates.

on the gloved finger on rectal examination. Laboratory tests revealed a normal white blood count and hemoglobin of 10.3 mg/dL with a normocytic normochromic peripheral blood smear. CT revealed circumferential thickening of a long segment of the distal colon with a proximal dilated segment suggestive of a stricture or a malignancy (Figure 1). He then underwent a colonoscopy which demonstrated a friable mass occupying up to half the circumference of the splenic flexure (Figure 2). Biopsy of the mass demonstrated acute colitis with marked fibrinopurulent exudates, consistent with acute ischemic colitis (Figure 3). He was treated with a regimen of bowel rest, intravenous fluids, and antibiotics, recovered completely and was discharged home.

DISCUSSION

Acute onset bleeding after anticoagulation originates from the colon in 25% of cases, commonly from polyps, diverticular disease, vascular malformations or cancer[3]. Rectal bleeding as a presentation of ischemic colitis is usually mild, not requiring transfusion[4]. CT scan is usually not performed for the diagnosis of ischemic colitis and can differentiate it from colon cancer in 75% of cases based on length and thickness of the thickened colonic segment, enhancement of the segment and the presence of target or double halo sign[5]. Colonoscopy or sigmoidoscopy is the preferred method for diagnosing ischemic colitis as it allows for direct visualization with tissue sampling[6]. However colonoscopy is preferred over sigmoidoscopy as lesions are found proximal to the sigmoid colon in 50% of cases except in the setting of aortic surgery where it is limited to the distal colon. Biopsies demonstrate vascular congestion, submucosal hemorrhage, interstitial edema, inflammatory infiltration, loss of superficial cells, and intravascular platelet thrombi. Acute ischemia is suggested by hyalinization of the lamina propria, full-thickness mucosal necrosis, the presence of atrophic microcrypts, and hemorrhage in the lamina propria whereas hemosiderin deposition, along with transmural fibrosis and mucosal atrophy, is a pathognomonic of a chronic phase of the ischemic colitis[7]. Colonoscopy demonstrates edematous, hemorrhagic, hyperemic or necrotic colonic mucosa in the acute phase, and strictures, or loss of haustrations in the chronic phase.

In 20% of the cases of ischemic colitis, a coexisting colonic carcinoma or another potentially obstructing lesion has been described[8]. In these cases the lesion is distal in location and separated from it by a variable segment of normal colon. The mechanism may involve an increased intra-colonic pressure proximal to the lesion with decreased colonic blood flow. Previous case reports[9-13] have described varied presentations of ischemic colitis as an ulcerated or submucosal mass or as a narrowed segment of colon with ulcerated mucosa on colonoscopy. The objective of our case report is to create awareness and early recognition of such varied presentations of a common condition in order to differentiate from a colonic carcinoma, avoid unnecessary surgery and related complications.

Treatment of mild cases involves bowel rest and parenteral fluids with antibiotics to cover usual bowel flora. Ischemic colitis involving the right side of the colon occurs in 23% of cases and these patients more often need surgical intervention[14]. Complications include bowel gangrene, ulcerations, strictures, and fulminant colitis. It has been suggested that in the rare scenario where colo-

noscopy also suggests a neoplasm with biopsy specimens negative for a tumor, repeating imaging and colonoscopy studies 7-10 d later may identify the evolving nature of the acute ischemic lesion and obviate the need for surgery.

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Self-expanding metallic esophageal stents: A long way to go before a particular stent can be recommended

Pankaj Jain

Pankaj Jain, Department of Gastroenterology, Fortis Modi Hospital, Vivekanand Nagar, 324010 Kota, Rajasthan, India
Author contributions: Jain P analyzed the data, reviewed the literature and wrote the paper.
Correspondence to: Pankaj Jain, MD, DM, Consultant Gastroenterologist, Department of Gastroenterology, Fortis Modi Hospital, Vivekanand Nagar, 324010 Kota, Rajasthan, India. panj2007@rediffmail.com
Telephone: +91-9829-564531 Fax: +91-0744-2473500
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Abstract

We agree that the covered self-expanding metal stents (SEMSs) fare better than the uncovered stents as recurrent dysphagia due to tumor ingrowth is common with uncovered stent. Recent American College of Gastroenterology Practice Guideline on the Role of Esophageal Stents in Benign and Malignant Diseases concludes that SEMSs cannot be routinely recommended in conjunction with chemo-radiation. The comparison of ultraflex and choostent in the Italian study found no difference in the palliation of dysphagia, rate of complications and survival rate.

Key word: Self-expanding metallic esophageal stents; Dysphagia; Esophageal stents

Peer reviewer: Dr. Josep M Bordas, MD, Department of Gastroenterology IMD, Hospital Clinic, Llusanes 11-13, Barcelona 08022, Spain

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TO THE EDITOR

We read with interest the article "Covered nitinol stents for the treatment of esophageal stricture and leaks" by Bona *et al*[1] in the May 14, 2010 issue of *World Journal of Gastroenterology*. We agree that covered self-expanding metal stents (SEMSs) fare better than uncovered stents as recurrent dysphagia due to tumor ingrowth is common with uncovered stent[2]. Partially covered SEMSs are superior to uncovered SEMSs in the palliation of dysphagia due to unresectable esophageal tumor[3].

Although the study discussed the positive role of temporary stent placement in patients undergoing neoadjuvant therapy, recent American College of Gastroenterology Practice Guideline on the Role of Esophageal Stents in Benign and Malignant Disease concludes that SEMSs cannot be routinely recommended in conjunction with chemo-radiation[4]. The data on use of SEMSs for gastroesophageal junction cancers with concomitant radiation are retrospective, discordant and limited[5,6].

The self-expanding plastic stents are preferable over SEMSs as temporary stent insertion in case of anastomotic complications or post-radiotherapeutic stricture because the option of retrieval is better, there is limited local tissue reaction and is of lower costs[7].

SEMSs are useful in patients with poor functional status who cannot tolerate chemotherapy or radiotherapy, who have advanced metastatic disease or in whom previous therapy has failed[8]. This data was lacking in the study, and it would have given a better way to compare ultraflex and choostent.

Bona *et al*[9] in their study of comparison of ultraflex and choostent found no difference in the palliation of dysphagia, rate of complications and survival rates. Both stents were safely removable in short term follow-up. The benefit of temporary insertion of both types of stents was documented in patients with esophageal carcinoma prior to chemotherapy or chemoradiotherapy and in those with anastomotic strictures or leaks. The ideal timing for metallic stent removal is not well defined and varies from

2 wk to 4 mo. However, it is safe to remove within 2 mo after stent placement[10].

So, further studies are required before firm recommendation regarding the choostent can be made.

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Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building, F09, The University of Sydney, Sydney NSW 2006, Australia

Marcelo A Beltran, MD, Chairman of Surgery, Hospital La Serena, PO Box 912, La Serena, IV Region, Chile

Alberto Biondi, PhD, Department of Surgery, 1st Surgical Division, Catholic University of Rome, Largo A. Gemelli 8, Rome 00168, Italy

Andrew Seng Boon Chua, MD, Department of Gastroenterology, Gastro Centre Ipoh, 1, lorong Rani, 31, lebuhraya Tmn Ipoh, Ipoh Garden South, IPOH 30350, Malaysia

Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

Wen-Hsin Huang, MD, Division of Hepatogastroenterology, Department of Internal Medicine, China Medical University Hospital, No 2, Yuh-Der Road, Taichung 404, Taiwan, China

Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Teng-Yu Lee, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taichung Veterans General Hospital, 160, Sec. 3, Taichung Harbor Road, Taichung 407, Taiwan, China

John K Marshall, MD, Associate Professor of Medicine, Division of Gastroenterology (4W8), McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

Yasushi Matsuzaki, Associated Professor, Division of Gastroenterology and Hepatology, Graduate School of Comprehensive Human Sciences and University Hospital, 1-1-1, Tennodai, Tsukuba 305-8575, Japan

Giuseppe Orlando, MD, PhD, Department of Health Sciences, Wake Forest Institute for Regenerative Medicine, 391 Technology Way, Winston Salem, NC 27101, United States

Freddy Penninckx, Professor, Department of Abdominal Surgery, University Clinic Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

Ji Kon Ryu, Professor, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, South Korea

Tokihiko Sawada, MD, PhD, Associate professor, Second Department of Surgery, Dokkyo Medical University, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan

Kazuaki Takabe, MD, PhD, Assistant Professor of Surgery and Assistant Professor of Biochemistry and Molecular Biology, Surgical Oncology, VCU Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, PO Box 980011, Richmond, VA 23298-0011, United States

Lin Zhang, PhD, Associate Professor, Department of Pharmacology & Chemical Biology, University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, UPCI Research Pavilion, Room 2.42d, Hillman Cancer Center, 5117 Centre Ave., Pittsburgh, PA 15213-1863, United States

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

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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 the 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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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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Title: Title should be less than 12 words.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Abstract

There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

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Authors: the name of the first author should be typed in boldfaced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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

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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).

Units

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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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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